Korean J. Medicinal Crop Sci. 13(5) : 233 – 236 (2005)

Up-regulation of Asiaticoside Biosynthesis by Methyl Jasmonate and Thidiazuron in *Centella asiatica* L. Urban

Ok Tae Kim*, Min Young Kim*, Jun Cheul Ahn, and Baik Hwang*†**

*Department of Biology and Institute of Plant Resources, Chonnam National University, Gwangju 500-757, Korea.
**Department of Life Sciences, Seonam University, Namwon 590-170, Korea.

ABSTRACT : *Centella asiatica* accumulates large amounts of triterpene saponin, such as centellasaponin, asiaticoside, madecassoside. We examined the effect of two candidates, MJ (Methyl jasmonate) and TDZ (thidiazuron), on asiaticoside production and the accumulation of bAS mRNA associated with asiaticoside biosynthesis in leaves of cultured whole plants. The growth of whole plants treated with 0.1 mM MJ was found to decrease significantly, however, the growth of whole plants treated with 0.1 mM MJ plus 0.025 ㎎/ℓ TDZ was better than that treated with MJ alone. When MJ alone was added to culture medium, asiaticoside contents in leaves were higher than that of control after 7 days of treatments. The maximum level of *bAS* (β-amyrin synthsae) mRNA in leaves of whole plant treated TDZ and MJ was transiently observed after exposure to 5 days. These results showed the up-regulation of *bAS* gene by adding TDZ and MJ at the molecular level, however, synergic effects of TDZ and MJ on asiaticoside biosynthesis were not testified.

Key words **:** β-amyrin synthase, asiaticoside, *Centella asiatica*, cycloartenol synthase, triterpene saponins

INTRODUCTION

Triterpene saponins, which are accumulated in their tissues, are common plant secondary metabolites. Physiological roles of saponins in plants are not clear, but several papers related to function of saponins have been reported. Papadopoulou *et al.* (1999) found that many saponins have potent anti-fungal activities, since they may serve as preformed phytoprotectants against fungal attack, as shown for avenacins in oats. Asiaticoside and other triterpene saponins share a common biosynthetic intermediate, β-amrin, which is synthesized by β-amyrin synthsae (*bAS*), an oxidosqualene cyclase (OSC) (Fig. 1). OSCs catalyze the cyclization of 2,3-oxidosqualene, a common intermediate of both phytosterol and triterpene biosyntheses (Abe *et al.*, 1993; Haralampidis *et al.*, 2001). The later enzymes of triterpene saponin biosynthesis are primarily cytochrome P450s and glycosyltransferases. Glucosyltransferase activity increase accorded to saponin increase in licorice cell cultures (Hayashi *et al.*, 2003). There are the facts that cytochrome P450s and glucosyltransferases play an important role on regulating triterpene saponin biosynthesis, but their functions remain to be characterized at the molecular level.

Centella asiatica accumulates large amounts of triterpene saponin, such as centellasaponin, asiaticoside, madecassoside and sceffoleoside (Kartnig & Hoffmann-Bohm, 1992; Matsuda *et al.*, 2001). The effect of methyl jasmonate (MJ) and thidiazuron (TDZ) on asiaticoside production was investigated from whole plant cultures of *C. asiatica*. It has been demonstrated that treatments with MJ and TDZ as a factor for enhancement of asiaticoside increased asiaticoside production (Kim *et al.*, 2004a; Kim *et al.*, 2004b). Recently several key enzymes that synthesize the triterpenes have been cloned and characterized from *C. asiatica* (Kim *et al.*, 2005a, Kim *et al.*, 2005b, Kim *et al.*, 2005c).

Therefore, we have chosen *C. asiatica* as a suitable species for a regulation approach to triterpene saponin biosynthesis in view of a critical intermediate (*bAS*) because of the enhancement of saponins by the up-regulation of *bAS*, which has been reported in other plants. In this paper, we identify the effect of two candidates, MJ and TDZ, on the accumulation of *bAS* mRNA in leaves of cultured whole plants.

