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Short Communication

Stimulation of calcium signal transduction results in enhancement of production of scopadulcic acid B by methyl jasmonate in the cultured tissues of *Scoparia dulcis*

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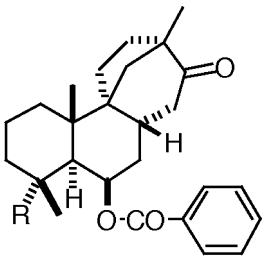
Abstract Signal transduction events which are involved in methyl jasmonate (MeJA)-enhanced production of the tetracyclic diterpene, scopadulcic acid B (SDB), were investigated in leaf organ cultures of *Scoparia dulcis*. Pretreatment of leaf organ cultures with Ca^{2+}-channel blocker, verapamil, resulted in a dose-dependent inhibition of MeJA enhanced SDB production. Treatments with Ca^{2+} ionophore, A23187, stimulated SDB production in the absence of MeJA. In addition, preincubation of the leaf organ cultures with calmodulin (CaM) antagonist, trifluoperazine (TFP), diminished MeJA enhanced SDB production. MeJA-enhanced production of SDB was suppressed by addition of protein kinase inhibitors such as staurosporine and 2,6-diamino-*N*-([1-(1-oxotridecyl)-2-piperdinyl]methyl)hexanamide (NPC-15437). An activator of protein kinase C, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), stimulated this production in the absence of MeJA. These results indicate that Ca^{2+} and CaM mediates signaling events leading to enhancement of SDB production upon MeJA treatment. It was also suggested that phosphorylation of protein by protein kinases might be involved in this signal pathway.

Key words: Calmodulin, diterpene, methyl jasmonate, scopadulcic acid B, *Scoparia dulcis*.

Scopadulcic acid B (SDB), which exhibits diverse biological activities, is one of aphidicolin-like tetracylic diterpenes isolated from a tropical medicinal plant *Scoparia dulcis* (Hayashi et al. 1999, 2000). Studies on the biosynthesis of diterpenes in *S. dulcis* revealed that they are mainly accumulated in young leaves (Hayashi et al. 1991) and are produced via mevalonate independent pathway (Hayashi et al. 1999). Their production is closely related to the differentiation level of leaves (Hayashi et al. 1993, 1997), and enhanced by methyl jasmonate (MeJA) (Nkembo et al. 2005). The stimulatory effects of MeJA on the production of several classes of secondary metabolites is well documented (Bulgakov et al. 2002; Zhao and Sakai 2003; Ketchum et al. 2003), but the most of targeted metabolites are phytoalexins, which are induced by stresses including various elicitors or signal molecules, and their signaling mechanisms are still poorly understood. Various components, including ion fluxes and calcium signaling, reactive oxygen species, ethylene, abscisic acid, salycylic acid and jasmonate, have been reported to mediate the signal transduction for production of secondary metabolites (Zhao et al. 2005). Protein kinases and phosphatases are proposed to be involved in these signaling pathways (Lecourieux et al. 2002), and some of them are regulated by Ca^{2+} binding. In this report, we describe the role of calcium-mediated signal transduction in MeJA-enhanced production of SDB in leaf organ cultures of *S. dulcis*.

Leaf organ cultures of *S. dulcis* were established as described (Hayashi et al. 1997). Approximately 400 mg of leaf segments (n=17–20) were transferred into a 100 ml-culture flask containing Murashige-Skoog (MS)



SDB: R = COOH
SDC: R = CH_2OH

Chart 1. Structures of SDB and SDC.

Abbreviations: CaM, calmodulin; NPC-15437, 2,6-diamino-*N*-([1-(1-oxotridecyl)- 2-piperdinyl]-methyl)-hexamide; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; SDB, scopadulcic acid B; MeJA, methyl jasmonate; TFP, trifluoperazine.

