Cloning and Molecular Analysis of cDNA Encoding Cycloartenol Synthase from *Centella asiatica* (L.) Urban

Ok-Tae Kim[1], Min-Young Kim[1], Sung-Jin Hwang[3], Jun-Cheul Ahn[4], and Baik Hwang[1,2]

[1] Department of Biology, Chonnam National University, Gwangju 500-757, Korea
[2] Institute of Plant Resources, Chonnam National University, Gwangju 500-757, Korea
[3] Department of Food & Biotechnology, Dongshin University, Naju 520-714, Korea
[4] Department of Life Sciences, Seonam University, Namwon 590-170, Korea

Abstract cDNA for oxidosqualene cyclase was cloned by a homology-based PCR method and sequenced from *Centella asiatica*. In a sequences analysis, the putative polypeptide of *C. asiatica* cycloartenol synthase (*CaCYS*) deduced from the 2,274 bp nucleotide sequence, consisted of 758 amino acids and had a molecular mass of 86.3 kD. The predicted amino acid sequence exhibited high homology to that of PNX (cycloartenol synthase) from *Panax ginseng* (89%). Southern blot analysis suggests that *CaCYS* may be present in one copy of the *C. asiatica* genome. If methyl jasmonate (MJ) is applied exogenously to plants, not only triterpene saponins are accumulated in tissues, but also it produces effects such as growth inhibition and the promotion of ethylene production. In order to investigate the effect of MJ and thidiazuron (TDZ), a cytokinin that plays a role as an antisenescence agent in several plants, on the level of *CaCYS* mRNA, we performed northern blot analysis. When MJ is alone treated by adding to culture medium, *CaCYS* transcripts were inhibited. However, sustained levels of the expression of *CaCYS*, by adding TDZ to the medium despite MJ treatments, were demonstrated in *C. asiatica* leaves.

Keywords: *Centella asiatica*, oxidosqualene cyclase, triterpenes saponins, phytosterols

INTRODUCTION

Oxidosqualene cyclase (OSC) catalyzes the cyclization of 2,3-oxidosqualene, a common precursor of sterols and triterpenoids, and it situates at the branching point for the sterol and triterpenoid pathway [1]. A plant produces cycloartenol as a precursor of phytosterols. In addition to cycloartenol synthase, higher plants contain other OSCs that convert oxidosqualene into a vast family of pentacyclic triterpenes such as lupeol, and α- and β-amyrins (Fig. 1). Phytosterols are known to play at least two critical roles in a plant: as membrane constituents, and as precursors for hormone biosynthesis in higher plants [2]. Recent progress in understanding the biosynthesis of phytosterols has been achieved by cloning the genes that encode cycloartenol synthase in *Arabidopsis thaliana* [3], *Glycyrrhiza glabra* [4], and *Costus speciosus* [5].

Centella asiatica (L.) Urban, a member of the *Umbelliferae* family, has been used for the treatment of leprosy, varicose veins, ulcers, lupus, and certain eczemas [6]. *C. asiatica* contains triterpene glycosides (otherwise known as triterpenoid saponins) such as centellasaponin, asiaticoside, madecassoside, and sceffoleoside, and triterpenic steroids such as stigmasterol and sitosterol [7,8]. We used whole plant cultures of *C. asiatica* as a model system to investigate the regulation of phytosterol and triterpenoid biosyntheses in higher plants. In addition to phytosterols, we were interested in increasing the accumula-

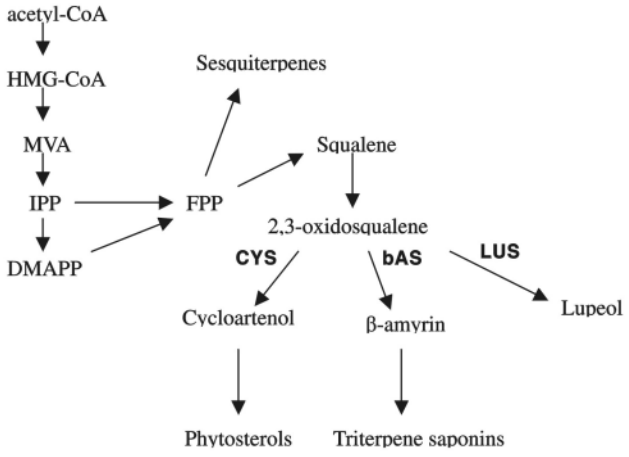

Fig. 1. Biosynthetic pathways of triterpenes in *C. asiatica*. DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-conenzyme A; IPP, isopentenyl pyrophosphate; MAV, mevalonate; CYS, cycloartenol synthase; bAS, β-amyrin synthase; LUS, lupeol synthase.

