Differential Expression of Three Oxidosqualene Cyclase mRNAs in *Glycyrrhiza glabra*

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The cultured cells and intact plants of *Glycyrrhiza glabra* (Fabaceae) produce betulinic acid and oleanane-type triterpene saponins (soyasaponins and glycyrrhizin). To elucidate the regulation of triterpenoid biosynthesis in *G. glabra*, the cDNA of lupeol synthase, an oxidosqualene cyclase (OSC) responsible for betulinic acid biosynthesis, was cloned, and expression patterns of lupeol synthase and two additional OSCs, β -amyrin synthase and cycloartenol synthase, were compared. The mRNA expression levels of lupeol synthase and β -amyrin synthase were consistent with the accumulation of betulinic acid and oleanane-type triterpene saponins, respectively. The transcript of lupeol synthase was highly expressed in the cultured cells and root nodules. The transcript of β -amyrin synthase, an OSC responsible for oleanane-type triterpene biosynthesis, was highly expressed in the cultured cells, root nodules and germinating seeds, where soyasaponin accumulates, and in the thickened roots where glycyrrhizin accumulates. In the cultured cells, the addition of methyl jasmonate up-regulated β -amyrin synthase mRNA and soyasaponin biosynthesis, but down-regulated lupeol synthase mRNA. Furthermore, the addition of gibberellin A₃ down-regulated β -amyrin synthase mRNA but not lupeol synthase mRNA in the cultured cells. The mRNA levels of cycloartenol synthase, an additional OSC responsible for sterol biosynthesis, in the intact plant and cultured cells were relatively constant in these experiments.

Key words β -amyrin synthase; *Glycyrrhiza glabra*; lupeol synthase; oxidosqualene cyclase; triterpene biosynthesis

Higher plants produce not only sterols but also various triterpenes, which are derived from six isoprene units,¹⁾ and some triterpenes are further glycosylated to produce various triterpene saponins.²⁾ These triterpenes and triterpene saponins are believed to participate in the plant defence systems against microbial pathogens or insects.^{3–5)} An interesting effect that has also been reported is that an exogenous triterpene saponin stimulates the growth of roots in several plants.^{6,7)} In addition to the benefit for plants themselves, these triterpenes and saponins are beneficial for human beings as medicines, sweeteners, detergents, and cosmetics. Although structural elucidation of triterpenes and saponins has been extensively studied,^{1,2)} our understanding about the regulation of their biosyntheses is quite limited at the molecular level.⁸⁾

Oxidosqualene cyclases (OSCs) catalyze the cyclization of 2,3-oxidosqualene, a common intermediate of both sterols and triterpenes. Lanosterol synthase, playing a crucial role in cholesterol biosynthesis, is a unique OSC in mammals. In higher plants, on the other hand, some OSCs responsible for triterpene biosynthesis co-exist with cycloartenol synthase corresponding to lanosterol synthase in mammals.^{8,9)} Up to now, cDNAs of OSCs including cycloartenol synthase, β -amyrin synthase, lupeol synthase and multifunctional triterpene synthase have been functionally characterized from various plant species.⁸⁾ After cloning of these OSC genes, our interest has shifted the regulatory roles of these OSCs in triterpene and saponin biosyntheses in higher plants.^{8,10–13)}

In our study, licorice (*Glycyrrhiza glabra* L., Fabaceae) was used as a model plant to elucidate the regulation of triterpene biosyntheses in higher plants (Fig. 1). Cultured licorice cells lacked the ability to produce glycyrrhizin, a sweet oleanane-type triterpene saponin produced in the roots

of the mother plant, but produced phytosterols and two structurally different triterpenoid constituents, soyasaponins and betulinic acid.^{15,16)} Glycyrrhizin is localized exclusively in the woody parts of the thickened roots and stolons, whereas soyasaponins, oleanane-type triterpene saponins, are localized mainly in the seeds and rootlets of licorice.¹⁷⁾ On the other hand, the content of betulinic acid, a lupane-type triterpene, is high in the cork layer of thickened licorice roots.¹⁵⁾ cDNAs of cycloartenol synthase, an OSC involved in sterol biosynthesis, and β -amyrin synthase, an OSC involved in both glycyrrhizin and soyasaponin biosynthesis, have been cloned from *G. glabra*.^{18,19)} In addition to these two OSCs, the third OSC, lupeol synthase, which is responsible for the biosynthesis of betulinic acid, most likely exists in cultured licorice cells. In the present study, an OSC cDNA for lupeol synthase was isolated by heterologous hybridization with that of olive lupeol synthase,²⁰⁾ and the patterns of mRNA expression of the three OSCs in the cultured cells and the intact plants of *G. glabra* were examined.