MATERIAL AND METHODS

Plant Materials and Culture Condition

Seeds of C. asiatica from Jeju island of Korea were sterilized with 3% sodium hypochlorite solution containing 0.1% Tween 20 for 10 min, and then rinsed twice with sterile distilled water. Whole plant cultures of C. asiatica were maintained in a 250-㎖ Erlenmeyer flask containing 50 ㎖ of liquid

† Corresponding author: (Phone) +82-62-530-3392 (E-mail) bhwang@chonnam.ac.kr
Received July 20, 2005 / Accepted October 20, 2005

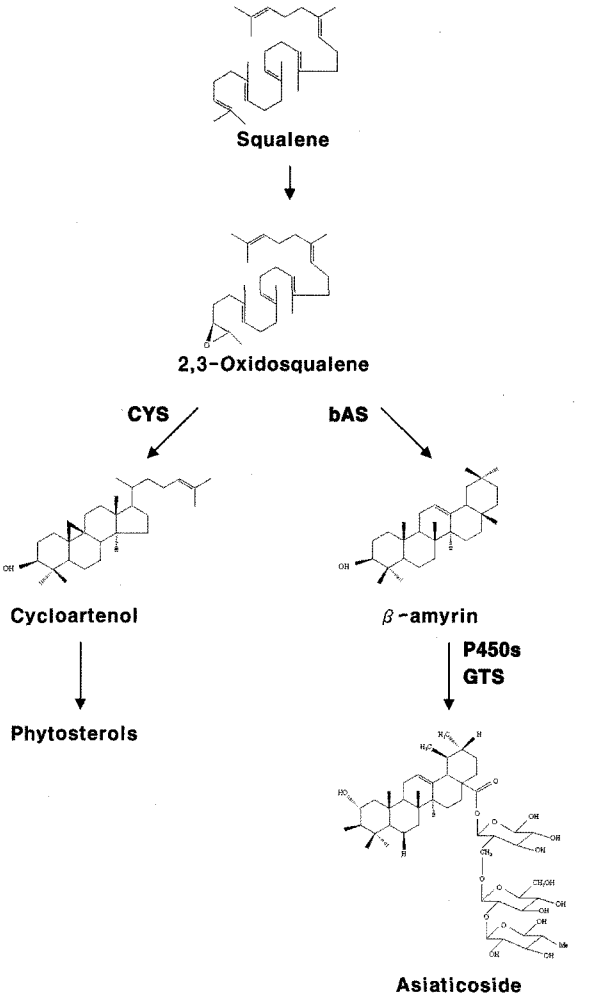

Fig. 1. Asiaticoside biosynthesis pathway. CYS, cycloartenol synthase; *bAS*, β-amyrin synthase; P450s, cytochrome P450s; GTS, glucosyltransferases.

B5 medium (Gamborg *et al.*, 1968) supplemented with 3% sucrose in the light at 25°C. Subculturing was carried out by inoculation of one node removed shoot and root of C. asiatica, obtained by cutting 6 weeks old whole plants as described by Kim *et al.* (2004a).

Addition of TDZ and MJ

MJ (TCI, Tokyo, Japan) was prepared as a stock solution in ethanol and filter-sterilized. After of 5 weeks of precultivation period, whole plants were collected from flasks and aseptically transferred to B5 liquid medium (50 mℓ) supplemented with TDZ, MJ alone or MJ plus TDZ, which were added in suitable amounts. Cultures were harvested at 1, 3, 5, 7, and 14 days after elicitation. TDZ (Sigma, St. Louis, Mo) was dissolved in dimethyl sulfoxide (DMSO) and was added to the medium before autoclaving. DMSO in the same amount was added to culture medium without TDZ to insure synchronous conditions. At the end of a culture period, whole plants from flasks were collected and the biomass was freeze-dried and the dry weight determined. The contents of asiaticoside from leaves were determined. For molecular work, cultured tissues were collected by filtration, frozen with liquid nitrogen and stored at −80°C.

Northern blot analysis

Northern blot analysis was performed with Sambrook's methods (1989). Small scale total RNA was isolated from leaves of *C. asiatica* using Trizol reagent according to the procedures of Invitrogen (Carlsbad, Calif.). Aliquotes of the RNA preparations (30 μg per lane) were fractionated by electrophoresis on 1.5% agarose gels containing formaldehyde and then blotting fragments onto nylon membrane (Bio-rad, Hercules, Calif.). This was followed by hybridization with cDNA probe that had been radiolabelled with α-[^{32}P]-dCTP by random primer kit (Roche, Indianapolis, Ind.). Probes for Northern analysis were generated as follows. The probe for *CabAS* (*C. asiatica* putative β-amyrin synthase, GenBank accession No. AY520818) was PCR-amplified from cDNA using the forward and reverse primers 5'-GATGGAGGATGGGGATTC-TAT-3' and 5'-AGAGACAACCCAA-CCCTGATCTTG3', respectively. The membrane was wash for 15 min at room temperature with 2X SSC/0.1% SDS and then for 15 min at room temperature with 0.5X SSC/0.1% SDS, followed by wash for 15 min at 43 ℃ with 0.1X SSC/0.1% SDS. They were then autoradiographed with an intensifying screen at −80℃ for 5 days.