***Corresponding author**
Tel: +82-62-530-3392 Fax: +82-62-530-3409
e-mail: bhwang@chonnam.ac.kr

tion of saponins in *C. asiatica* by genetic manipulation of the enzymes because of the economical value of saponins. Since both phytosterols and tirterpene saponins share the common biosynthetic intermediate, 2,3-oxidosquelene, which is synthesized by OSCs, we decided to isolate the gene that encodes cycloartenol synthase in *C. asiatica* as a first step in understanding phytosterols and the triterpene saponin pathway.

Several papers of triterpene saponins production from plant cell or tissue cultures have been reported [9,10]. Elicitors have been found to induce triterpene saponin accumulation in plants. Among elicitors, it has been reported that exogenously applied methyl jasmonate (MJ) induces the biosynthesis of many secondary metabolites [11], including terpenoids [12-14]. However, if MJ is applied exogenously to plants, it produces effects such as growth inhibition, the induction of leaf senescence [15, 16], and the promotion of ethylene production [17]. Therefore, we estimated the effect of MJ and TDZ (thidiazuron, 1-phenyl-3-(1,2,3-thidiazol-5-yl)urea), a cytokinin that plays a role as an antisenescence agent in several plants, on cycloartenol synthase mRNA in whole plant cultures of *C. asiatica*.

This paper reports the isolation of the cDNA of oxidosqualene cyclase, which encodes cycloartenol synthase from *C. asiatica*. Although this study was not able to elucidate the functional expression of this gene in a yeast mutant (*erg*7), the deduced amino acid sequence from this clone showed that OSC encodes cycloartenol synthase. This was confirmed by analysis of the constructed phylogenetic tree. A Northern blot analysis showed the effect of MJ on the levels of *CaCYS* mRNA, which is a key enzyme for the regulation of phytosterol biosynthesis.

MATERIALS AND METHODS

Plant Materials and Culture Condition

Whole plant cultures of *C. asiatica* were maintained in a 250-mL Erlenmeyer flask containing 50 mL of liquid B5 medium [18] supplemented with 3% sucrose in the light at 25°C, and subcultured at intervals of 6 weeks. For the experiments, three of the nodes were cultured in a 250-mL Erlenmeyer flask containing 50 mL of the B5 standard medium on a rotary shaker at 100 rpm. After a precultivation period of 5 weeks, TDZ, and MJ alone or MJ plus TDZ were treated by addition of those to whole plant cultures as described by Kim *et al.* [19]. Cultures were harvested at 1, 3, 5, 7 and 14 days after elicitation. The leaves of the cultured whole plants were collected by filtration, frozen with liquid nitrogen, and stored at -80°C.

RNA Extraction and cDNA Synthesis

After 6 weeks of cultivation in a flask, total RNA was extracted from leaves of *C. asiatica* using Trizol reagent according to the procedures of Invitrogen (Carlsbad, CA, USA). mRNA was purified by the Poly(A)$^+$ RNA purification kit (Qiagen, Hilden, Germany). mRNA was reverse transcribed using an oligo(dT) primer (RACE 32, 5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTT-3') as described in the literature [20,21], and AMV reverse transcriptase (Invitrogen) with dNTP at 37°C according to the manufacturer's protocol.

Cloning of Cycloartenol Synthase

Our cloning strategy was based on a reverse transcription polymerase chain reaction (RT-PCR) using degenerate primers designed from the highly conserved regions of the known OSCs. The nucleotide sequences of these primers were as follows: coreS = 5'-CCIATGWSITAYYT ITAYGGIAAR-3' (PMSYLYGK), and coreA=5'-CCCAISW ICCITMCCAISWICCRTC-3' (DGSW(Y/E)G(C/S) W(G/A)). Firstly, PCR was carried out with coreS and coreA primers using Ex-Taq DNA polymerase (TaKaRa Kyoto, Japan) with dNTP in a final volume of 100 μL according to the manufacturer's protocol. PCR was carried out for 30 cycles using the GeneAmp PCR system 2700 (Tropix, Applied Biosystems, Foster City, CA, USA) with a program (94°C, 1 min, 42°C, 2 min, 72°C, 3 min, and a final extension at 72°C, 10 min). Secondly, PCR was carried out again with the same primers as with the first PCR product (5 μL) as a template under the same conditions as the first PCR, and given a 1,000-bp fragment. The PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into DH5α *Escherichia coli* competent cells. Plasmid DNA was prepared and sequenced by the Automatic Genetic Analyzer 3100 (Applied Biosystems).