MATERIALS AND METHODS

Plant Materials and Culture Conditions The seeds of *G. glabra* were collected in Van, Turkey in 1986, and Zaragoza, Spain in 1996, and stored at -20°C . The seeds were germinated after treatment with conc. H_2SO_4 for 40 min. For the time course experiment of germination, germinating seeds (derived from Spain) were grown under permanent light ($60\ \mu\text{mol}/\text{m}^2\text{s}$) at 25°C . Cultivation of plants (derived from Turkey) in pots was done in Niigata, Japan, as previously reported,²¹⁾ and a thickened root from each three-year plant was collected every month from February to November in 1996. Harvested plant materials were frozen with

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Fig. 1. Biosynthetic Pathways of Triterpenoid and Saponin in *Glycyrrhiza glabra*
bAS, β -amyrin synthase; LUS, lupeol synthase; CAS, cycloartenol synthase.

liquid nitrogen and stored at -80°C . Naturally formed root nodules were collected in June from *G. glabra* plants (derived from Turkey) cultivated in pots outdoors.

Cell suspension cultures of *P. glabra*, strain RNS-1B¹⁶⁾ derived from a seed collected in Turkey, were maintained in a 300 ml Erlenmeyer flask containing 60 ml Linsmaier–Skoog (LS) medium²²⁾ 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 fresh weight) 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 1 μM 6-benzyladenine (producing medium). Cultured cells were collected by filtration through Miracloth, frozen with liquid nitrogen and stored at -80 °C.

Cloning of a Lupeol Synthase cDNA from *G. glabra* A 1263-bp digoxigenin (DIG)-labeled DNA probe, corresponding to amino acid residue numbers 160—580 of olive OEI lupeol synthase,²⁰⁾ was prepared by PCR using two primers, 5'-GAAGATGGAGGTTGGGG-3' and 5'-GTGTCCTGGATGCAACTGCTT-3', as previously reported.¹⁸⁾ A cDNA library prepared from cultured licorice cells was screened by the DIG-labeled probe under conditions of low stringency as reported.¹⁸⁾ Nine positive clones, isolated from 2×10⁵ plaques, were subcloned into pBluescript SK (–) by *in vivo* excision. Partial sequencing of the 5' and 3'-ends of the cDNAs revealed that the sequences of two positive clones were different from those of GgCAS1 cycloartenol synthase cDNA and GgbAS1 β -amyrin synthase cDNA cloned so far from *G. glabra*,^{18,19)} and that the other seven clones had sequences identical to that of GgbAS1 cDNA. Since both new OSC clones have an 3'-identical sequence, the longer clone was sequenced in both strands. Nucleotide sequences were determined by the dideoxy chain termination method using a model 373A DNA sequencer (PE Biosystems). Nucleotide and amino acid sequence analyses were conducted using

Genetyx-Mac software (Genetyx Corporation, Japan).

Functional Expression of cDNA in Yeast PCR was carried out for 25 cycles with 40 s at 94 °C, 40 s at 50 °C, and 2 min at 72 °C using GgLUS1 as a template, *Pfu* DNA polymerase, and two primers, 5'-TCGGTACCGAGGCAATTAAAGCGAG-3' (*Kpn*I site underlined) and 5'-CCTCTAGATGTGTCATAACTGTGAGCAC-3' (*Xba*I site underlined), corresponding to the 5' and 3'-termini of the deduced ORF of GgLUS1. The PCR product obtained was digested with *Kpn*I and *Xba*I, and ligated to the *Kpn*I and *Xba*I sites of pYES2 (Invitrogen, U.S.A.) to construct an expression plasmid, pYES2-GgLUS1, in which the open reading frame of the cDNA was downstream of the galactose-inducible GAL1 promoter. The nucleotide sequence of pYES2-GgLUS1 was confirmed by sequencing in both strands. Yeast strain GIL77,²³⁾ an ERG7-deficient mutant, was transformed with pYES2-GgLUS1 or pYES2 by the lithium acetate method.²⁴⁾ The protocol for the culture conditions, induction by galactose, and preparation of the triterpene mono-alcohol fraction were exactly the same as described.²³⁾ The triterpene mono-alcohol fraction was analyzed by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC-APCIMS) using a LCQ (Thermo Quest) under the following HPLC conditions: column, Super-ODS (diameter 4.6 mm, length 200 mm; Tosoh); solvent system, 95% acetonitrile aq.; flow rate, 1 ml min⁻¹; column temperature, 40 °C; detection, UV 202 nm; retention time for lupeol, 18.0 min.