HPLC analysis of asiaticoside

Extraction of asiaticoside from leaves on whole plants was carried out as described by Kim *et al.* (2004b). Quantitative determinations of asiaticoside were accomplished by HPLC. Asiaticosides were quantified after separation on a Waters C_{18} μ-Bondapak column using a 60 : 40 methanol-H_2O mixture mobile phase at a flow rate of 0.8 mℓ min^{-1} and monitored at 214 nm.

RESULTS AND DISCUSSION

Effect of TDZ and MJ on Whole Plant Growth

To investigate the effect of chemicals on the growth of whole plants, 5-weeks precultivated whole plants were treated by adding chemicals during 7 days. As shown in Fig. 2, TDZ was the most effective for enhancing growth in whole plants, however, the growth of whole plants treated with 0.1 mM MJ was found to decrease significantly. Because MJ stimulates ethylene biosynthesis and leaves senescence (Satler & Thi-

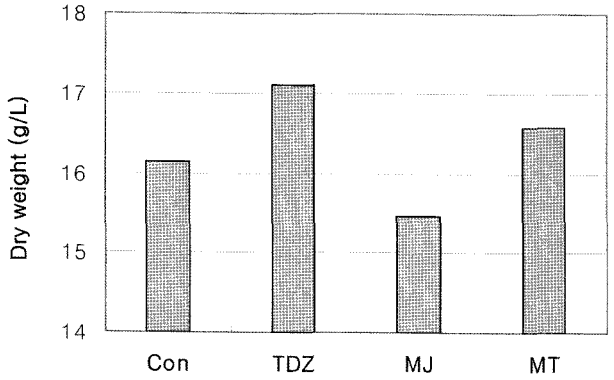

Fig. 2. The effect of chemicals on growth of *Centella asiatica* whole plants treated with 0.025 ㎎/ℓ TDZ (TDZ), 0.1 mM MJ (MJ), 0.1 mM MJ with 0.025 ㎎/ℓ TDZ (MT), or control without chemicals (Con) 7 days after treatments.

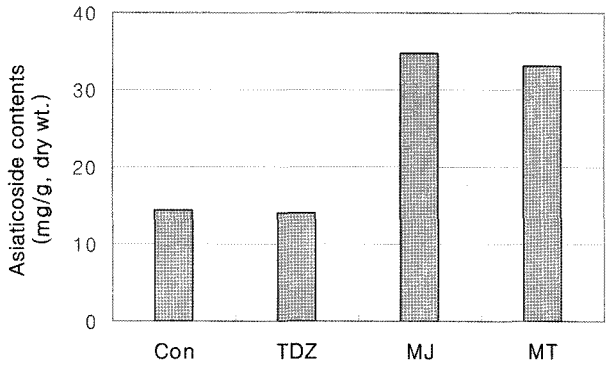

Fig. 3. The effect of chemicals on asiaticoside contents from *Centella asiatica* leaves of whole plant cultures treated with 0.025 ㎎/ℓ TDZ (TDZ), 0.1 mM MJ (MJ), 0.1 mM MJ with 0.025 ㎎/ℓ TDZ (MT), or control without chemicals (Con).

mann, 1981; Saniewski *et al.*, 1987). The similar results for the detrimental effect of MJ on the growth rate have been reported (Szabo *et al.*, 1999; Zhao *et al.*, 2001; Singh *et al.*, 1998). The growth of whole plants treated with 0.1 mM MJ plus 0.025 ㎎/ℓ TDZ was better than that treated with MJ alone. Wedhase *et al.* (1987) found that exogenous cytokinins countered the degradation chlorophyll induced by MJ. Therefore, our results suggest that TDZ prevented the negative effects of MJ on whole plant growth.