Rapid Amplification of cDNA Ends PCR

Both a 5' rapid amplification of the cDNA ends (RACE) and 3'RACE PCR were performed with gene-specific primers designed against the partial sequences obtained through RT-PCR. The primer sequences were as follows: 5'RACE 5'-GGTGCGAAGAGCTTTCTCTCTCA A-3' and the nested primer 5'-GGTGCGAAGAGCTTTCTCTCTC AA-3'; 3'RACE 5'-TCCACTGCTGATCAT-GGATGGCC C-3' and the nested primer 5'-GCAGGGGAAAAGGCAG ATGTTGAGCGA-3'. PCR was carried out for 30 cycles with a program (94°C, 1 min, 58°C, 1 min, 72°C, 3 min, and a final extension at 72°C, 10 min). Firstly, products were amplified with the RACE32 primer and filtered products were used as a template for the second PCR with a nested and anchor primer (5'-GACTCGAGTCGA CATCGA-3'). The full-length cDNA of *CaCYS* was obtained by PCR using the *N*-terminal (5'-TTGAGGTACCA TGTGGAAGCTCAAAGTCGCT-3', *Kpn*I site underlined) and *C*-terminal primer (5'-TTAGCTCGAGTCA TGGCG CCAGAAGTAC ACG-3', *Xho*I site underlined). The obtained full-length cDNA clone, *CaCYS*, was sequenced and this sequence was submitted to EMBL, GenBank and the DDBJ sequence-databases.

Phylogenetic Tree of OSCs

The accession numbers of the sequences in EMBL,

GenBank and the DDBJ sequence-data banks used in this analysis are as follows, β-amyrin synthases: BPY, *Betula platyphylla* (AB055512); GgbAS, *Glycyrrhiza glabra* (AB037203); PNY, *Panax ginseng* (AB009030), MtAMY1, *Medicago truncatula* (AJ430607); and PSY, *Pisum sativum* (AB03802); multifunctional triterpene synthase: PSM, *Pisum sativum* (AB034803); lupeol synthases: LUP1, *Arabidopsis thaliana* (U49919); OEW, *Olea europaea* (AB025343); and TRW, *Taraxacum officinale* (AB025 345); cycloartenol synthases: GgCAS1, *Glycyrrhiza glabra* (AB025968); CAS1, *Arabidopsis thaliana* (U02555); PSX, *Pisum sativum* (D89619); BPX, *Betula platyphylla* (AB05 5510); and PNX, *Panax ginseng* (AB009029). A phylogenetic analysis was carried out with the CLUSTAL W program based on a neighbor-joining method. The distances between each clone and group are as follows: (((((((GgCAS1:0.06004, PSX:0.06419):0.03064, ((CaCYS: 0.06032, PNX:0.05143):0.04679, BPX:0.08463): 0.00966): 0.01482, CAS1:0.11032):0.19192, (OEW:0.11336, TRW: 0.12785):0.09843):0.13694, LUS1:0.22790):0.02692, (((MtAMY1:0.02666, PSY:0.02566):0.03033, GgbAS: 0.04452):0.02102, PSM:0.15673):0.04266):0.02270, BPY: 0.07386, PNY:0.08577). Based on these values, a phylogenic tree was created with the TreeView program [22].

Southern Blot Analysis

Genomic DNA was isolated from leaves of *C. asiatica* using the cetyldimethylethylammonium bromide procedure [23]. Southern and northern blot analyses were performed according to Sambrook *et al*. [24]. The Southern blot method involved digesting 10 μg of leaf genomic DNA with *Eco*RI, *Bam*HI, *Hin*dIII,or *Xba*I, and electrophoresing the digested products on a 0.8% agarose gel (25 V, overnight). This was followed by hybridization with the probe cDNA that had been radiolabelled with α-[^{32}P]-dCTP by a random primer kit (Roche, Indianapolis, Ind, USA). Probes for the Southern and Northern analyses were generated as follows. The probe for *CaCYS* was PCR-amplified from cDNA using the forward and reverse primers 5'- GATGGTGGGTGGGGTTTACAC-3' and 5'-TCCTTCGGCTGTACAATCTGA-3', respectively. The blot was first washed two times with 5% SDS containing 1 mM EDTA and 40 mM Na_2HPO_4 for 15 min at 65°C, followed by two washes (15 min each) with 1% SDS containing 1 mM EDTA and 40 mM Na_2HPO_4 at 65°C.

Northern Blot Analysis

Small scale total RNA was isolated from the tissue of *C. asiatica* using Trizol reagent according to the procedures of Invitrogen. Aliquotes of the RNA preparations (30 μg per lane) were fractionated by electrophoresis on 1.5% agarose gels containing formaldehyde, and then fragments were blotted onto a nylon membrane (Bio-Rad, Hercules, CA, USA). The membrane was washed 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 a wash for 15 min at 43°C with 0.1× SSC/0.1% SDS. The membranes were then autoradiographed with an intensifying screen at -80°C for 5 days.