Northern Blot Analysis RNA extraction, preparation of digoxigenin (DIG) labeled RNA probes, and Northern blot hybridization analysis were performed as previously reported.¹⁹⁾ The DIG labeled RNA probes were prepared from truncated cDNAs of GgLUS1, GgbAS1,¹⁹⁾ and GgCAS1¹⁸⁾ using T7 RNA polymerase (Invitrogen) and DIG RNA labeling mix (Roche Diagnostics), according to the manufacturer's manual. These DIG-labeled RNA probes specifically hybridized to the respective cDNA under conditions of high

Fig. 2. LC-APCIMS Analysis of the Triterpene Mono-alcohol Fraction from Yeast Strain GIL77 Transformed with pYES2-GgLUS1

(A) HPLC profile (UV absorption at 205 nm) of the triterpene mono-alcohol fraction of pYES2-GgLUS1 transformant; (B) mass chromatogram of m/z 409 of the triterpene mono-alcohol fraction of pYES2-GgLUS1 transformant; (C) MS/MS (precursor ion at m/z 409) fragmentation patterns of the peak at 18.0 min of pYES2-GgLUS1 transformant; (D) MS/MS (precursor ion at m/z 409) fragmentation patterns of authentic lupeol.

stringency.

Quantitative Analysis of Constituents Quantitative analysis of soyasaponins, betulinic acid, and total phytosterols was performed by gas chromatography as previously reported.¹⁴⁾ HPLC analysis of glycyrrhizin was performed as reported.¹⁴⁾

RESULTS

Cloning and Functional Expression of a Lupeol Synthase cDNA from *G. glabra* A λ -ZAP cDNA library prepared from cultured licorice cells¹⁸⁾ was screened by olive OEW lupeol synthase cDNA.²⁰⁾ The positive clones were subcloned into pBluescript SK (–) by *in vivo* excision and sequenced. Although most of them have identical sequences to that of GgbAS1 β -amyrin synthase cDNA, the sequences of two positive clones were different from those of the GgCAS1 cycloartenol synthase cDNA and GgbAS1 β -amyrin synthase cDNA cloned thus far from *G. glabra*.^{18,19)} Since partial sequencing of the 3' non-coding region revealed that both clones have an identical sequence, the longer clone was sequenced in both strands.²⁵⁾ The 2657-bp cDNA contained an open reading frame for a polypeptide of 758 amino acids. The relatively high identities (77–73%) of the deduced protein with lupeol synthases²⁰⁾ suggested that the new OSC cDNA encodes a lupeol synthase of *G. glabra*. To elucidate the function of the cDNA, its open reading frame was inserted into pYES2 (Invitrogen), a yeast expression plasmid with a galactose-inducible GAL1 promoter. The plasmid obtained, designated pYES2-GgLUS1, was introduced into a yeast mutant GIL77,²³⁾ which lacks a functional lanosterol synthase gene and produces no detectable lanosterol or triterpene mono-alcohols. The transformed yeast was treated with galactose to induce expression of the recombinant protein, and the triterpene mono-alcohol fraction was

prepared and then analyzed by LC-APCIMS. As shown in Fig. 2, a major peak at 18.0 min corresponding to standard lupeol was observed in the HPLC profile. This main peak produced an identical MS/MS (precursor ion at m/z 409) fragmentation pattern to that of authentic lupeol. As the control pYES2 transformant produced no triterpene mono-alcohol, accumulation of lupeol in the pYES2-GgLUS1 transformant indicates that the new OSC cDNA, termed GgLUS1, encodes lupeol synthase of *G. glabra*.

