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Effects of Precursor and Thidiazuron on Triterpene Saponin Biosynthesis in *Centella asiatica* (L.) Urban

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ABSTRACT : Plants have been known to accumulate a very diverse range of triterpene saponins. We have investigated the regulation of saponin biosynthesis in higher plants using *Centella asiatica* (L.) Urban as a model plant. Effects of a feeding precursor on asiaticoside production from leaves and on the level of two-type OSCs mRNA were investigated. As a feeding precursor, squalene negatively affected the levels of CYS and bAS mRNA, but it also decreased the production of asiaticoside from whole plants. Plant hormones regulate secondary metabolism, and in plant tissue cultures they could affect both culture growth and secondary metabolite production. Although enhancement of asiaticoside production from whole plant cultures by addition of TDZ (thidiazuron) has been reported, the positive effect of TDZ on the levels of OSCs transcripts was not observed.

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

INTRODUCTION

Plants have been known to accumulate a very diverse range of triterpene saponins. The natural role of triterpene saponins in plants is likely to be in defense against attack by rooting-infecting fungus (Osbourn *et al.*, 1994; Bowyer *et al.*, 1995). These molecules are also economically important as drugs, detergents, sweetenersand cosmetics (Hostettmann & Marston, 1995). Although structural elucidation of triterpene saponins has been externsively studied, our understanding about the regulation of their biosyntheses is quite limited at the molecular level.

In the biosynthesis of triterpene saponins, a little is known about enzymes responsible for the production of the different intermediates. Two molecules of farnesyl diphosphate (FPP) are combined to form squalene to which as epoxide group is added to produce 2,3-oxidosqualene. One of the important branchpoints of triterpens and sterols biosynthesis, between primary and secondary metabolism, is the cyclization of 2,3-oxidosqualene (Fig. 1). Triterpenes and sterols are synthesized from the isoprenoid pathway by 2,3-oxidosqualene cyclaes (OSCs) (Abe *et al.*, 1993). Recently several key enzymes that synthesize the triterpenes have been cloned and characterized from *Centella asiatica* (Kim *et al.*, 2005a, 5b, 2005c). The leaves of *C. asiatica* contain large amount of triterpene saponin, such as centellasaponin, asiaticoside, madecassoside and sceffoleoside (Kartnig & Hoffmann-Bohm, 1992; Matsuda *et al.*, 2001). Therefore, *C. asiatica* could be a candidate involved in the understanding of triterpene saponin biosynthesis. To understand genes function may enable a molecular approach for improvement of triterpene saponin production from plants.

The effects of precursors and plant growth regulators on secondary metabolites of plants have been reported. Effects of thidiazuron (TDZ) on asiaticoside production from whole plant of *C. asiatica* were investigated (Kim *et al.*, 2004a), but the application of squalene as a precursor for activation of the triterpene biosynthesis has not been found.

In this paper, the effect of a feeding precursor on triterpene saponins production from *C. asiatica* and on the level of two-

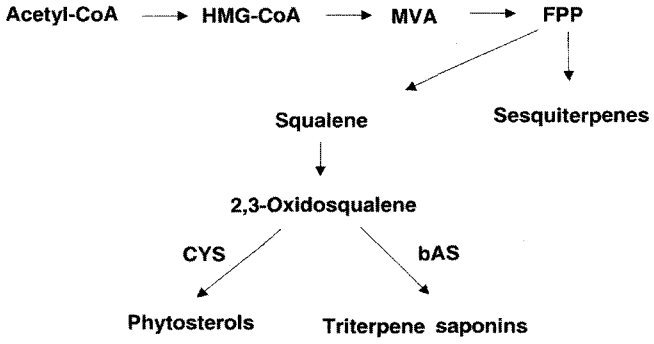

Fig. 1. Triterpenoid pathway. HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonic acid; FPP, farnesyl diphosphate; CYS, cycloartenol synthase; bAS, β-amyrin synthase.

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type OSCs mRNA will be discussed. In addition, the effect of TDZ addition on the key enzymes involved in the tritepene saponin biosynthesis will be discribed.