RESULTS AND DISCUSSION

Cloning of Full-length cDNA of CaCYS

cDNA was prepared from the mRNA of leaves of 6-week-old cultured whole plants. PCRs for cloning were carried out using degenerate primers designed from the highly conserved regions of the known OSCs from *Lotus japonicus* (AF478455), *Panax ginseng* (AB009030), *Arabidopsis thaliana* (U02555), *Costus speciosus* (AB058508) and *Taraxacum officinale* (AB025345). The forward degenerate primer was based on a consensus sequence (PMSYLYGK) and the reverse primer was based on a consensus sequence (DGSW(Y/E)G(C/S)W(G/A)). The resulting amplification product was about 1,000 bp and it was then cloned and sequenced. 20 clones indicated the presence of the characteristic sequences of OSC, and its corresponding full-length cDNA was named CaCYS. Full-length cDNAs were obtained by 5'- and 3'-RACE using specific primers. The open reading frames of CaCYS (GenBank accession no. AY5208179) contained ORFs of 2,274 bp, which codes for 758 amino acids with a predicted molecular mass of 86.3 kD (Fig. 2). CaCYS amino acids are highly identical to the ginseng cycloartenol synthase PNX (98%) [21]. The CaCYS amino acids revealed 84, 79, and 73% identity with cycloartenol synthase *G. glabra* (AB025968), *A. thaliana* (U02555) and *Avena strigosa* (AJ31190), respectively. CaCYS has the amino acid motif DCTAE [25] and four of the QW motifs that are characteristic of the OSC superfamily [26].

Comparison of Amino Acid Sequences of CaCYS

A number of OSCs and their functions have recently been reported. Amino acid sequence comparisons can show the relation among OSCs. In order to clarify the relationship with 14 other OSC sequences from plants, sequence homologies were calculated (Table 1) and a phylogenetic analysis of CaCYS was carried out with a web version of the CLUSTAL W program [27] based on a neighbor-joining method. CaCYS and other cycloartenol synthase clones (GgCAS1, PNX, BPX, and CAS1) show high identities (79~89%), but other OSCs are only identical (53~61%). Amino acid sequence comparisons indicate that the triterpene synthases related to β-amyrin and lupeol, are clearly distinct from cycloartenol synthases. The results of the phylogenetic tree revealed that CaCYS is clustered with the cycloartenol synthases of the other plants (Fig. 3). Also, CaCYS is very closely related to the *P. ginseng* cycloartenol synthase, PNX [21], as expected. To solve the exact function of the *CaCYS* gene, it must be elucidated by an overexpression in a yeast mutant (*erg7*) that lacks lanosterol synthase. This is because, to date, all previous attempts to express OSC in *E. coli* have been unsuccessful [28]. Although we failed to obtain a yeast mutant (*erg7*), further experiments are necessary to clar-

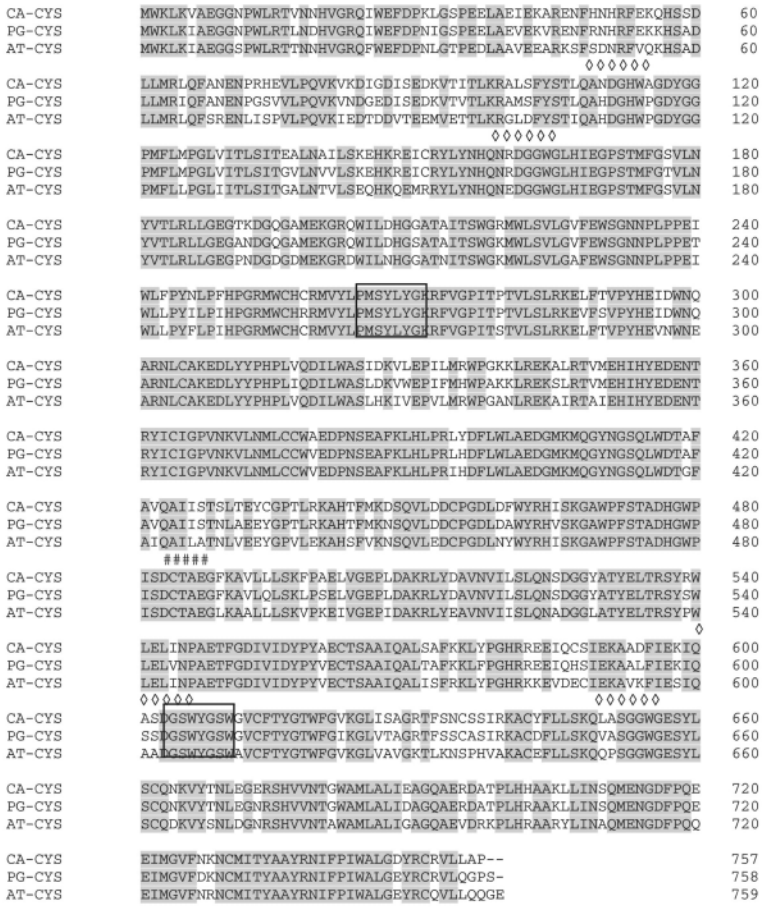

Fig. 2. Alignment of the deduced amino acid sequences of CaCYS from *C. asiatica* with other plants. GeneBank accession numbers: CA-CYS, *C. asiatica* cycloartenol synthase *CaCYS* (AY520819); PG-CYS, *P. ginseng* cycloartenol synthase *PNX* (AB009029); AT-CYS, *A. thaliana* cyclortenol synthase *CAS1* (U02555). Boxes indicate the degenerate primer of the core fragment for PCR. #, DCTAE motif. ◊, QW[QXXXGXW] motif. The shaded colors of the residues correspond to the identity.