The phylogenetic tree constructed from the deduced amino acid sequences of plant OSCs is shown in Fig. 3. The deduced amino acid sequence of the *G. glabra* GgLUS1 lupeol synthase exhibits 80%, 77%, 73%, 61%, and 61% identity to those of *Betula platyphylla* BPW lupeol synthase,²⁶⁾ *O. europea* OEW lupeol synthase,²⁰⁾ *Taraxacum officinale* TRW lupeol synthase,²⁰⁾ *G. glabra* GgbAS1 β -amyrin synthase,¹⁹⁾ and *G. glabra* GgCAS1 cycloartenol synthase,¹⁸⁾ respectively. It is noteworthy that the deduced amino acid sequence of GgLUS1 exhibits the highest amino acid sequence identity (87%) to that of MtN18 from *Medicago truncatula*, which is a putative and partial-length OSC cDNA induced during root nodule development.^{13,27)}

Differential Expression of Three OSC mRNAs in the Cultured Cells and Intact *G. glabra* The three OSCs, namely, cycloartenol synthase, β -amyrin synthase and lupeol synthase, are situated at a crucial branching step for the biosynthesis of sterols, soyasaponins and betulinic acid, respectively, in cultured licorice cells. β -Amyrin synthase also plays an important role in the biosynthesis of glycyrrhizin in the thickened roots and stolons. In addition, it is reported that transcript of MtN18, which is suggested to be a lupeol synthase cDNA of *M. truncatula*, accumulates during the development of root nodules.^{13,27)} Thus, the mRNA expression levels of three OSCs in the cultured cells, thickened roots and root nodules were compared by Northern blot analysis (Fig.

4) with the contents of soyasaponins, glycyrrhizin and betulinic acid, which were determined by GC or HPLC analysis (Table 1).

High levels of β -amyrin synthase mRNA were observed in the cultured cells, especially in the producing medium, and the levels were also high in the thickened main roots and root nodules. These results were consistent with the high-level accumulation of soyasaponins in the cultured cells (in the producing medium) and root nodules, and that of glycyrrhizin in the thickened main roots (Table 1). Although the mRNA ex-

pression level of β -amyrin synthase in the cultured cells (harvested on day 8) in standard medium was very low (Fig. 4), the levels became higher in the late growth stages of the cultured cells (data not shown). Thus, a considerable amount of soyasaponins was also detected in the cultured cells in standard medium, which were harvested on day 24 (Table 1). The lupeol synthase mRNA was detected only in the cultured cells (in the producing medium) and root nodules, in which a relatively high level of betulinic acid was detected. In contrast, the mRNA level of cycloartenol synthase was relatively constant in the cultured cells, root nodules and thickened main roots. These results suggest independent regulation of two triterpene synthases, β -amyrin synthase and lupeol synthase, and the accumulation of oleanane-type triterpene saponins (soyasaponin and glycyrrhizin) and betulinic acid. It is noteworthy that the levels of their mRNAs correlated with the accumulation of the respective end products, suggesting that the transcript levels of OSCs are important regulatory steps for triterpene biosynthesis but not for sterol biosynthesis.

Differential Expression of Three OSC mRNAs in the Germinating Seedlings of *G. glabra* β -Amyrin synthesis was reported to be very active during the germination of pea seeds, while sterol synthesis is insignificant during the early stage of germination.²⁸⁾ β -Amyrin synthase activity was also reported to be maximum on the 2nd day after germination of pea seeds, whereas cycloartenol synthase activity reached a maximum on the 4th day.²⁹⁾ Recently, it has also been re-

Fig. 3. Phylogenetic Tree Constructed from the Deduced Amino Acid Sequences of *Glycyrrhiza* OSCs and Other Plant OSCs

