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# Up-regulation of Soyasaponin Biosynthesis by Methyl Jasmonate in Cultured Cells of *Glycyrrhiza glabra*

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**Exogenously applied methyl jasmonate (MeJA) stimulated soyasaponin biosynthesis in cultured cells of *Glycyrrhiza glabra* (common licorice). mRNA level and enzyme activity of β-amyrin synthase (bAS), an oxidosqualene cyclase (OSC) situated at the branching point for oleanane-type triterpene saponin biosynthesis, were up-regulated by MeJA, whereas those of cycloartenol synthase, an OSC involved in sterol biosynthesis, were relatively constant. Two mRNAs of squalene synthase (SQS), an enzyme common to both triterpene and sterol biosyntheses, were also up-regulated by MeJA. In addition, enzyme activity of UDP-glucuronic acid: soyasapogenol B glucuronosyltransferase, an enzyme situated at a later step of soyasaponin biosynthesis, was also up-regulated by MeJA. Accumulations of bAS and two SQS mRNAs were not transient but lasted for 7 d after exposure to MeJA, resulting in the high-level accumulation (more than 2% of dry weight cells) of soyasaponins in cultured licorice cells. In contrast, bAS and SQS mRNAs were coordinately down-regulated by yeast extract, and mRNA accumulation of polyketide reductase, an enzyme involved in 5-deoxyflavonoid biosynthesis in cultured licorice cells, was induced transiently by yeast extract and MeJA, respectively.**

**Key words**: *Glycyrrhiza glabra* — Licorice — Methyl jasmonate — Oxidosqualene cyclase — Triterpene saponin.

Abbreviations: bAS β-amyrin synthase; Triterpene synthase; DIG, digoxigenin; JA, jasmonate; LUS, lupeol synthase; MeJA, methyl jasmonate; OSC, oxidosqualene cyclase; PKR, polyketide reductase; SQS, squalene synthase; UGT, UDP-glucuronic acid: soyasapogenol B glucuronosyltransferase.

## Introduction

Chemically diverse triterpene saponins are accumulated in higher plants, but their physiological roles in plants are not clear. Since many saponins have potent anti-fungal activities, they may serve as preformed phytoprotectants against fungal attack, as shown for avenacins, oleanane-type triterpene saponins in oats (Papadopoulou et al. 1999). On the other hand, these saponins are of economical importance as drugs, detergents, sweeteners and cosmetics (Hostettmann and Marston 1995). Although structural elucidation of triterpene saponins has been extensively studied (Mahato et al. 1988, Mahato and Nandy 1991), our understanding about the regulation of saponin biosynthesis is quite limited (Chappell 1995, Haralampidis et al. 2002). We have investigated the regulation of saponin biosynthesis in higher plants using *Glycyrrhiza glabra* L. (common licorice) as a model plant. The roots and stolons of *G. glabra* accumulate a large amount of glycyrrhizin, an oleanane-type triterpene saponin, which is a well-known natural sweetener as well as an anti-inflammatory drug (Gibson 1978, Shibata 2000). In our previous work, cell suspension cultures established from rootlets of *G. glabra* did not produce glycyrrhizin (Hayashi et al. 1988), but produced another oleanane-type triterpene saponins, soyasaponins I and II (Hayashi et al. 1990). Soyasaponins were detected in various leguminous plants, such as soybean (Kitagawa et al. 1974, Kudou et al. 1993) and pea (Yokota et al. 1982, Tsurumi et al. 1992). Soyasaponins were also detected by LC-MS from *Medicago truncatula* (Huhman and Sumner 2002), a model legume. In the intact plant of *G. glabra*, soyasaponins are localized in the seeds and rootlets, where glycyrrhizin does not accumulates (Hayashi et al. 1993).

Both glycyrrhizin and soyasaponins share a common biosynthetic intermediate, β-amyrin (Fig.1), which is synthesized by β-amyrin synthase (bAS), an oxidosqualene cyclase (OSC). OSCs catalyze the cyclization of 2,3-oxidosqualene, a common intermediate of both triterpene and phytosterol biosyntheses (Abe et al. 1993, Haralampidis et al. 2002). In *G. glabra*, three OSCs: bAS, lupeol synthase (LUS) and cycloartenol synthase (CAS) are situated at the branching step for biosynthesis of oleanane-type triterpene saponins, lupane-type triterpene (betulinic acid) and phytosterols, respectively (Fig. 1). cDNAs of bAS (Hayashi et al. 2001) and CAS (Hayashi et al. 2000a) have already been isolated from the cultured licorice cells. In addition, two cDNAs for squalene synthase (SQS), another enzyme common to both sterol and triterpene biosyntheses, have also been isolated from the cultured licorice cells (Hayashi et al. 1999). Molecular cloning of these genes provides useful tools for studying the regulation of the saponin biosynthesis in *G. glabra*.