Table 1. Amino acid identity between CaCYS from *C. asiatica* and other plant OSCs

Enzyme	GenBank accession No.	Amino acid identity (%)
		CaCYS
BPY, β-amyrin synthase (*B. platyphylla*)	AB055512	58
GgbAS, β-amyrin synthase (*G. glabra*)	AB037203	57
PNY, β-amyrin synthase (*P. ginseng)*	AB009030	59
MtAMY1, β-amyrin synthase (*M. truncatula*)	AJ430607	57
PSY, β-amyrin synthase (*P. sativum*)	AB03802	57
PSM, multifunctional triterpene synthase (*P. sativum*)	AB034803	53
LUP1, lupeol synthase (*A. thaliana*)	U49919	60
OEW, lupeol synthase (*O. europaea*)	AB025343	61
TRW, lupeol synthase (*T. officinale*)	AB025345	60
GgCAS1, cycloartenol synthase (*G. glabra*)	AB025968	84
CAS1, cycloartenol synthase (*A. thaliana*)	U02555	79
PSX, cycloartenol synthase (*P. sativum*)	D89619	82
BPX, cycloartenol synthase (*B. platyphylla*)	AB055510	83
PNX, cycloartenol synthase (*P. ginseng*)	AB009029	89

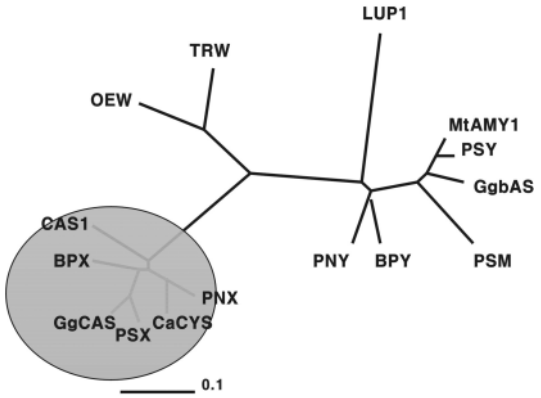

Fig. 3. Phylogenetic tree constructed from the deduced amino-acid sequences of CaCYS from *C. asiatica* and OSCs from other plants. β-amyrin synthases: BPY, *B. platyphylla*; GgbAS, *G. glabra*; PNY, *P. ginseng*, MtAMY1, *M. truncatula*; PSY, *P. sativum*; multifunctional triterpene synthase: PSM, *P. sativum*; lupeol synthases: LUP1, *A. thaliana*; OEW, *O. europaea*; TRW, *T. officinale*; cycloartenol synthases: GgCAS1, *G. glabra*; CAS1, *A. thaliana*; PSX, *P. sativum*; BPX, *B. platyphylla*; PNX, *P. ginseng*. A gray circle indicates a group for cycloartenol synthases.

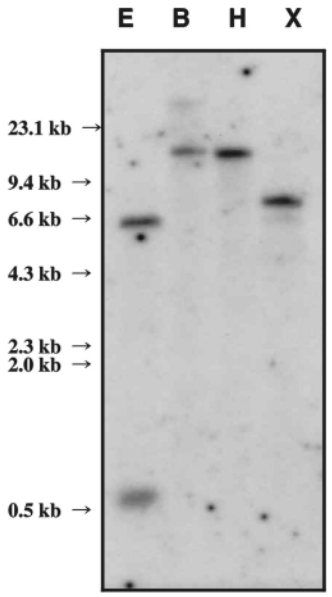

Fig. 4. Southern blot analysis of *CaCYS*. Genomic DNA of *C. asiatica* was digested with the restriction enzymes *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H) or *Xba*I (X) and Southern blotted (10 μg of DNA/track). Blots were hybridized to the appropriate radiolabelled homologous probes under high stringency conditions. The molecular weight marker is indicated on the left.

ify the function of the *CaCYS* gene by an overexpression in a yeast mutant (*erg7*).

