

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

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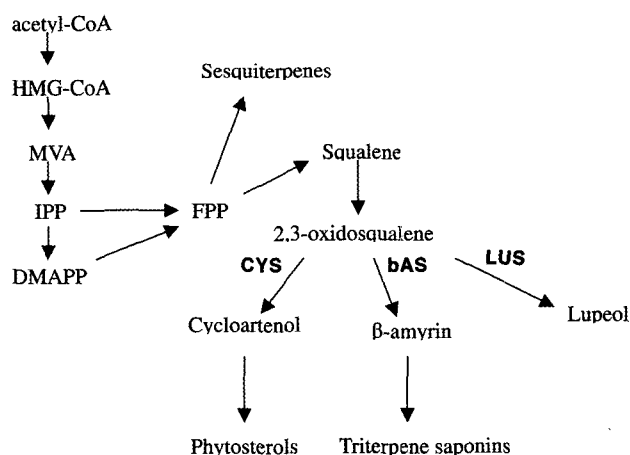
**Abstract** cDNA for oxidosqualene cyclase was cloned by a homology-based PCR method and sequenced from *Centella asiatica*. In a sequences analysis, the putative polypeptide of *C. asiatica* cycloartenol synthase (*CaCYS*) deduced from the 2,274 bp nucleotide sequence, consisted of 758 amino acids 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 antisenesescence 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  $\alpha$ - and  $\beta$ -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 scaffeoleoside, and triterpenic steroids such as stigmasterol and sitosterol [7,8]. We



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

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-

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tion of saponins in *C. asiatica* by genetic manipulation of the enzymes because of the economical value of saponins. Since both phytosterols and triterpene saponins share the common biosynthetic intermediate, 2,3-oxidosqualene, 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-thiadiazol-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 (*erg7*), 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)<sup>+</sup> RNA purification kit (Qiagen, Hilden, Germany). mRNA was reverse

transcribed using an oligo(dT) primer (RACE 32, 5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-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'-CCIATGWSITAYYTITAYGGIAAR-3' (PMSYLYGK), and coreA = 5'-CCCAISWICCITMCCAISWICCRTC-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'-GGTGCGAAGAGCTTTCTCTCTCAA-3'; 3'RACE 5'-TCCACTGCTGATCAT-GGATGGCC C-3' and the nested primer 5'-GCAGGGAAAAGGCCAGATGTTGAGCGA-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'-GACTCGAGTCGACATCGA-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 TGGCC CAGAAGTAC 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,  $\beta$ -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* (AB025345); cycloartenol synthases: GgCAS1, *Glycyrrhiza glabra* (AB025968); CAS1, *Arabidopsis thaliana* (U02555); PSX, *Pisum sativum* (D89619); BPX, *Betula platyphylla* (AB055510); 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 phylogenetic tree was created with the TreeView program [22].

### Southern Blot Analysis

Genomic DNA was isolated from leaves of *C. asiatica* using the cetyltrimethylammonium bromide procedure [23]. Southern and northern blot analyses were performed according to Sambrook *et al.* [24]. The Southern blot method involved digesting 10  $\mu$ g of leaf genomic DNA with *Eco*RI, *Bam*HI, *Hind*III, 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  $\alpha$ - $^{32}$ 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<sub>2</sub>HPO<sub>4</sub> for 15 min at 65°C, followed by two washes (15 min each) with 1% SDS containing 1 mM EDTA and 40 mM Na<sub>2</sub>HPO<sub>4</sub> 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. Aliquots of the RNA preparations (30  $\mu$ g 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 $\times$  SSC/0.1% SDS and then for 15 min at room temperature with 0.5 $\times$  SSC/0.1% SDS, followed by a wash for 15 min at 43°C with 0.1 $\times$  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

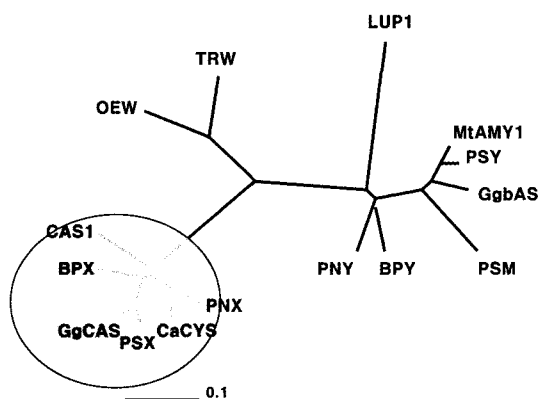
A number of OSCs and their functions have recently been reported. Amino acid sequence comparisons can show the relation among OSCs. In order to clarify the relationship with 14 other OSC sequences from plants, sequence homologies were calculated (Table 1) and a phylogenetic analysis of *CaCYS* was carried 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  $\beta$ -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-