Jasmonate (JA) and its methyl ester (MeJA), are generally considered to modulate many physiological events in higher plants, such as defence responses, flowering and senescence, and are regarded as a new class of phytohormone (Creelman and Mullet 1997, Wasternack and Parthier 1997). In

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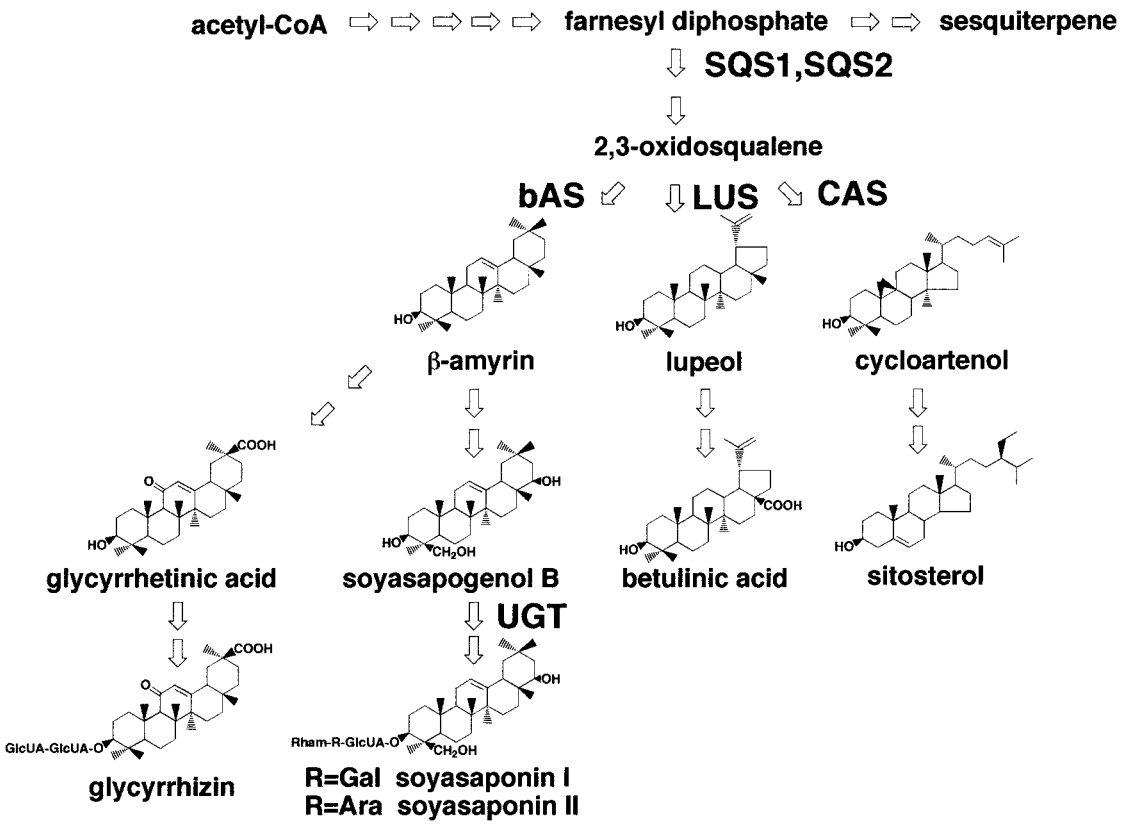


**Fig. 1** Biosynthetic pathways of triterpenoids and saponins in *Glycyrrhiza glabra*. bAS, β-amyrin synthase; CAS, cycloartenol synthase; LUS, lupeol synthase; SQS, squalene synthase; UGT, UDP-glucuronic acid: soyasapogenol B glucuronosyltransferase.

addition, it is reported that exogenously applied MeJA induce the biosynthesis of many secondary metabolites (Gundlach et al. 1992), including terpenoids (Yukimine et al. 1996, Mandujano-Chavez et al. 2000, Martin et al. 2002). MeJA was also reported to stimulate saponin production in cultured ginseng cells (Lu et al. 2001) and *Bupleurum falcatum* root fragments (Aoyagi et al. 2001), but the detailed mechanism of its stimulatory process remains poorly understood.