liquid medium (50 ml) and incubated on a rotary shaker at 140 rpm at 26°C. MeJA, verapamil hydrochloride, and trifluoperazine (TFP) were obtained from Wako Pure chemicals (Osaka, Japan). A23187, staurosporine aglycon, 2,6-diamino-*N*-([1-(oxotridecyl)-2-piperidinyl]) methyl)hexanamide (NPC-15437) and 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) were obtained from Sigma-Aldrich Japan (Tokyo, Japan). MeJA and other chemicals were dissolved in EtOH and DMSO, respectively, and sterilized by Millipore LCR filter (Millipore Corp., Billerica, MA). Six-day-old leaf organ cultures were used in all experiments. In a single treatment, a known volume of filter-sterilized solution of either A23187 or OAG was added to leaf tissue cultures at a final concentration ranging from 0 to 100 μM. In a combined treatment with MeJA, TFP, verapamil, staurosporine, and NPC-15437 were added to the cultured tissues 6 h prior to addition of MeJA. In all treatments, the control received only the same volume of solvent. The leaf organ cultures were harvested after 2 and 4 days for A23187, and 4 days later for other treatments. Freeze-dried leaf organ cultures were extracted three times with $CHCl_3$–MeOH (3 : 1) under sonication for 20 min. SDB in the medium was extracted three times with $CHCl_3$. The combined extracts were concentrated to dryness and the residue was dissolved in $CHCl_3$, which was then subjected to a solid phase extraction using Bondesil-SI cartridge (500 mg, Varian, USA). The cartridge was successively washed with $CHCl_3$ (5 ml) and then with MeOH (5 ml), and SDB present in the MeOH phase was subjected to HPLC as described (Hayashi et al. 1991). Briefly, the sample was loaded to a $5C_{18}$-AR-II HPLC column (4.6×150 mm, Nacalai Tesque, Kyoto, Japan) and eluted with a solvent system of 0.02 M H_3PO_4/MeOH (1 : 3) at 1.0 ml min^{-1} by monitoring at 230 nm. Phytol and β-sitosterol in the $CHCl_3$ fraction were extracted into *n*-hexane after treatment with 6% KOH in MeOH. The *n*-hexane extract was then subjected to solid phase extraction, and phytol and β-sitosterol were eluted with MeOH–$CHCl_3$ (1 : 1) solution. After drying, the residue was dissolved in *n*-hexane containing cholesterol (1 mg ml^{-1}) as internal standard. Gas chromatography (GC) was performed using a GC-353 gas chromatograph equipped with a SPB-1 fused silica capillary column (30 m×0.32 mm×0.25 mm film thickness, Supelco Inc., USA), and N_2 carrier gas with a flow rate of 1.8 ml min^{-1}. The oven temperature was maintained at 180°C for 5 min and then programmed at 10°C min^{-1} to 280°C and kept for 24 min, and peaks were detected by flame ionization detector (FID). Each experiment was repeated at least twice and the results are expressed as mean±SE. Differences between means were tested for significance using paired Student's *t*-test.

Calcium mobilization is a characteristic component in plant cellular signaling pathways (Sander et al. 1999; Scrase-Field and Knight 2003). To examine whether calcium is involved in MeJA signal transduction in *S. dulcis*, cultured tissues were treated with verapamil, a Ca^{2+}-channel blocker. Pretreatment of leaf organ cultures with verapamil did not affect their growth (Table 1) and resulted in a dose-dependent inhibition of SDB production upon MeJA treatment (Figure 1A). To determine if the decreased content of SDB in verapamil-treated tissues was related to an increased release of SDB into culture medium, the content of SDB in each culture medium was examined. Results showed no difference in culture media of both control and verapamil-treated tissues (Table 1). To evaluate the effect of verapamil on the production of other isoprenoids, accumulation of β-sitosterol biosynthesized via mevalonate pathway and phytol via mevalonete-independent pathway was examined. Verapamil promoted the inhibitory effect of MeJA on β-sitosterol production (Figure 1B) but had no effect on phytol production (Figure 1C). These result suggested that Ca^{2+} influx was involved in the MeJA-enhanced production of SDB. To confirm the requirement of Ca^{2+} influx for MeJA-enhanced production of SDB, the leaf organ cultures were treated with calcium ionophore A23187 for two or four days in the absence of MeJA. As shown in Figure 2A, A23187 was sufficient to enhance the production of SDB in the cultured tissues of *S. dulcis*. When leaf organ cultures were treated with A23187 the SDB production was more enhanced with 10 μM than with 50 μM. In addition, A23187 induced faster SDB production than MeJA. Its potency was comparative with that of 10 μM MeJA-treated tissues on day 4 (Figure 2A). These results suggest that influx of extracellular Ca^{2+} plays an

Table 1. Effect of verapamil on tissue growth and content of SDB in cultured tissues and culture media.

Culture Conditions		Growth (mg dry weight)	SDB content (μg)	
MeJA (10 μM)	Verapamil (μM)		Tissues	Medium
−	−	200.0±30.0	3138.4±110.6	59.9±8.6
+	−	146.7±15.3	3926.4±171.4	50.3±2.5
+	50	146.7±11.5	2980.2±268.0*	54.4±12.7
+	100	143.3±15.3	2260.6±61.5**	55.2±11.3

Leaf organ cultures of *S. dulcis* were treated for four days with different concentrations of verapamil in the presence of 10 μM MeJA six days after inoculation. SDB content was calculated as total amount per flask and medium (50 ml). Data are expressed as mean±S.D., n=3. Significant difference was calculated from MeJA-treated tissues. *$p<0.05$, **$p<0.001$.