Southern Blot Analysis

Hayashi *et al.* has indicated that at least two copies of cycloartenol synthase may exist in the genome of *G. glabra* [4]. To estimate the copy number of the cycloartenol synthase gene in the *C. asiatica* genome, genomic DNA digested with *Eco*RI, *Bam*HI, *Hin*dIII or *Xba*I, was probed with the 1.0 kb cDNA. As shown in Fig. 4, two signals of the cycloartenol synthase gene were observed in the *Eco*RI lane. The two bands observed in the *Eco*RI lane (there is no *Eco*RI site in the cDNA) may be explained by possible existence of an additional homologous gene. Southern blot analysis suggests that *CaCYS* may be present in one copy of the *C. asiatica* genome. Further experiments are required to elucidate whether or not the other homologus gene is cross-hybridized.

Northern Blot Analysis

To investigate the levels of *CaCYS* mRNA in various tissues, total RNAs were extracted from different tissues of a cultured whole plant after 6 weeks of cultivation. The expression of *CaCYS* was analyzed by a Northern blot analysis with a partial 1.0 kb probe. *CaCYS* was expressed in all of the tissues except in the roots, with the highest transcript levels in the leaf. The signal for the transcript of *CaCYS* was not detected in the roots (Fig. 5). The growth of roots *in vitro* was attained at the stationary stage after 6 weeks of cultivation, whereas the growth of shoots including the node continued for 8 weeks (data not shown). Therefore, the levels of *CaCYS* mRNA may reveal a strong signal expression in the shoots. An OSC

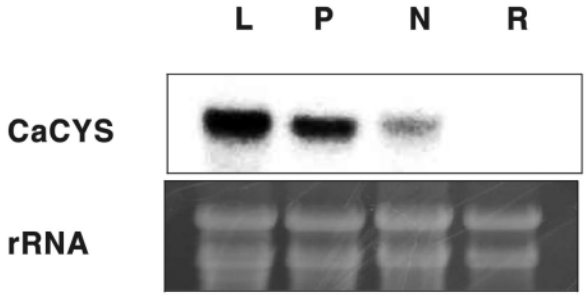

Fig. 5. Expression of mRNA *CaCYS* in various organs from *C. asiatica*. After 6 weeks of cultivation, total RNA was isolated from the tissues shown, resolved by agarose gel electrophoresis, blotted, and probed with *CaCYS*. Relative transcript abundance was calculated by dividing the intensity of each transcript to that of the corresponding rRNA transcript level. L, leaves; P, petiols; N, nodes; R, roots.

encoded cycloartenol synthase is indirectly or directly associated with the cell growth of plants because of their membrane constituents. The increase of cell growth in Tobacco cell suspension cultures has been shown to increase sterol contents [29].

Several studies have shown that the enhancement of triterpene saponins is induced by MJ treatments [30,31], as well, the transcript levels and activity of an OSC encoded β-amyrin synthase, parallel increasing saponin contents [32]. However, the down-regulation of cycloartenol synthase transcripts, following an exposure to MJ, has

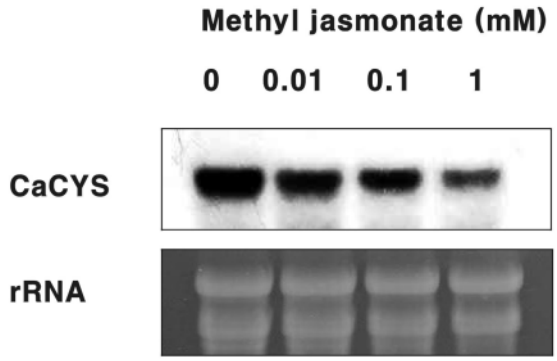

Fig. 6. Effect of MJ concentration on mRNA levels of *CaCYS* in leaves of cultured *C. asiatica* whole plants. MJ was added to media at 5 weeks of the culture. After 7 days of elicitation, total RNA was isolated from the leaves shown, resolved by agarose gel electrophoresis, blotted, and probed with *CaCYS*. Relative transcript abundance was calculated by dividing the intensity of each transcript to that of the corresponding rRNA transcript level.

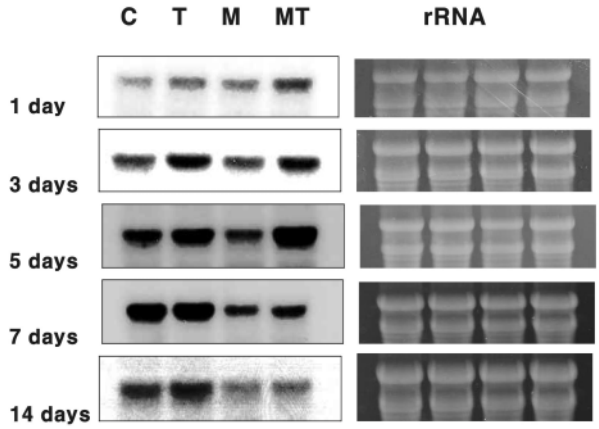

Fig. 7. Effect of MJ and MJ plus TDZ on the expression of *CaCYS* mRNA in the leaves of cultured whole plants from *C. asiatica*. 5-week-old whole plants were treated with the control without chemical (C), TDZ (T), MJ (M), or MJ plus TDZ (MT). After 1, 3, 5, 7 or 14 days of treatment, total RNA was isolated from the leaves, resolved by agarose gel electrophoresis, blotted, and probed with *CaCYS*. Relative transcript abundance was calculated by dividing the intensity of each transcript to that of the corresponding rRNA transcript level.