In the present study, we examined the effect of two elicitors, MeJA and yeast extract, on the accumulation of bAS mRNA in cultured licorice cells, and we found that exogenously applied MeJA induced accumulation of bAS and SQS transcripts, resulting in the accumulation of the end-product, soyasaponin. In contrast, levels of bAS and SQS mRNAs were down-regulated by yeast extract, which was known as an elicitor for flavonoid biosynthesis in cultured licorice cells (Ayabe et al. 1986, Nakamura et al. 1999).

## Results

### *Up-regulation of SQS and bAS mRNAs by MeJA in cultured licorice cells*

Previously we reported that the mRNA level of bAS, a specific cyclase producing the oleanane skeleton, was much higher in the licorice cells cultured in the soyasaponin-producing medium than that in the standard medium (Hayashi et al. 2001). This suggests a crucial role in the regulation of soyasaponin biosynthesis. Thus the effect of MeJA on the accumulation of bAS mRNA was examined first. In this experiment, MeJA was added to 10-day-old licorice cells cultured in the standard medium, and Northern hybridization analysis was

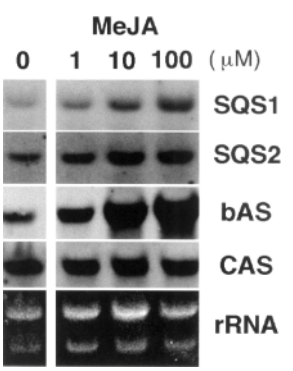


**Fig. 2** Effect of MeJA on mRNA levels of SQS1, SQS2, bAS and CAS in cultured licorice cells. MeJA was added to media on day 10 of culture, and the cells were harvested after 24 h incubation for Northern blot analysis.

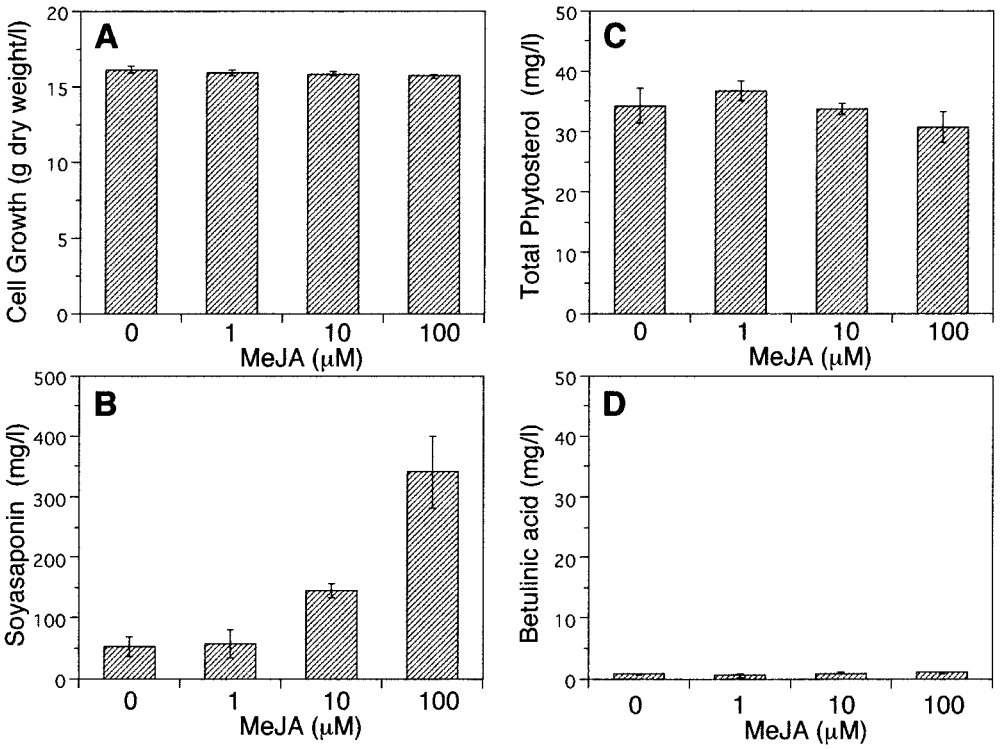


**Fig. 3** Effect of MeJA on cell growth (A) and accumulation of soyasaponins (B), total phytosterol (C) and betulinic acid (D) in cultured licorice cells. MeJA (100 μM) was added to media on day 10 of culture, and the cells were harvested on day 24. Mean of three replicates. Bar indicates standard deviation.

performed with gene-specific digoxigenin (DIG)-labeled RNA probes for SQSs and OSCs, cloned from *G. glabra*. Fig. 2 shows the effect of MeJA on the mRNA levels of two SQSs, bAS and CAS in cultured licorice cells. The bAS transcript was accumulated following addition of 10–100 μM of MeJA to the culture medium, whereas the mRNA level for CAS, which is responsible for sterol biosynthesis, was relatively constant. In addition to bAS mRNA, both SQS1 and SQS2 mRNAs were up-regulated by MeJA.