Effect of TDZ and MJ on Asiaticoside Production

Fig. 3 shows the effect of chemicals on asiaticoside content in leaves of *C. asiatica* whole plants. When MJ alone was added to culture medium, asiaticoside contents in leaves were higher than that of control after 7 days of treatments. However, asiaticoside contents were not significantly changed by adding TDZ to medium including MJ comparing to MJ treatment alone. Any change of asiaticoside contents was not observed in leaves of whole plants treated with TDZ alone. We have now determined conditions for rapid induction of triterpene biosynthesis in the whole plant cultures following exposure to MJ but TDZ did not affect it. Jasmonates are important stress signaling molecules known to be good elicitors for a wide range of secondary metabolites such as a polyamines, coumaryl conjugates, anthraquinones, naphthoquinones, polysaccharides, terpenoids, alkaloids, and phenylpropanoid from different plant origins (Memelink *et al.*, 2001). Our results also showed that maximum asiaticoside content was achieved following treatment with 0.1 mM MJ alone (43 ㎎/g, dry wt.)

Up-regulation of bAS mRNA

Recently, the enhancement of triterpene saponin production correlates with the increased level of *bAS* mRNA in various

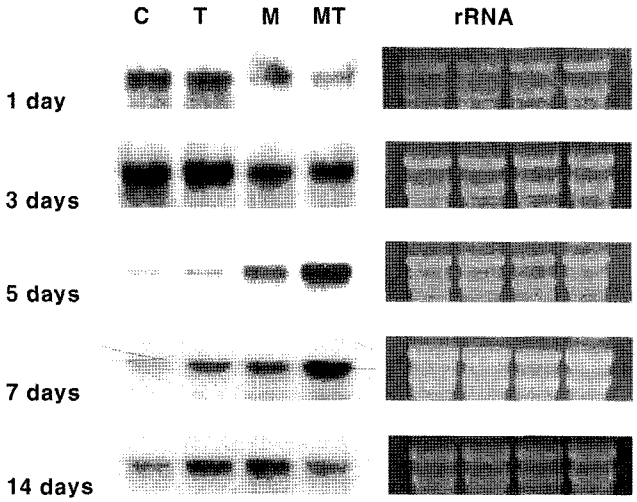

Fig. 4. Effect of MJ and MJ plus TDZ on the expression of *bAS* mRNA in the leaves of cultured whole plants from *C. asiatica*. 5-week-old whole plants were treated with the control without chemical (C), TDZ (T), MJ (M), or MJ plus TDZ (MT). After 1, 3, 5, 7 or 14 days of treatment, total RNA was isolated from the leaves, resolved by agarose gel electrophoresis, blotted, and probed with putative *bAS* associated with asiaticoside biosynthesis.

plants, *Glycyrrhiza glabra* (Hayashi *et al.*, 2003), *Medicago trucatula* (Suzuki *et al.*, 2002), and *Centella asiatica* (Kim *et al.*, 2005b). In the present study, in order to identify the effect of TDZ or MJ, TDZ plus MJ treatments on the level of *bAS* mRNA, total RNA was isolated from leaves of whole plants. As shown in Fig. 4, the levels of *bAS* transcripts were found to be increased in leaves treated with TDZ or MJ treatment, and the high level of that mRNA was maintained for 2 weeks after TDZ or MJ treatment. The result showed that the enhancement of *bAS* mRNA by MJ was associated with asiaticoside produc-

tion, but asiaticoside biosynthesis was not affected by TDZ treatment despite of increasing the level of *bAS* mRNA.

When both TDZ and MJ were added to the medium, the level of *bAS* mRNA in leaves was greater than that by TDZ or MJ alone 1 week after treatments. But transcript level of *bAS* was returned to the level of that mRNA of control without TDZ or MJ treatment after 2 weeks. The maximum level of *bAS* mRNA in leaves of whole plant treated with TDZ and MJ was transiently observed after exposure at 5 days. These results showed the up-regulation of *bAS* gene by adding TDZ and MJ at the molecular level, however, synergic effects of TDZ and MJ on asiaticoside biosynthesis were not testified. The biosynthetic flux by phytohormone may be affected by certain factors related with other biosynthetic pathways, although up-regulation of saponins production by MJ was demonstrated. Finally, MJ and TDZ are new candidates in the regulation of triterpene saponins biosynthesis in *C. asiatica* whole plant cultures.

ACKNOWLEDGEMENT

This work was supported by a Korea Industrial Technology Foundation Grant (2004).

LITERATURE CITED

Abe I, Rohmer M, Prestwich GD (1993) Enzymatic cyclization of squalene and oxidosqualene to sterols and triterpenes. Chem. Rev. 93:2189-2206.

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension culture of soybean root cells. Exp. Cell Res. 50:195-202.