been observed in *Medicago* cell suspension cultures [33]. In the case of a licorice cell, a significant change of the transcript levels for cycloartenol synthase (*GgCAS1*) by MJ treatments has not been shown and their activity corresponds to the transcripts levels [32]. Also, these levels are consistent with the cell growth of licorice. Since MJ can have an effect on phytosterols production, we investigated the effect of MJ concentration on the levels of *CaCYS* mRNA in leaves. Total RNA was isolated from leaves treated with different MJ concentrations. As shown in Fig. 6, the levels of *CaCYS* transcripts were found to be down-regulated in leaves treated with MJ 7 days after the treatment. The more the MJ concentration was increased, the more the *CaCYS* transcripts were down-regulated. These results indicated that MJ treatments could negatively affect cell growth and phytosterol biosynthesis in leaves of whole plants, although the northern blot analysis does not provide a truly quantitative measure of the expression.

In our previous paper, we reported that the production of asiaticoside as saponins from whole plants treated with MJ plus TDZ is superior to that of an untreated control [19]. The results of that study indicated that asiaticoside production is enhanced by an MJ treatment and that TDZ addition sustained plant growth by the inhibition of senescence is caused by a MJ treatment. In other words, the enhancement of asiaticoside production is due to an increase in shoot growth, where asiaticoside is mainly synthesized, rather than the stimulation of secondary metabolites. When 0.025 mg/L TDZ was added to the media containing 0.1 mM MJ, these chemicals could affect the mRNA levels of the genes related to phytosterol biosynthesis pathways. The levels of *CaCYS* mRNA, an OSC involved in phytosterol biosythtesis, were higher 5 days after a MJ plus TDZ treatment than by a MJ treatment alone (Fig. 7). These transcripts in the leaves treated by MJ with TDZ, rather than those treated by MJ alone, lasted from 1 to 5 days, but they were not shown after 7 days. These results suggest that the increases of the mRNA levels of *CaCYS* by adding TDZ with MJ can be evidence for the reported results [19] that showed sustainment of shoot growth despite MJ treatments.

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REFERENCES

[1] Iturbe-Ormaetxe, I., K. Haralampidis, K. Papadopoulou, and A. E. Osbourn (2003) Molecular cloning and characterization of triterpene synthases from *Medicago truncatula* and *Lotus japonicus*. *Plant Mol. Biol.* 51: 731-743.

[2] Bach, T. J. and P. Benveniste (1997) Cloning of cDNAs or genes encoding enzymes of sterol biosynthesis from plants and other eukaryotes: Heterologous expression and complementation analysis of mutations for functional characterization. *Prog. Lipid Res.* 36: 197-226.

[3] Corey, E. J., S. P. T. Matsuda, and B. Bartel (1993) Isolation of *Arabidopsis thaliana* encoding cycloartenol synthase by functional expression in a yeast mutant lacking lanosterol synthase by the use of a chromatographic screen. *Proc. Natl. Acad. Sci. USA* 90: 11628-11632.

[4] Hayashi, H., N. Hiraoka, Y. Ikeshiro, T. Kushiro, M. Morita, M. Shibuya, and Y. Ebizuka (2000) Molecular cloning and characterization of a cDNA for *Glycyrrhiza glabra* cycloartenol synthase. *Biol. Pharm. Bull.* 23: 231-234.

[5] Kawano, N., K. Ichinose, and Y. Ebizuka (2002) Molecular cloning and functional expression of cDNAs encoding

oxidosqualene cyclase from *Costus speciosus. Biol. Pharm. Bull.* 25: 477-482.

[6] Kartnig T. (1988) Clinical application of *Centella asiatica* (L.) Urb. In: L. E. Craker and J. E. Simon (eds.). *Recent Advance in Botany*. Horticulture and Pharmacology. Vol. 3. Oryx Press, Phoenix.

[7] Matsuda, H., T. Morikawa, H. Ueda, and M. Yoshikawa (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.

[8] Brinkhaus, B., M. Lindner, D. Schuppan, and E. G. Hahn (2000) Chemical, pharmacological and clinical profile of the east Asian medical plant *Centella asiatica*. *Phytomedicine* 7: 427-448.

[9] Chattopadhyay, S., S. Farkya, A. K. Srivastava, and V. S. Bisaria (2002) Bioprocess considerations for production of secondary metabolites by plant cell suspension cultures. *Biotechnol. Bioprocess Eng*. 7: 138-149.