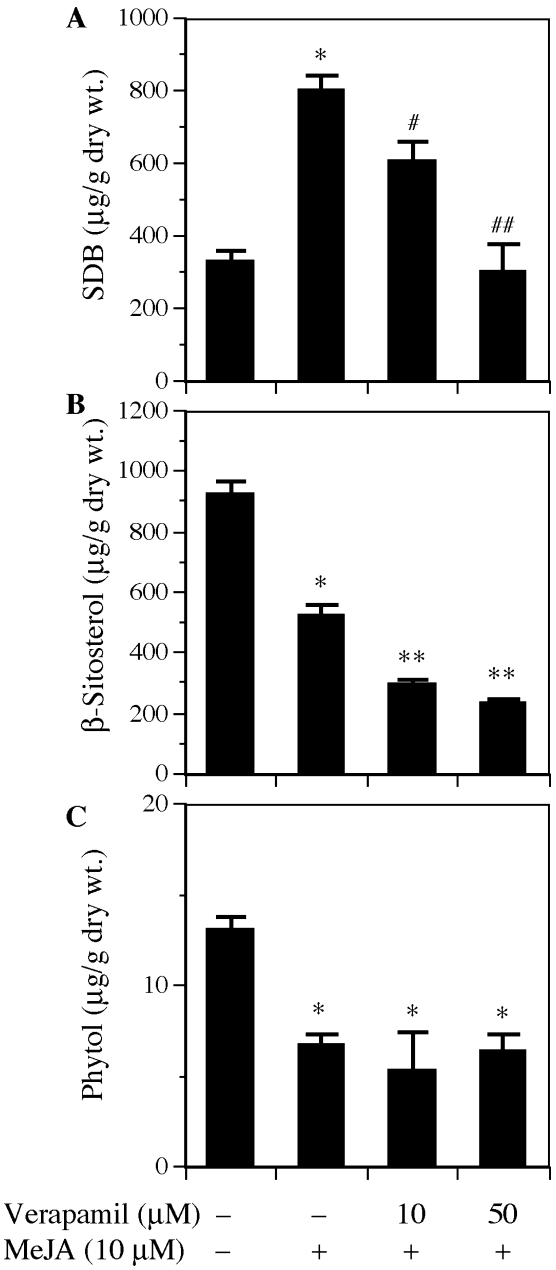


Figure 1. Effect of verapamil on production of isoprenoids in cultured tissues. 6-day-old leaf organ cultures were pretreated with 50 and 100 μM verapamil for 6 h before addition of 10 μM MeJA. The cultured tissues were harvested after 4 days and analyzed for their SDB (A), β-sitosterol (B), and phytol (C) contents. Data are expressed as mean±SE, n=3. Significant differences were calculated from the control, *$p<0.01$, **$p<0.001$ and MeJA-treated tissues, $^{\#}p<0.05$, $^{\#\#}p<0.01$.