|        |   |     |
|--------|---|-----|
| CA-CYS | MWKLKVAEGGNPWLRTVNNHVGRQIWEFDPKLGSPPEELAEIEKARENFNHHRFEKQHS   | 60  |
| PG-CYS | MWKLKIAEGGNPWLRTLNNDHVGKQIWEFDPNTGSPPEELAEVEKVKRENFRNHRFEKHS  | 60  |
| AT-CYS | MWKLKIAEGGSPWLRTTNNHVGRQIWEFDPNLGTPEDLAAVEEARAKSFSDNRFVQKHS   | 60  |
|        | 0 0 0 0 0   |     |
| CA-CYS | LLMRLOFANENPRHEVLPQVKVDIGDISSEKVTITLKRALSFYSTLQANDGHWAGDYGG   | 120 |
| PG-CYS | LLMRIQFANENPGSVVLPQVKVNDGEDISEKVTITLKRAMSFYSTLQANDGHWPGDYGG   | 120 |
| AT-CYS | LLMRLOFSRENLI SPVLPQVKIEDTDDVTEEMVETTLKRGDFVSTIQANDGHWPGDYGG  | 120 |
|        | 0 0 0 0 0   |     |
| CA-CYS | PMFLMPGLVITLSITPEALNAILSKEHKREICRYLYNHQNRDGGWGLHIEGPSTMFQSVLN | 180 |
| PG-CYS | PMFLMPGLVITLSITGVLVNVL SKEHKREICRYLYNHQNRDGGWGLHIEGPSTMFQTVLN | 180 |
| AT-CYS | PMFLLPCLITLSITGALNTVLSQHQKQEMRRYLYNHQNEEDGGWGLHIEGPSTMFQSVLN  | 180 |
| CA-CYS | YVTLRLLEGEGTKDGGQAMEKGRQWLDHGGAITTSWGRMWSVLGVFEMSGNNPLFPEI    | 240 |
| PG-CYS | YVTLRLLEGEGANDGGQAMEKGRQWLDHGSAITTSWGRMWSVLGVFEMSGNNPLFPEI    | 240 |
| AT-CYS | YVTLRLLEGEGENDGGQAMEKGRQWLDHGGAITTSWGRMWSVLGVFEMSGNNPLFPEI    | 240 |
| CA-CYS | WLLPYPYLPIHFGRMCHCRVYVLPMSYLYGRFVGPITPTVLSLRKELFTVPYHEIDVWQ   | 300 |
| PG-CYS | WLLPYPYLPIHFGRMCHCRVYVLPMSYLYGRFVGPITPTVLSLRKELFTVPYHEIDVWQ   | 300 |
| AT-CYS | WLLPYPYLPIHFGRMCHCRVYVLPMSYLYGRFVGPITPTVLSLRKELFTVPYHEIDVWQ   | 300 |
| CA-CYS | ARNLCAKEDLYYPHPLVQDILWASIDKVEPTLMRWPGKRLREKALRTVMEHIHYEDENT   | 360 |
| PG-CYS | ARNLCAKEDLYYPHPLIQDILWASLDKVEPTLMRWPAKRLREKSLRTVMEHIHYEDENT   | 360 |
| AT-CYS | ARNLCAKEDLYYPHPLVQDILWASLHKIVPEVLMRWPGANLREKALRTVMEHIHYEDENT  | 360 |
| CA-CYS | RYICIGPVNKLNMLCCWAEDPNSEAFKLHLPRLYDFLWLAEDGMKQGYNGSOLWDTAF    | 420 |
| PG-CYS | RYICIGPVNKLNMLCCWVEDPNSEAFKLHLPRLHDFLWLAEDGMKQGYNGSOLWDTAF    | 420 |
| AT-CYS | RYICIGPVNKLNMLCCWVEDPNSEAFKLHLPRHDFLWLAEDGMKQGYNGSOLWDTGF     | 420 |
| CA-CYS | AVQAIISTNLAEYGPTRLKAHTFMKNSQVLDLDCPGDLDFWYRHISRGAWPFSTADHGWP  | 480 |
| PG-CYS | AVQAIISTNLAEYGPTRLKAHTFMKNSQVLDLDCPGDLDAWYRHVSAGAWPFSTADHGWP  | 480 |
| AT-CYS | AVQAIISTNLAEYGPTRLKAHTFMKNSQVLDLDCPGDI NYWYRHISRGAWPFSTADHGWP | 480 |
|        | #####   |     |
| CA-CYS | ISDCTAEGFKAVLLSKFPAELVGEPLDAKRLYDAVNVILSLONSDGGYATYELTRSYRW   | 540 |
| PG-CYS | ISDCTAEGFKAVLLSKLPSELVGEPLDAKRLYDAVNVILSLONSDGGYATYELTRSYRW   | 540 |
| AT-CYS | ISDCTAEGFKAAVLLSKVPKEIVGEPIDAKRLYEAENVVILSLQADGGGLATYELTRSYRW | 540 |
|        | 0 0 0 0 0   |     |
| CA-CYS | LELNPARTFGDIVIDYPPVECTSAAIQALSAFKKLYPGRHREIEQCSI EKAADFIEKIQ  | 600 |
| PG-CYS | LELNPARTFGDIVIDYPPVECTSAAIQALSAFKKLYPGRHREIEQCSI EKAALFIEKIQ  | 600 |
| AT-CYS | LELNPARTFGDIVIDYPPVECTSAAIQALISPRKLYPGRHREIEQCSI EKAALFIEKIQ  | 600 |
|        | 0 0 0 0 0   |     |
| CA-CYS | ASDGSWYGSWAVCFVTYGTWFGVXGLISAGRTFSSNCSSIRKACYFLLSKQASGGWGESYL | 660 |
| PG-CYS | SSDGSWYGSWAVCFVTYGTWFGVXGLISAGRTFSSNCSSIRKACDFLLSKQASGGWGESYL | 660 |
| AT-CYS | ASDGSWYGSWAVCFVTYGTWFGVXGLISAGRTFSSNCSSIRKACDFLLSKQASGGWGESYL | 660 |
| CA-CYS | SCQNKVYTNLEGRSHVNTAWAMLALIEAGQAERDATPLHRAAKLLINSQENGDFFPQ     | 720 |
| PG-CYS | SCQNKVYTNLEGRSHVNTAWAMLALIEAGQAERDATPLHRAAKLLINSQENGDFFPQ     | 720 |
| AT-CYS | SCQDKVYTNLEGRSHVNTAWAMLALIEAGQAERDRKPLHRAARYLINAQENGDFFPQ     | 720 |
| CA-CYS | EIMGVFNKNCMI TYAAYRNIFPIWALGDYRCRVLAP--                       | 757 |
| PG-CYS | EIMGVFNKNCMI TYAAYRNIFPIWALGEYRCRVLQGPS-                      | 758 |
| AT-CYS | EIMGVFNKNCMI TYAAYRNIFPIWALGEYRCQVLLQQGE                      | 759 |