The phylogenetic tree was constructed using the unweighted pair group method using the arithmetic average⁴⁸⁾ by Genetyx-Mac software (Ver. 10, Software Development, Japan). The GenBank database accession numbers used in this analysis are as follows, AB055512 (*Betula BPY*), AB009030 (*Panax PNY*), AB014057 (*Panax PNY2*), AJ430607 (*Medicago BAS*), AB034802 (*Pisum PSY*), AB037203 (*Glycyrrhiza GgbAS1*), AF78455 (*Lotus LjAMY2*), AB034803 (*Pisum PSM*), AB058643 (*Luffa LcIMS1*), AC002986 (*Arabidopsis YUP8H12R.43*), AC007152 (*Arabidopsis F1019.4*), U49919 (*Arabidopsis LUP1*), AC007260 (*Arabidopsis T30F21.16*), AJ311789 (*Avena AsbAS1*), AB025343 (*Olea OEW*), AB025345 (*Taraxacum TRW*), AB055511 (*Betula BPW*), AB116228 (*Glycyrrhiza GgLUS1*), AB009031 (*Panax PNZ*), AB025346 (*Taraxacum TRV*), AB033335 (*Luffa OSC2*), AB058508 (*Costus CSV*), AJ311790 (*Avena AsCS1*), AB058507 (*Costus CSI*), AB025353 (*Allium AMX*), AB025968 (*Glycyrrhiza GgCAS1*), D89619 (*Pisum PSX*), AB025344 (*Olea OEX*), AB055510 (*Betula BPX2*), AB009029 (*Panax PNX*), U02555 (*Arabidopsis CAS1*), AB055509 (*Betula BPX*), AB033334 (*Luffa LcCAS1*). bAS, β -amyrin synthase; CAS, cycloartenol synthase; IMS, isomultiflorenol synthase; LUS, lupeol synthase; MFS, multifunctional triterpene synthase; OSC, oxidosqualene cyclase.

Table 1. Contents of Glycyrrhizin, Total Soyasaponins and Betulinic Acid in Cultured Cells in Standard Medium and Producing Medium, Root Nodules and Thickened Roots of *Glycyrrhiza glabra*

	Contents (% of dry weight) of		
	Glycyrrhizin	Soyasaponins	Betulinic acid
Cultured cells in SM (24-d-old)	n.d.	0.231	0.002
Cultured cells in PM (24-d-old)	n.d.	0.377	0.285
Root nodules	n.d.	0.978	0.060
Thickened Roots	0.695	0.035	0.002

SM: standard medium, PM: producing medium, n.d.: not detectable. Root nodules and thickened root, which were derived from plants cultivated outdoors in pots, were collected in June.

Fig. 4. mRNA Levels of β -Amyrin Synthase (bAS), Cycloartenol Synthase (CAS) and Lupeol Synthase (LUS) in Cultured Cells (Harvested on Day 8) in Standard Medium (SM) or Producing Medium (PM), Root Nodules (RN) and Thickened Roots (TR) of *G. glabra*

The ethidium bromide staining of the gel before transfer is shown below.

Fig. 5. Time Course of mRNA Expression Levels and Soyasaponin Content in Germinating Licorice Seeds

(A) Time course of germination; (B) time course of mRNA levels for β -amyrin synthase (bAS), cycloartenol synthase (CAS), and lupeol synthase (LUS). The ethidium bromide staining of the gel before transfer is shown below. (C) Time course of soyasaponin content. Mean of five replicates. Bar indicates SE.

ported that the transcript levels of β -amyrin synthase peaked at day two and then declined during germination.¹³⁾ In order to investigate these phenomena in germinating licorice seeds, the time course of the three OSC mRNA levels was examined (Fig. 5). The level of β -amyrin synthase mRNA was maximum on day two during germination, and the mRNA level decreased rapidly after day two (Fig. 5B). In the seedlings on day two, the mRNA expression level of β -amyrin synthase was much higher in the roots and hypocotyls of seedlings than in the cotyledons (data not shown). On the other hand, the level of cycloartenol synthase mRNA was relatively constant during germination. Quantitative analysis of the germinating seeds revealed that the soyasaponin content increased from day one to day four during germination (Fig. 5C), whereas no significant accumulation of β -amyrin and soyasapogenol B, the sapogenol of soyasaponins I and II, was detected by GC analysis. These results indicate that the transient increase of β -amyrin synthase mRNA level results in the increase of soyasaponin content in the germinating licorice seedlings. Furthermore, the lack of accumulation of intermediates suggests that the steps beyond β -amyrin synthase are not likely to be limiting steps in the soyasaponin

Fig. 6. Seasonal Variation of mRNA Expression Levels for β -Amyrin Synthase (bAS) and Cycloartenol Synthase (CAS) in the Thickened Main Roots

The ethidium bromide staining of the gel before transfer is shown below.

biosynthesis in the seedlings. In contrast to the mRNA levels of β -amyrin synthase and cycloartenol synthase, the mRNA level of lupeol synthase was low in developing seedlings, since they did not accumulate betulinic acid.