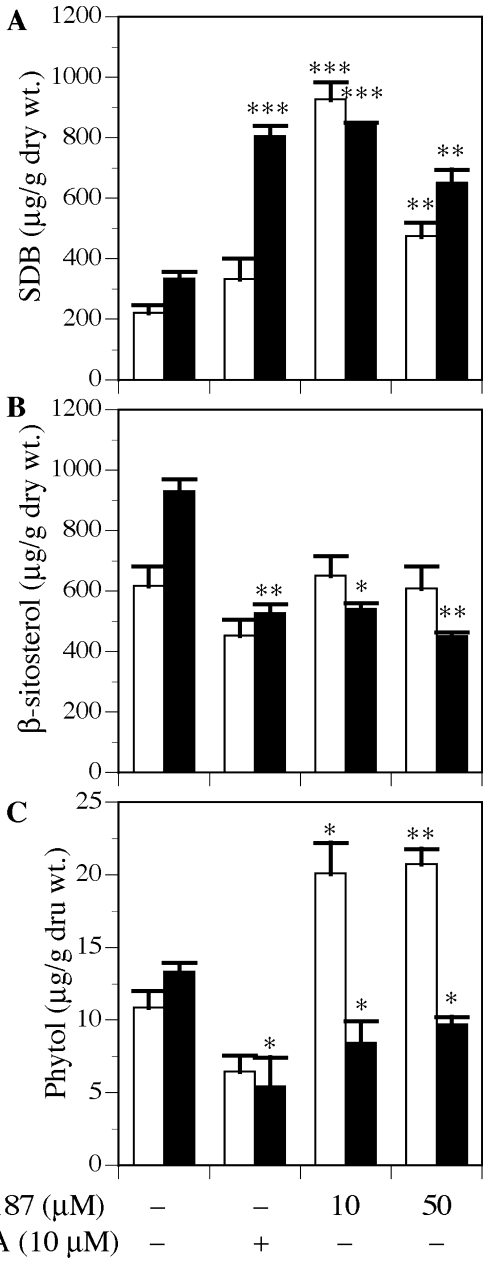


Figure 2. Effect of A23187 on production of isoprenoids in cultured tissues. 6-day-old leaf organ cultures were treated with A23187 as indicated in the absence of 10 μM MeJA for 2 days (white bar) and 4 days (black bar). Data are expressed as mean±SE, n=3. Significant differences were calculated from the control, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

important role in the production of SDB. In contrast, A23187 inhibited β-sitosterol production in time-dependent manner (Figure 2B), while it transiently stimulated phytol production (Figure 2C). Calmodulin (CaM) is one of targets of calcium signals in plant cells (Sanders et al. 1999). This lead us to test the effect of specific CaM antagonist TFP on MeJA-induced enhancement of SDB production, and found that pretreatment with TFP indeed reduced MeJA-stimulated SDB production (Figure 3A), while the same treatment promoted the MeJA-induced reduction of β-sitosterol and phytol production (data not shown). Although results obtained with TFP should be interpreted with caution due to possible inhibition through other processes such as cAMP cycle (Cheung, 1980), these data suggest that CaM or CaM-like proteins participate in MeJA signal transduction and therefore that Ca^{2+} might be involved in this transducing pathway. Protein phosphorylation has

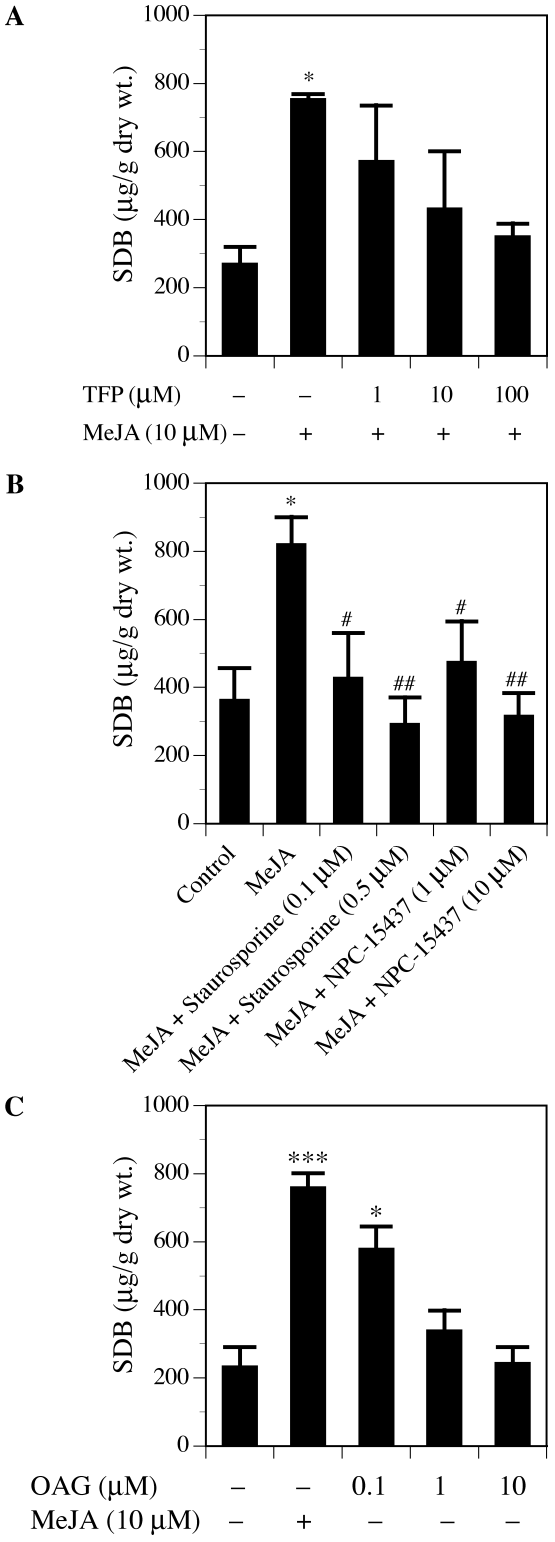


Figure 3. Effect of inhibitors and activator on production of SDB in cultured tissues. 6-day-old leaf organ cultures were treated with different concentrations of TFP (A), staurosporine or NPC-15437 (B) 6 h before addition of MeJA, and OAG (C) in the absence of MeJA. Data are expressed as mean±SE, n=3. Significant differences were calculated from the control, $*p<0.05$, $**p<0.01$, $***p<0.01$, and MeJA-treated tissues, $^{\#}p<0.005$, $^{\#\#}p<0.001$.