*Effect of MeJA on the cell growth and the accumulation of soyasaponins in cultured licorice cells*

Fig. 3 shows the effect of MeJA on the cell growth and accumulation of soyasaponins, phytosterols and betulinic acid in the licorice cells cultured in the standard medium. MeJA (100 μM) added to the medium stimulated accumulation of soyasaponin up to sixfold without affecting the cell growth, and the maximum content of soyasaponin reached 2% of dry weight of cells, which was much higher than that in the cells cultured in the soyasaponin-producing medium (Hayashi et al. 1990). By contrast, no stimulatory effect of MeJA on the accumulation of total phytosterols and betulinic acid was observed, and the content of betulinic acid was very low in the present experiment. Furthermore, glycyrrhizin, the major oleanane-type triterpene saponin in the thickening roots and stolons of *G. glabra*, was detected in neither control nor MeJA-treated cultured cells by HPLC analysis.

*Effect of MeJA on the activities of OSCs and UGT in cultured licorice cells*

To determine the level of activities of three OSCs, the microsomal fraction was prepared from the MeJA-treated or control cells. As shown in Fig. 4, bAS activity in MeJA-treated cells was eleven times higher than that of control cells (Fig. 4B). In contrast to bAS activity, CAS activity was almost the same in MeJA-treated and control cells (Fig. 4A). LUS activity, which is responsible for betulinic acid biosynthesis, was much lower than those of bAS and CAS in the cells cultured in the standard medium, corresponding to low production of betulinic acid (Fig. 3D) and was not detected in the MeJA-treated cells (Fig. 4C). These results indicate that only bAS is up-regulated by MeJA.

The activity of UDP-glucuronic acid: soyasapogenol B glucuronosyl-transferase (UGT), which is situated at the later step of soyasaponin biosynthesis, was reported to be detected in the microsomal fraction of the cultured licorice cells (Hayashi et al. 1996a) and soybean seedlings (Kurosawa et al. 2002). Thus, the effect of MeJA on UGT activity in the microsomal fraction was also examined. As shown in Fig. 4D, UGT activity in the MeJA-treated cells was two times higher than