Seasonal Variation of β -Amyrin Synthase and Cycloartenol Synthase mRNA Levels in the Thickened Main Roots The site of glycyrrhizin biosynthesis is localized in the thickened roots and stolons of *G. glabra*.^{21,30)} It is also noteworthy that seasonal variation of the incorporation of [¹⁴C]mevalonic acid into the glycyrrhizin fraction by the root segments was observed. The incorporation rate was high in May, June and September, and low in August and winter.²¹⁾ Thus, Northern blot analysis was performed to determine the seasonal variation of OSC mRNA levels in the thickened main roots of three-year-old plants. As shown in Fig. 6, the highest level of β -amyrin synthase mRNA was observed in May, when the aerial parts were rapidly growing. High levels of β -amyrin synthase mRNA were also observed in June, July and September, when the aerial parts were growing. The level of β -amyrin synthase mRNA was very low in August, when many leaves abscised, probably due to the high temperature and high humidity in Japan. This result was also observed in another experiment. No signal for β -amyrin synthase mRNA was detected during winter when the aerial parts were inactive. These were consistent with those of the incorporation rate of [¹⁴C]mevalonic acid into the glycyrrhizin fraction by the root segments and the seasonal variation of glycyrrhizin content.²¹⁾ In contrast to β -amyrin synthase mRNA, the level of cycloartenol synthase mRNA was relatively constant. It is noteworthy that a significant level of cycloartenol synthase mRNA was observed in winter, when no leaves remained. These results seem to indicate that cycloartenol synthase is a housekeeping enzyme, whereas β -amyrin synthase is expressed during the growing season when thickened roots manufacture glycyrrhizin. The level of lupeol synthase mRNA was not detectable in this experiment (data not shown).

Differential Regulation of Three OSC mRNAs by MeJA and Gibberellin A₃ (GA₃) in Cultured *G. glabra* Cells Since the mRNA levels of β -amyrin synthase and lupeol synthase were highly regulated in the intact plant of *G. glabra*, it is of interest to elucidate the signal molecules for regulation of triterpene biosynthesis. Thus, the effects of various plant hormones were examined using the cultured licorice cells, and we have already reported that exogenous methyl jasmonate (MeJA) up-regulates soyasaponin biosynthesis and mRNA levels of β -amyrin synthase in cultured licorice

Fig. 7. Effects of Jasmonate (JA), Methyl Jasmonate (MeJA) and Gibberellin A₃ (GA₃) on the mRNA Levels for β -Amyrin Synthase (bAS), Cycloartenol Synthase (CAS) and Lupeol Synthase (LUS)

Jasmonates and gibberellin were added to the media on day 10 of culture, and the cells were harvested after 24 h incubation. Ethidium bromide staining of the gel before transfer is shown below.

cells.¹⁴) Furthermore, we have found that exogenous GA₃ down-regulates the mRNA level of β -amyirin synthase in cultured licorice cells. Thus, the effects of MeJA and GA₃ on the mRNA levels of three OSCs were examined. These two hormones were added to 10-day-old licorice cells cultured in the producing medium, in which the mRNA levels of all three OSCs are high, and Northern blot analysis was performed (Fig. 7). The mRNA level of β -amyirin synthase was up-regulated by 100 μ M of MeJA, but was down-regulated by 10–100 μ M of GA₃. On the other hand, the lupeol synthase transcript was down-regulated by 100 μ M of MeJA, whereas the level was not changed by 10–100 μ M of GA₃. By contrast, the level of cycloartenol synthase mRNA was relatively constant in these experiments. These results showed that the mRNA levels of the three OSCs were differentially regulated by MeJA and GA₃.