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 ( <i>B. platyphylla</i> )               | AB055512              | 58                      |
| GgbAS, β-amyrin synthase ( <i>G. glabra</i> )                  | AB037203              | 57                      |
| PNY, β-amyrin synthase ( <i>P. ginseng</i> )                   | AB009030              | 59                      |
| MtAMY1, β-amyrin synthase ( <i>M. truncatula</i> )             | AJ430607              | 57                      |
| PSY, β-amyrin synthase ( <i>P. sativum</i> )                   | AB03802               | 57                      |
| PSM, multifunctional triterpene synthase ( <i>P. sativum</i> ) | AB034803              | 53                      |
| LUP1, lupeol synthase ( <i>A. thaliana</i> )                   | U49919                | 60                      |
| OEW, lupeol synthase ( <i>O. europaea</i> )                    | AB025343              | 61                      |
| TRW, lupeol synthase ( <i>T. officinale</i> )                  | AB025345              | 60                      |
| GgCAS1, cycloartenol synthase ( <i>G. glabra</i> )             | AB025968              | 84                      |
| CAS1, cycloartenol synthase ( <i>A. thaliana</i> )             | U02555                | 79                      |
| PSX, cycloartenol synthase ( <i>P. sativum</i> )               | D89619                | 82                      |
| BPX, cycloartenol synthase ( <i>B. platyphylla</i> )           | AB055510              | 83                      |
| PNX, cycloartenol synthase ( <i>P. ginseng</i> )               | AB009029              | 89                      |



**Fig. 3.** Phylogenetic tree constructed from the deduced amino-acid sequences of CaCYS from *C. asiatica* and OSCs from other plants.  $\beta$ -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.