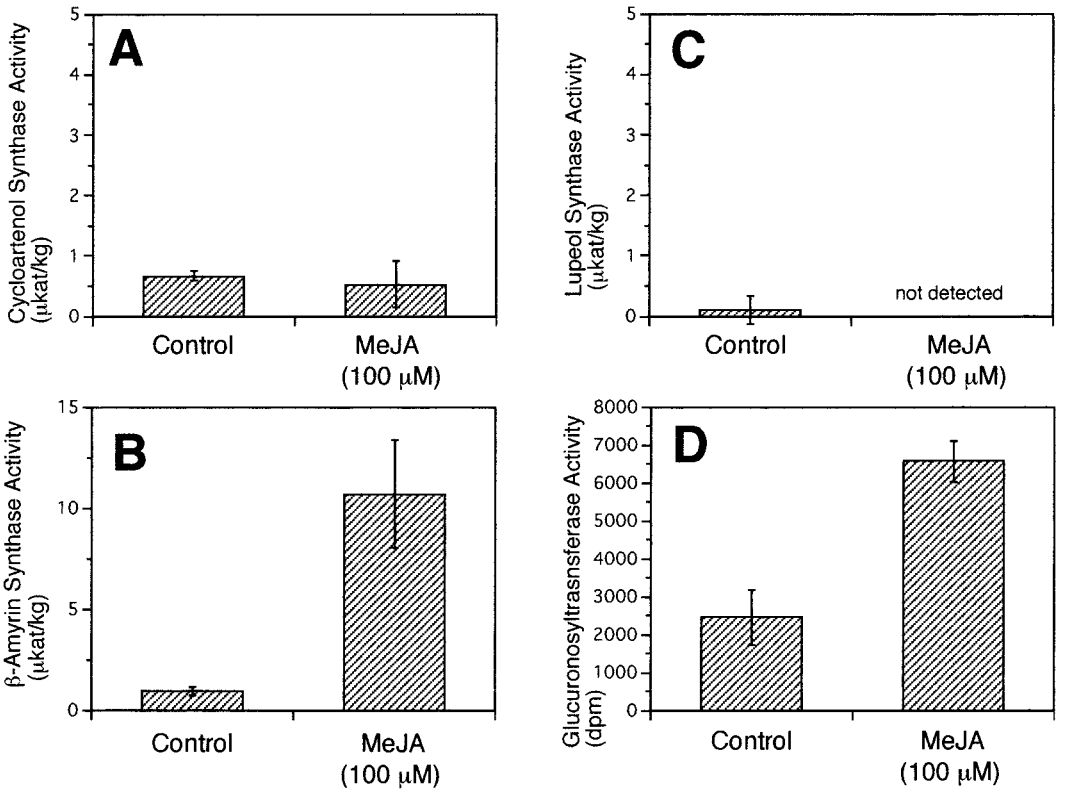


**Fig. 4** Effect of MeJA on three OSC activities (A, CAS; B, bAS; C, LUS) and UDP-glucuronic acid: soyasapogenol B glucuronosyltransferase activity (D, UGT) in cultured licorice cells. MeJA (100 μM) was added to media on day 10 of culture, and the cells were harvested after 24 h incubation for the determination of enzyme activity. Mean of three replicates. Bar indicates standard deviation.

that in the control cells, suggesting that not only bAS and SQS but also UGT, another enzyme for soyasaponin biosynthesis, were up-regulated by MeJA.

*Time course of mRNA accumulation during MeJA treatment*

Fig. 5 shows time course of mRNA accumulation of two SQSs, bAS and CAS in the MeJA-treated cells. mRNA level of polyketide reductase (PKR, chalcone reductase), which participates in the biosynthesis of 5-deoxy-(iso)flavonoids in cultured licorice cells (Hayashi et al. 1996b), was also determined. mRNA levels of both SQSs and bAS were coordinately up-regulated by addition of MeJA to the culture medium, and higher levels of these mRNAs lasted for 7 d after MeJA treatment. The maximum mRNA levels for two SQS and bAS were observed 3 d after MeJA treatment. mRNA levels of CAS, an OSC involved in phytosterol biosynthesis, was relatively constant in these experiment. In contrast, mRNA level of PKR were up-regulated by MeJA, although the increase of PKR transcript was transient. The maximum mRNA level for PKR was observed 9 h after exposure to MeJA.

*Down regulation of bAS and SQS mRNAs by yeast extract*

The jasmonates were proposed to be signaling molecules synthesized in response to fungal elicitor (Gundlach et al. 1992, Mueller et al. 1993). It was also reported that yeast extract stimulates flavonoid biosynthesis in cultured licorice cells (Ayabe et al. 1986, Nakamura et al. 1999). In addition, yeast extract and MeJA stimulated saponin production in cultured ginseng cells (Lu et al. 2001). To elucidate the effect of yeast extract on bAS mRNA, yeast extract was added to the soyasaponin-producing medium containing 10-day-old licorice cells (high-level of bAS mRNA). Fig. 6 shows time course of mRNA accumulation of two SQSs, bAS, CAS and PKR in the yeast extract-treated cells in the soyasaponin-producing medium. In contrast to up-regulation by MeJA, mRNA levels of bAS and two SQSs were down-regulated by yeast extract, and no signal was detected 24 h after application. CAS mRNA level was also slightly decreased by yeast extract. On the other hand, PKR transcript was up-regulated by yeast extract, reaching the maximum mRNA level 9 h after exposure to yeast extract.

## Discussion

Clearly, the most attractive outcome in the present study is the overproduction of triterpene saponins, which are pharmaceutically important compounds. Metabolic engineering of saponin-producing plants is also an attractive approach in the future. Up-regulation of mRNA levels of bAS and two SQS

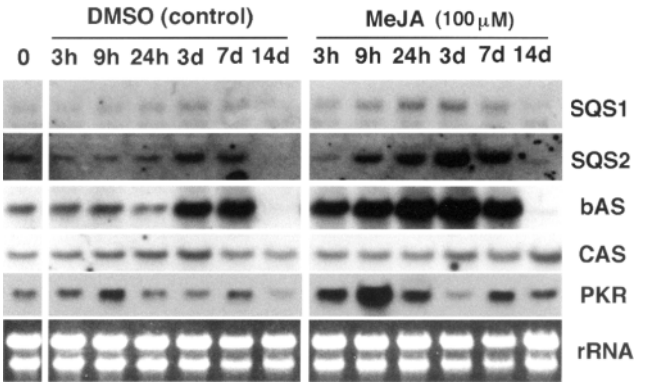


**Fig. 5** Time course of accumulation of SQS1, SQS2, bAS, CAS and PKR mRNAs in cultured licorice cells treated with MeJA. MeJA (100 μM) was added to media on day 10 of culture.