[10] Hwang, S. J., K. S. Kim, B. S. Pyo, and B. Hwang (1999) Saponin production by hairy root cultures of *Panax ginseng* CA Meyer: Influence of PGR and polyamines. *Biotechnol. Bioprocess Eng*. 4: 309-312.

[11] Gundlach, H., M. J. Muller, T. M. Kutchan, and M. H. Zenk (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. *Proc. Natl. Acad. Sci. USA* 89: 2389-2393.

[12] Yukimine, Y., J. Tabata, Y. Higashi, and Y. Hara (1996) Methyl jasmonate-induced overproduction of paclitaxel and baccatin III in *Taxus* cell suspension cultures. *Nat. Biotechnol.* 14: 1129-1132.

[13] Mandujano-Chavez, A., M. A. Schoenbeck, L. F. Ralston, E. Lozoya-Gloria, and J. Chappell (2000) Differential induction of sesquiterpene metabolism in tobacco cell suspension cultures by methyl jasmonate and fungal elicitor. *Arch. Biochem. Biophys.* 381: 285-294.

[14] Liu, C., Y. Wang X. Xu, F. Ouyang, H. Ye, and G. Li (1999) Improvement of artemisinin accumulation in hairy root cultures of *Artemisia annua* L by fungal elicitor. *Bioprocess Eng.* 20: 161-164.

[15] Satler, S. O. and K. V. Thimann (1981) Le jasmonate de methyle: Nouveau et puissant promoteur de la senescence des feuilles. *C. R. Acad. Sci. S.* 293: 735-740.

[16] Weidhase, R. A., J. Lehmann, H. Kramell, G. Sembder, and B. Parthier (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.

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

[18] Gamborg, O. L., R. A. Miller, and K. Ojima (1968) Nutrient requirements of suspension culture of soybean root cells. *Exp. Cell. Res.* 50: 195-202.

[19] Kim, O. T., M. Y. Kim, M. H. Hong, J. C. Ahn, and B. Hwang (2002) Stimulation of asiaticoside accumulation in the whole plant cultures of *Centella asiatica* (L.) Urban by elicitors. *Plant Cell Rep.* 23: 339-344.

[20] Frohman M. A., M. K. Dush, and G. R. Martin (1988) Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* 85: 8998-9002.

[21] Kushiro, T., M. Shibuya, and Y. Ebizuka (1998) Beta-amyrin synthase: Cloning of oxidosqualene cyclase that catalyzes the formation of the most popular triterpene among higher plants. *Eur. J. Biochem.* 256: 238-244.

[22] Page R. D. M. (1996) TreeView: An application to display phylogenetic trees on personal computer. *Compt. Appli. Biosci.* 12: 357-358.

[23] Doyle, J. J. and J. L. Doyle (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19:11-15.

[24] Sambrook, J., E. F. Fritsch, and T. Maniatis (1989) *Molecular Cloing: A Laboratory Manual*. 2nd ed., Cold spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

[25] Abe, I. and G. D. Prestwich (1995) Identification of the active site of vertebrate oxidosqualene cyclase. *Lipids* 30: 231-234.

[26] Poralla, K., A. Hewelt, G. D. Prestwich, I. Abe, I. Reipen, and G. Sprenger (1994) A specific amino acid repeat in squalene and oxidosqualene cyclases. *Trends Biochem. Sci.* 19: 157-158.

[27] Thompson, J. D., D. G. Higgins, and T. J. Gibson (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673-4680.

[28] Corey, E. J., H. Cheng, C. H. Baker, S. P. T. Matsuda, D. Li, and X. Song (1997) Methology for the preparation of pure recombinant *S. cerevisiae* lanosterol synthase using a baculovirus expression system. Evidence that oxirane cleavage and A-ring formation are concerted in the biosynthesis of lanosterol from 2,3-oxidosqualene. *J. Am. Chem. Soc.* 119: 1277-1288.

[29] Wentzinger, L. F., T. J. Bach, and M. A. Hartmann (2002) Inhibition of squalene synthase and squalene epoxidase in Tobacco cells triggers an up-regulation of 3-hydroxy-3-methylglutaryl coenzyme a reductase. *Plant Physiol.* 130: 334-346.

[30] Aoyagi, H., Y. Kobayashi, K. Yamada, K. Yokoyama, K. Kusakari, and H. Tanaka (2001) Efficient production of saikosaponins in *Bupleurum falcatum* root fragments combined with signal transducers. *Appl. Microbiol. Biotechnol.* 57: 482-488.

[31] Lu, M. B., H. L. Wong, and W. L. Teng (2001) Effects of elicitation on the production of saponin in cell culture of *Panax ginseng*. *Plant Cell Rep.* 20: 674-677.

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

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

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