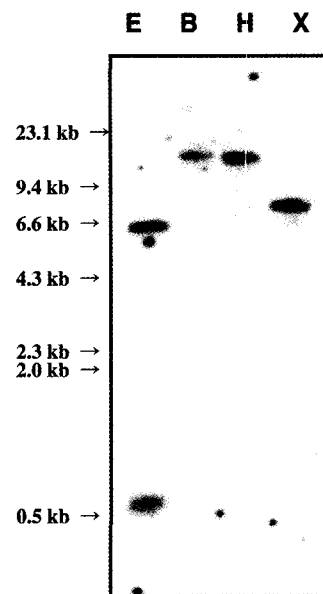
ify the function of the *CaCYS* gene by an overexpression in a yeast mutant (*erg7*).

#### Southern Blot Analysis

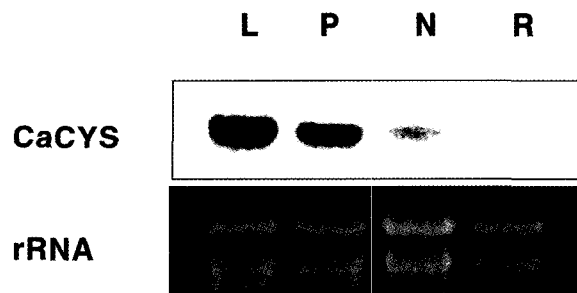
Hayashi *et al.* has indicated that at least two copies of cycloartenol synthase may exist in the genome of *G. glabra* [4]. To estimate the copy number of the cycloartenol synthase gene in the *C. asiatica* genome, genomic DNA digested with *EcoRI*, *BamHI*, *HindIII* or *XbaI*, was probed with the 1.0 kb cDNA. As shown in Fig. 4, two signals of the cycloartenol synthase gene were observed in the *EcoRI* lane. The two bands observed in the *EcoRI* lane (there is no *EcoRI* 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 homologous 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



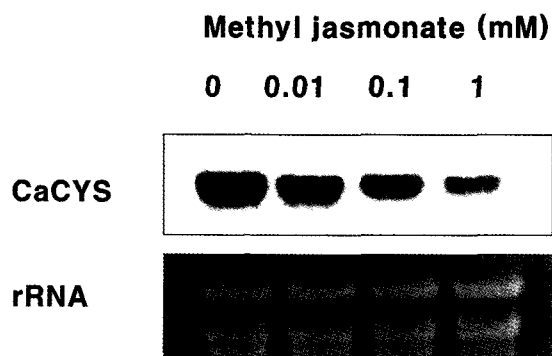
**Fig. 4.** Southern blot analysis of *CaCYS*. Genomic DNA of *C. asiatica* was digested with the restriction enzymes *EcoRI* (E), *BamHI* (B), *HindIII* (H) or *XbaI* (X) and Southern blotted (10  $\mu$ g of DNA/track). Blots were hybridized to the appropriate radiolabelled homologous probes under high stringency conditions. The molecular weight marker is indicated on the left.



**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, petioles; 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].

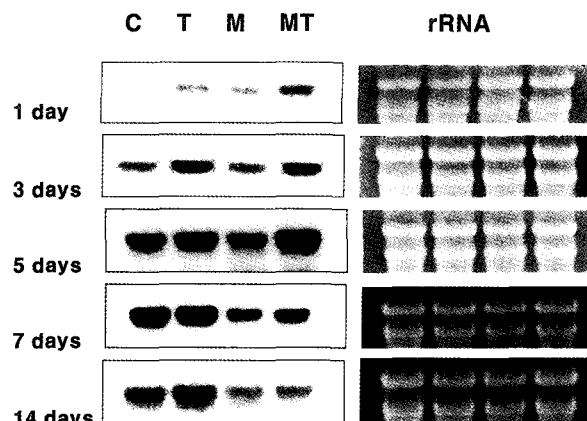
Several studies have shown that the enhancement of triterpene saponins is induced by MJ treatments [30,31], as well, the transcript levels and activity of an OSC encoded  $\beta$ -amyrin synthase, parallel increasing saponin contents [32]. However, the down-regulation of cycloartenol synthase transcripts,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

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 biosynthesis, 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,



**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.

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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