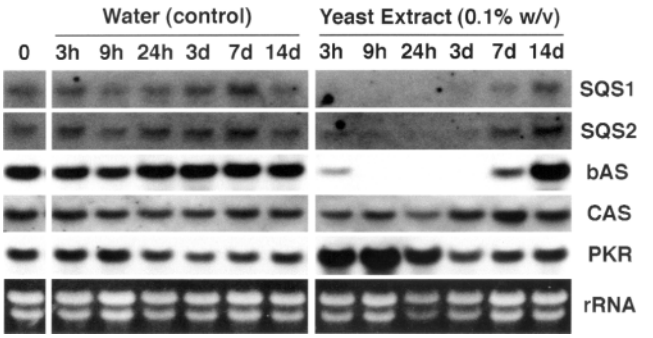


**Fig. 6** Time course of accumulation of SQS1, SQS2, bAS, CAS and PKR mRNAs in cultured licorice cells treated with yeast extract. Yeast extract (0.1% w/v) was added to media on day 10 of culture.

isozymes by MeJA reported here awakened our expectation that transcriptions of other enzymes involved in oxidation and glycosylation of β-amyrin leading to saponins, which remain to be characterized at the molecular level (Haralampidis et al. 2002), might be up-regulated by MeJA in cultured licorice cells. In fact, the enzyme activities of UGT, an enzyme situated downstream of bAS in soyasaponin biosynthesis (Hayashi et al. 1996a, Kurosawa et al. 2002), were enhanced in the microsomal fraction of the MeJA-treated licorice cells. More recently, induction of bAS mRNA and saponin accumulation by MeJA have also been reported in cultured cells of *M. truncatula* (Suzuki et al. 2002), a model legume. Identification of MeJA-inducible genes by micro-array analysis in the model legumes, such as *M. truncatula* (Cook 1999) and *Lotus japonicus* (Handberg and Stougaard 1992), might be a powerful tool to identify genes involved in oxidation and glycosylation steps in saponin biosynthesis.

To date, many MeJA-induced genes had been identified in the model plant, *Arabidopsis thaliana*, but accumulation of most of those MeJA-induced genes was transient (Sasaki et al. 2001). In contrast, long-lasting mRNA accumulations of geranylgeranyl diphosphate synthase and taxadiene synthase, which participate in Taxol™ (paclitaxel) production, was observed in MeJA-treated cell suspension cultures of *Taxus canadensis* (Hefner et al. 1998). In the present study, higher levels of bAS and SQS mRNAs lasted for more than 3 d after exposure to MeJA, whereas mRNA accumulation of PKR, involved in 5-deoxyflavonoid biosynthesis, was transient. This long-lasting mRNA accumulation of bAS and SQS endorses the high-level accumulation of soyasaponins in MeJA-treated cells. The contents of soyasaponins were reported to be very high in soybean seed hypocotyls (Tani et al. 1985, Shiraiwa et al. 1991) and pea seedlings (Tsurumi et al. 1992). In addition, the amounts of jasmonates were found to be very high in seeds and germinating seeds of soybean (Creelman and Mullet 1995). Up-regulation of soyasaponin biosynthesis by MeJA shown in the present study suggested that jasmonates may participate in up-regulation of saponin biosynthesis in germinating seeds of leguminous plants.

Yeast extract was shown to induce the flavonoid biosynthesis in cultured licorice cells (Ayabe et al. 1986, Nakamura et al. 1999). In the present study, mRNA levels of PKR, an enzyme for flavonoid biosynthesis, were transiently up-regulated by both yeast extract and MeJA in agreement with the previous observations that MeJA is a signal molecule in elicitor-induced cells (Gundlach et al. 1992, Mueller et al. 1993). However, mRNA levels of SQSs and bAS were down-regulated by yeast extract in the present study. The opposite effects of MeJA and yeast extract on the levels of bAS and SQS mRNAs suggested that the signaling pathway for MeJA is different from that for yeast extract, at least in cultured licorice cells. The levels of SQS and bAS mRNA were reported to be slightly up-regulated by yeast extract in cultured cells of *M. truncatula* (Suzuki et al. 2002), and the saponin biosynthesis in cultured ginseng cells was also up-regulated by yeast extract (Lu et al. 2001). These results suggest that the signaling pathway leading to activation of saponin biosynthesis in the cultured licorice cells is different from those in ginseng and *M. truncatula*. It is noteworthy that transcription factors involved in MeJA-inducible indole alkaloid biosynthesis have been characterized in *Catharanthus roseus* (Memelink et al. 2001). Such similar stress responsive transcription factors are suggested to be involved in saponin biosynthesis in legumes, and might shed some light on the distinct regulation of saponin biosynthesis in *G. glabra*.