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 mℓ Erlenmeyer flask containing 50 mℓ of liquid B5 medium (Gamborg *et al.*, 1968) supplemented with 3% sucrose in the light at 25°C Subculture 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 Squalene

Culture media were prepared with B5 medium supplemented with 3% sucrose (w/v) and appropriate amount of TDZ (thidiazuron, 1-phenyl-3-(1,2,3-thidiazol-5-yl)urea) or squalene. TDZ and squalene were purchased from Sigma-Aldrich (St Louis, Mo.). TDZ was added to the medium and pH was adjusted to 5.7 before autoclaving. Squalene stock solution was prepared in ethanol and filter-sterilized. For TDZ treatment, nodes as inoculum were used. For squalene treatment, cultured whole plants during 5 weeks were used as inoculum. The serial concentrations of TDZ (0, 0.005, 0.01, 0.025, 0.05, and 0.1 mg/ℓ) and squalene (0.1, 1, and 10 mM) were prepared in order to investigate their effects on the levels of OSC mRNA. After respective treatments, cultures were harvested to determine asiaticoside contents. For molecular work, cultured tissues were collected by filtration, and then were frozen with liquid nitrogen. The cultured tissues were stored at -80°C until used.

Northern blot analysis

Northern blot analysis was performed with Sambrook's methods (1989). Small scale total RNA was isolated from the 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. The blotting fragments were transferred onto nylon membrane (Bio-rad, Hercules, Calif.). This was followed by hybridization with cDNA probe that had been radiolabelled with a α-[^{32}P]-dCTP by a random primer kit (Roche, Indianapolis, Ind.). Probes for Northern analysis were generated as follows. The probe for *CaCYS* (*C. asiatica* cycloartenol synthse, GenBank accession No. AY520819) was PCR-amplified from cDNA using the forward and reverse primers 5'-GATGGTGGGT-GGGGTTTACAC-3' and 5'-TCCTTCGGCTGTACAATCTGA-3', respectively. The probe for *CabAS* (*C. asiatica* β-amyrin synthse, GenBank accession No. AY520818) was PCR-amplified from cDNA using the forward and reverse primers 5'-GATGGAGGATGGGGATTCTAT-3' and 5'-AGAGACAAC-CCAACCCTGATCTTG-3', respectively. The membrane was wash for 15 min at room temperature with 2×SSC/0.1% SDS and then for 15 min at room temperature with 0.5×SSC/0.1% SDS, followed by wash for 15 min at 43°C with 0.1×SSC/0.1% SDS. They were then autoradiographed with an intensifying screen at -80°C 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 (Waters C_{18} μ-Bondapak column, 60:40 methanol-H_2O mixture, 0.8°C min^{-1}, UV 214 nm).

RESULTS AND DISCUSSION

Effect of feeding a precursor on asiaticoside biosynthesis

Cycloartenol synthase (CYS), as a key enzyme for the regulation of sterols biosynthesis, has been reported through gene cloning (Corey *et al.*, 1993; Bach & Benveniste, 1997; Kim *et al.*, 2005c). Asiaticoside in *C. asiatica* shares a common biosynthetic intermediate with β-amyrin, which is synthesized by β-amyrin synthase (bAS), an OSC (Kim *et al.*, 2005b).

We were interested in the regulation of triterpenoid biosynthetic pathways after feeding of squalene which has been known as a precursor of oxidosqualene as branching point that lead to the formation of different types of triterpenoid. Enhancement of secondary metabolismby feeding precursors in cell suspension cultures has been reported (Contin *et al.*, 1999; Boitel-Conti *et al.*, 2000; El-Sayed & Verpoorte, 2002).

In order to investigate the effect of a feeding precursor on the OSCs mRNA levels, northern blot analysis was conducted with respective probes for bAS and CYS. Different concentrations of squalene were added to culture medium. Total RNA was extracted from leaves of cultured whole plants treated with squalene for 7 days. As shown in Fig. 2, the levels of bAS mRNA were decreased. Feeding of a precursor did not change CYS transcript levels. When 10 mM squalene was added to medium, the bAS transcripts were not induced. These results indicated that squalene, as a feeding precursor, have a negative effect on the levels of bAS mRNA, and also on a

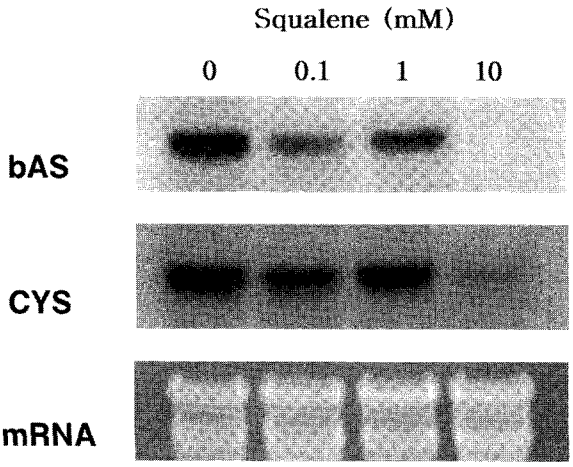

Fig. 2. Expression of bAS and CYS mRNA in the leaves of whole plant cultures after squalene treatment as a feeding precursor. Squalene was added to the culture medium and then plants were harvested after 7 days of treatment. Total RNA was isolated from leavesshown, resolved by agarose gel electrophoresis, blotted, and probed with bAS (β-amyrin synthase) or CYS (cycloartenol synthase).