Haralampidis K, Bryan G, Qi X, Papadopoulou K, Bakht S, Melton R, Osbourn A (2001) A new class of oxidosqualene cyclases directs synthesis of antimicrobial phytoprotectants in monocots. Proc. Natl. Acad. Sci. USA 98:13431-13436.

Hayashi H, Huang PY, Inoue K (2003) Up-regulation of soyasaponin biosynthesis by methyl jasmonate in cultured cells of *Glycyrrhiza glabra*. Plant Cell Physiol. 44:404-411.

Kartnig T, Hoffmann-Bohm K (1992) Centella. In: Hansel R, Keller K, Rimpler H, Schneider (eds) Hager's Handbuch der Pharmazeutischen Praxis. Band 4. Springer Verlag, Berlin

Kim OT, Kim MY, Huh SM, Ahn JC, Seong NS, Hwang B (2004a) Effect of growth regulators on asiaticoside production in whole plant cultures of *Centella asiatica* (L.) Urban. J. Plant Biol. 47:361-365.

Kim OT, Kim MY, Hong MH, Ahn JC, Hwang B (2004b) Stimulation of asiaticoside production from *Centella asiatica* (L.) Urban whole plant cultures by elicitors. Plant Cell Rep. 23:339-344.

Kim OT, Ahn JC, Hwang SJ, Hwang B (2005a) Isolation and characterization of squalene synthase cDNA from *Centella asiatica* (L.) Urban. J. Plant Biol. (In press).

Kim OT, Kim MY, Huh SM, Bai DG, Ahn JC, Hwang B (2005b) Cloning of a cDNA probably encoding oxidosqualene cyclase associated with asiaticoside biosynthesis from *Centella asiatica* (L.) Urban. Plant Cell Rep. 24:304-311.

Kim OT, Kim MY, Ahn JC, Hwang SJ, Hwang B (2005c) Cloning and molecular analysis of cDNA encoding cycloartenol synthase from *Centella asiatica* (L.) Urban. Biotechnol. Bioprocess Eng. 10:16-22.

Matsuda H, Morikawa T, Ueda H, Yoshikawa M (2001) Medicinal Foodstuffs. XXVII. Saponin constituents Gotu Kola (2): Structures of new ursane- and oleanane-type triterpene oligoglycosides, centellasaponin B, C, and D, from *Centella asiatica* cultivated in Sri Lanka. Chem. Pharm. Bull. 49: 1368-1371.

Memelink J, Verpoorte R, Kijne JW (2001) Organization of jasmonte-responsive gene expression in alkaloid metabolism. Trends Plant Sci. 6:212-219.

Papadopoulou K, Melton RE, Leggett M, Daniels MJ, Osbourn AE (1999) Compromised disease resistance in saponin-deficient plants. Proc. Natl. Acad. Sci. USA 96: 12923-12928.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold spring Harbor Laboratory Press, Plainview, N.Y.

Saniewski M, Nowacki J, Czapski J (1987) The effect of methyl jasmonate on ethylene production and ethylene-forming enzyme activity in tomatoes. J. Plant Physiol. 129:199-203.

Satler SO, Thimann KV (1981) Le jasmonate de methyle: Nouveau et puissant promoteur de la senescence des feuilles. C R Acad. Sci. Series 293:735-740.

Singh G, Gavrieli J, Oakey, Curtis WR (1998) Interaction of methyl jasmonate, wounding and fungal elicitation during sesquiterpene induction in *Hyoscyamus muticus*in root cultures. Plant Cell Rep. 17:391-395.

Suzuki H, Achnine L, Xu R, Matsuda SPT, Dixon RA (2002) A genomic approach to the early stages of triterpene saponin biosynthesis in *Medicago truncatula*. The Plant J. 32:1033-1048.

Szabo E, Thelen A, Petersen M (1999) Fungal elicitor perparations and methyl jasmonate enhance rosmarinic acid accumulation in suspension cultures of *Coleus blumei*. Plant Cell Rep. 18:485-489.

Weidhase RA, Lehmann J, Kramell H, Sembder G, Parthier B (1987) Degradation of ribulose-1,5-biphosphate carboxylase and chlorophyll in senescing barley leaf segments tirggered by jasmonic acid methyl ester and counteraction by cytokinin. Physiol. Plant 69:161-166.

Zhao J, Fujita K, Yamada J, Sakai K (2001) Improved β-thujaplicin production in *Cupressus lusitanica* suspension cultures by fungal elicitor and methyl jasmonate. Appl. Microbiol. Biotechnol. 55:301-305.