The cultured cells of *G. glabra* produce two functional SQS isozymes, and genomic Southern blot analysis suggested that three SQS genes exist in the licorice genome (Hayashi et al. 1999). The present study demonstrated that both SQS1 and SQS2 mRNAs were up-regulated by MeJA, and that the cell growth and phytosterol level were not down-regulated by MeJA. These results suggest two possible explanations for the roles of these SQS isozymes. One is that SQS1 and SQS2 participate in triterpene and/or phytosterol biosyntheses to supply the carbon flow into MeJA-induced soyasaponin biosynthesis and/or to maintain the carbon flow into phytosterol biosynthe-

sis. In addition, the third SQS (SQS3), which has not yet characterized from *G. glabra*, might not participate in soyasaponin and phytosterol biosyntheses in cultured licorice cells. The other is that both SQS1 and SQS2 are responsible for activation of triterpene saponin biosynthesis, and that SQS3 is responsible for phytosterol biosynthesis. In the latter case, the mRNA level of SQS3 might be relatively constant as observed for CAS in the present study.

Soyasaponins and their congeners were known to have several physiological activities to plant (Yokota et al. 1982, Tsurumi and Wada 1995), but the general role of soyasaponin in legumes has not yet been established. MeJA is reported to be a signal molecule for activation of plant responses against insect herbivory (McConn et al. 1997), and the level of bAS mRNA was estimated to be higher in insect-damaged leaves than in developing leaves based on relative abundance of *M. truncatula* ESTs (Suzuki et al. 2002). Thus, it might be also possible that jasmonates play important roles as intermediate signals for activation of saponin biosynthesis by insect attack. The accumulated saponins may act as anti-insect compounds. *G. glabra* produce not only soyasaponin but also glycyrrhizin which is a sweet triterpene saponin exclusively localized in the thickening roots and stolons. Our next interest is focused on the effect of MeJA on the biosynthesis of glycyrrhizin.

## Materials and Methods

*Plant materials*

Cell suspension cultures of *G. glabra*, strain RNS-1B (Hayashi et al. 1990), were maintained in a 300-ml Erlenmeyer flask containing 60 ml Linsmaier–Skoog (LS) medium (Linsmaier and Skoog 1965) supplemented with 100 μM 1-naphthaleneacetic acid and 1 μM 6-benzyladenine (standard medium) in the dark at 25°C, and subcultured at intervals of 4 weeks. For experiments, the cells (1 g FW) were cultured in a 100-ml Erlenmeyer flask containing 30 ml of the standard medium or LS medium containing 1 μM 1-naphthaleneacetic acid and 10 μM 6-benzyladenine (soyasaponin-producing medium). Cultured cells were collected by filtration through Miracloth, frozen with liquid nitrogen and stored at –80°C.

*Chemicals*

Oxidosqualene was synthesized from squalene by method of Nadeau and Hanzlik (1969). Cycloartenol was obtained from Prof. Y. Ebizuka (The University of Tokyo). Other chemicals were obtained from Wako Pure Chemicals, Funakoshi Ltd. or Nacalai Tesque.

*Northern blot analysis*

RNA extraction, preparation of DIG-labeled RNA probes and Northern blot hybridization analysis were performed according to the procedure of Hayashi et al. (2001). The DIG-labeled RNA probes were prepared from *Bam*HI-digested GgbAS1 (probe length 1.0 kb) (Hayashi et al. 2001), *Nco*I-digested GgCAS1 (probe length 0.5 kb) (Hayashi et al. 2000a) and *Hin*dIII-digested GgPKR1 (probe length 0.6 kb) (Hayashi et al. 1996b) using T7 RNA polymerase (Invitrogen) and DIG RNA labeling mix (Roche Diagnostics), according to the manufacturer's manual. For gene-specific RNA probes of SQS genes, their 3′-cDNA ends were amplified by PCR using Taq DNA polymerase (Takara Shuzo), GgSQS1 or GgSQS2 (Hayashi et al. 1999), as a template, and two primers, 5′-AATACGACTCACTATAG-3′ (T7 primer) and 5′-GCGAATTCTTTGCTTATCTCTCTGC-3′. Amplified fragments were digested with *Eco*RI and *Xho*I, and ligated into pSP73 vector (Promega). The obtained plasmids were digested with *Eco*RI and used as templates for DIG-RNA probes for GgSQS1 (probe length 0.4 kb) and GgSQS2 (probe length 0.2 kb). These DIG-labeled RNA probes specifically hybridized to the respective cDNA under condition of high stringency.