been implicated in plant responses to various stimuli such as elicitor treatments and pathogen attacks (Menke et al. 1999; Peck 2003). To examine possible participation of protein kinases in MeJA-related prodution of SDB, staurosporine (a serine/threonine kinase inhibitor) and NPC-15437 (a specific inhibitor of protein kinase C) were tested for the possibility to interfere the SDB production. Treatments with different concentrations of staurosporine (0.1 and 0.5 μM) or NPC-15437 (1 and 10 μM) resulted in suppression of MeJA-related SDB production (Figure 3B). In contrast, treatment with a specific activator of PKC, OAG (a synthetic analogue of diacyl glycerol), remarkably promoted production of SDB at 0.1 μM in the absence of MeJA. Its stimulatory effect was lower at higher concentrations (Figure 3C). The inhibitory effect of staurosporine and NPC-15437, and the stimulatory effect of OAG suggested that protein kinases are involved in signal transduction pathway for SDB production in *S. dulcis*.

Ca^{2+} mobilization has been shown to mediate the plant response to a number of stimuli including cold, heat shocks, pathogen challenges, elicitor, and hormone treatments (Knight et al. 1996; Kurosaki 1997; Liu et al. 2003; Zhao and Sakai 2003). The present results suggest that calcium influx is important for enhancement of MeJA-mediated SDB production. Cancellation of MeJA-enhanced SDB production by calcium channel blocker, verapamil, and CaM antagonist, TFP, suggested that extracellular calcium influx and CaM and/or calcium dependent kinases are involved in this pathway. These Ca^{2+}-related reagents have so far been shown to exhibit no or very little effect on constitutive production of secondary metabolism im plants (Kurosaki et al. 1992; unpublished result), suggesting that the present findings are not the results of toxic effects of these reagents. This is also supported by an observation that administration of A23187 alone enhanced the SDB. The SDB production was more rapidly induced by A23187 than by MeJA. Thus it was concluded that the enhancement of SDB production by MeJA is involved in the procedure of a calcium signaling pathway.

Menke et al. (1999) demonstrated the implication of protein phosphorylation in fungal elicitor- and MeJA-induced terpenoid indole alkaloid biosynthetic gene expression in *Catharanthus roseus*. In the present work, staurosporine and NPC-15437 significantly lowered the production of SDB in the presence of MeJA. In contrast, OAG stimulated the produciton of SDB at 0.1 μM in the absence of MeJA. These results indicate that MeJA-enhanced production of SDB requires protein phosphorylation. Effects of NPC-15437 and OAG suggest PKC to participate in MeJA-related signal transduction in the leaf organ cultures of *S. dulcis*.

The inhibitory effect of MeJA on β-sitosterol

production was not relieved by pretreatment with verapamil and TFP, suggesting them to additively suppress β-sitosterol production. Since calcium influx also appears to be involved in β-sitosterol production, MeJA and calcium might independently function the modulator of β-sitosterol biosynthesis under different mechanism from SDB production. In the case of phytol, effect of verapamil was not additive to MeJA, while TFP was. A23187 transiently stimulated phytol production. These results suggested that phytol production is resulted not only by calcium and CaM but also by some other undetermined mechanism(s). Jasmonates have been reported to induce chlorophyllase and to reduce phytosynthetic activities and chlorophyll degradation (Tsuchiya et al. 1999; Jung 2004). It is conceivable the reduced production of phytol is one of consequences of pertubations in the photosynthetic machinery caused by MeJA.

The stimulatory effect of MeJA on the production of secondary metabolites is well documented. However signaling elements which transduce its action are still poorly understood. From the present work, Ca^{2+}- and Ca^{2+}-CaM mediate the signal pathway was suggested to lead to MeJA-enahnced production of SDB in the cultured tissues of *S. dulcis*. There are still many open questions, for example, how MeJA regulates the flux of geraylgeranyl diphosphate (GGDP) towards biosynthesis of SDB. Our study showed that calcium influx stimulates and inhibits production of SDB and β-sitosterol, respectively, and that changes in cytoplasmic free Ca^{2+} could be an important signal leading to complex kinetics in plant cells.

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