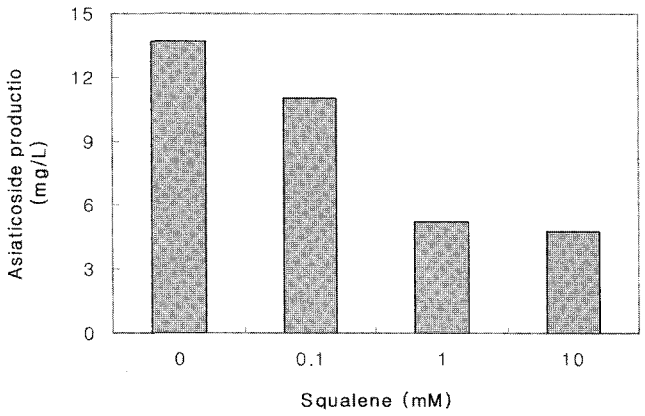

Fig. 3. The effect of squalene concentration on asiaticoside production from *C. asiatica* whole plant.

decrease of asiaticoside production from whole plants (Fig. 3). Thus, the feeding of aprecursor could have a negative effect on the flux into the triterpene saponins biosynthesis. This might be explained by the inhibition of related enzymes activity by feedback.

Effects of TDZ concentration on the levels of OSCs mRNA

Plant hormones regulate secondary metabolism, and in plant tissue cultures they affect both culture growth and secondary metabolite production. The effects of cytokinins depend on the products. For example, synthesis of anthocyanin is increased by kinetin in *Haplopappus gracilis*, but it is inhibited by BA in *Daucus carota* (Endress, 1994).

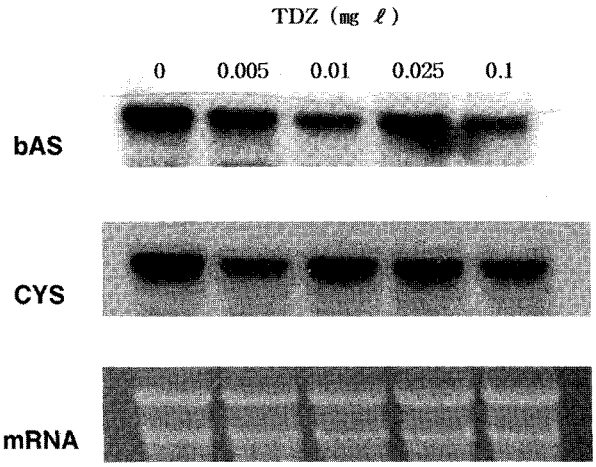

Fig. 4. Effect of TDZ concentrations on expression of bAS and CYS in leaves of *C. asiatica*. After 5 weeks of cultivation on liquid B5 medium supplemented with different concentrations of TDZ, total RNA was isolated from the tissues shown, resolved by agarose gel electrophoresis, blotted, and probed with bAS (β-amyrin synthase) or CYS (cycloartenol synthase).

Kim *et al.* (2004a) showed that TDZ as cytokinin affected asiaticoside production from whole plant cultures. Inorder to elucidate relationship between TDZ as cytokinin and OSCs transcripts on the mRNA level, total RNAs were extracted from leaves of whole plants treated with different concentration. Northern hybridization was performed with 1.0 kb probe for bAS and CYS, cloned from *C. asiatica*. Fig. 4 shows down-regulation of bAS mRNA at all concentrations of TDZ. In case of CYS mRNA, the positive effect of TDZ on the levels of transcription was not observed. Enhancement of asiaticoside production from whole plant cultures by addition of TDZ has been reported (Kim *et al.*, 2004a), but we did not testify to the effect of TDZ on the levels of transcripts of bAS and CYS. Therefore, this experiment is necessary to clarify the effect of TDZ on other genes related to triterpene biosynthesis pathway.

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