*Quantitative analysis of soyasaponins, total sterols, betulinic acid and glycyrrhizin*

Quantitative analysis of soyasaponins, betulinic acid and total phytosterols was performed by gas chromatography. Freeze-dried cells (50 mg) were extracted with ethyl acetate (4 ml twice, 60°C for 1 h). Cholesterol (0.5 mg) was added to the extract as an internal standard, and the dried sample was used for the determination of betulinic acid and phytosterols. The residue after the ethyl acetate extraction was re-extracted with methanol (4 ml twice, 60°C for 1 h), and the dried extract was methanolyzed with 1 ml of 9% (w/v) HCl-methanol (65°C for 3 h). After addition of diethyl ether (4 ml) and cholesterol (0.5 mg), the solution was neutralized with $Ag_2CO_3$. The supernatant was evaporated and dried, and the residue was used for determination of soyasaponins. All dried samples were dissolved in a mixture of pyridine (50 μl) and *N*,*O*-bis(trimethylsilyl)acetamide (50 μl), and incubated at 60°C for 2 h. An aliquot (1 μl) of the solution was analyzed by GC: capillary column: Ultra ALLOY-17 (15 m × 0.5 mm, film thickness 0.5 μm, Frontier Lab. Koriyama, Japan), carrier gas: He (4 ml $min^{-1}$), column temperature: 200–300 (20°C $min^{-1}$), detector: FID. The contents of sterols, betulinic acid and soyasapogenol B were calculated from the ratio of the peak area of the respective compound to that of the internal standard. The total soyasaponin content (estimated as soyasaponin II) was calculated from the quantity of soyasapogenol B. The total phytosterol content was the total of the stigmasterol, sitosterol and campesterol contents.

HPLC analysis of glycyrrhizin was performed as previously reported (Hayashi et al. 2000b).

*Preparation of microsomal fraction*

All enzyme preparation operations were carried out at 0–4°C. Harvested cells (16 g) were homogenized in 32 ml of 0.1 M Tris-HCl buffer (pH 7.5) containing 2 mM EDTA, 20 mM mercaptoethanol, 0.25 M sucrose and 8 g PVPP with a Teflon homogenizer. The homogenate was centrifuged at 12,000×*g* for 20 min. The supernatant was centrifuged at 100,000×*g* for 30 min. The pellet was resuspended in 2 ml of 0.1 M Tris-HCl buffer (pH 7.5) containing 2 mM EDTA, 20 mM mercaptoethanol and 20% (v/v) glycerol. This suspension was used as a microsomal fraction. Protein determinations were performed according to the procedure of Bradford (1976).

*Enzyme assay of OSC and UGT*

OSC activity was measured by a modification of the procedure previously reported (Cho et al. 1993). Oxidosqualene (0.4 μmol) was dispersed with Triton X-100 (1 mg) and added to the microsomal fraction (1 ml). After incubation for 60 min at 30°C, the reaction was terminated by addition of 6% (w/v) KOH in ethanol (1 ml). Reaction products were extracted with cyclohexane (5 ml twice), and the triterpene mono-alcohol fraction was separated by preparative silica gel TLC (benzene-ethyl acetate, 3 : 1). Cholesterol (40 μg) was added to the triterpene mono-alcohol fraction as an internal standard, and the solvent was dried in vacuo. The dried sample was dissolved in a mixture of pyridine (10 μl) and *N*,*O*-bis(trimethylsilyl)acetamide (10 μl), and incubated at 60°C for 1 h. An aliquot (1 μl) of the solution was analyzed by gas-chromatography (GC) for triterpene mono-alcohols.

GC analysis was performed under the same conditions mentioned above. The contents of cycloartenol, β-amyrin and lupeol were calculated from the ratio of the peak area of the respective compound to that of the internal standard. The reaction mixture containing heat-denatured protein was used to determine background of triterpene mono-alcohols in the microsomal fraction.

UGT activity was measured according to the procedure of Hayashi et al. (1996a). The standard assay mixture (total 300 μl) contained 75 μg protein (microsomal fraction), 0.1 M K-$P_i$ buffer (pH 6), 2 mM EDTA, 20 mM mercaptoethanol, 50 μM soyasapogenol B, 10 mM $MgCl_2$ and 12.5 nCi UDP-[$^{14}$C-U]glucuronic acid (ARC, 300 mCi mmol$^{-1}$). The mixture was incubated for 60 min at 30°C, and the reaction was terminated by addition of 1 M HCl (30 μl). Reaction products were extracted with 400 μl of *n*-butanol. The radioactivity of 100 μl aliquot of the *n*-butanol extract was measured with a liquid scintillation counter. The reaction mixture containing heat-denatured protein was used to determine background radioactivity in *n*-butanol fraction.

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