DISCUSSION

Although 13 OSC genes were identified in the *Arabidopsis thaliana* genome,³¹ five OSC genes, which have been functionally identified, encode one cycloartenol synthase³² and four multifunctional triterpene synthases,^{31,33–35} respectively. The multifunctional triterpene synthase produces multi-triterpene-products from 2,3-oxidosqualene, the common substrate for OSCs, and this type of OSC was also cloned from pea,³⁶ *Costus speciosus*³⁷ and *Lotus japonicus*.¹³ It is noteworthy, however, that the three OSCs cloned from *G. glabra* were monofunctional triterpene synthases, each of which produces a sole major triterpene product.^{18,19} Although the existence of additional copies for OSC genes in the licorice genome has been suggested by genomic Southern blot analysis,^{18,19} screening of the β -amyirin synthase gene from a licorice cDNA library prepared from the thickened main roots resulted in cloning of only the same β -amyirin synthase cDNA (Hayashi *et al.* unpublished results). Thus, it is likely that the single β -amyirin synthase gene is responsible for both glycyrrhizin and soyasaponin biosynthesis. In addition, extensive chemical studies have revealed that licorice

contains many oleanane-type triterpenes and triterpene saponins derived from β -amyirin, but ursane-type triterpenes have never been detected.³⁸ These findings suggest that cycloartenol synthase, β -amyirin synthase and lupeol synthase play important roles as the major OSCs responsible for the biosynthesis of sterol, oleanane-type triterpene saponins, and lupane-type triterpene, respectively, in licorice.

Lupeol synthase is responsible for the biosynthesis of lupane-type triterpenes, such as betulin and betulinic acid, which are widely distributed in higher plants. These lupane-type triterpenes are often localized at the interface of aerial and underground parts of higher plants, and probably act as a hydrophobic and antibiotic barrier.³⁹ In fact, betulinic acid is accumulated in the root bark of licorice.¹⁵ In the present study, a high level of lupeol synthase mRNA was detected in root nodules of licorice, which correlated well with the accumulation of betulinic acid. It is noteworthy that betulin was accumulated in the outer cortex of the root nodules of broad bean,⁴⁰ and that the putative lupeol synthase mRNA was induced during the root nodule development of a model legume, *M. truncatula*.^{13,27} Taken together, it is likely that lupane-type triterpenes play important roles as hydrophobic and antibiotic barriers in the development of symbiosis in legumes.

The present study showed that the transient increase of β -amyirin synthase mRNA level during the early stages of germination resulted in the increase of soyasaponin content in the seedlings of *G. glabra*. The soyasaponin content was reported to be very high in soybean seed hypocotyls^{41,42} and pea seedlings.⁴³ These results suggest that the induction of soyasaponin biosynthesis during germination is common in these legumes. In addition, the β -amyirin synthase mRNA level and soyasaponin production were up-regulated by MeJA in cultured cells of *M. truncatula* and *G. glabra*.^{12,14} It is also noteworthy that the jasmonate level is high in germinating seeds of soybean,⁴⁴ suggesting that jasmonates are a signal molecule to induce soyasaponin biosynthesis in legumes.

On the other hand, the thickened roots and stolons of *G. glabra* contain glycyrrhizin instead of soyasaponin. The present study showed that the β -amyirin synthase mRNA level in the thickened roots was very high when the aerial parts were active, whereas the level was very low when the aerial parts were inactive. This result indicates that the existence of the active aerial parts is necessary for the expression of β -amyirin synthase and glycyrrhizin biosynthesis in the thickened roots, and jasmonates are suggested to be a candidate positive regulator.

In addition to up-regulation of β -amyirin synthase mRNA and soyasaponin biosynthesis by jasmonates,^{12,14} the present study showed that exogenous GA₃ down-regulated mRNA levels of β -amyirin synthase but not those of lupeol synthase and cycloartenol synthase, suggesting that gibberellins may be negative regulators of the oleanane-type triterpene saponin biosynthesis in *G. glabra*. Gibberellins are known to play an important role during germination, but the mRNA expression level of β -amyirin synthase was higher in germinating seedlings in the present study. This contradiction may be explained by the fact that the β -amyirin synthase mRNA level was much higher in the roots and hypocotyls of seedlings than in the cotyledons. In addition, gibberellins are known to

be a potential negative regulator in potato tuber development, whereas exogenous jasmonates are inducing factors for potato tuberization.⁴⁵⁾ It is noteworthy that glycyrrhizin accumulates exclusively in the thickened roots and stolons of *G. glabra*, both of which are storage organs of this perennial plant species. Furthermore, the glycyrrhizin content increases during the thickening of the underground parts.^{17,21)} We are also interested in the effects of gibberellins and jasmonates on the biosynthesis of glycyrrhizin in the thickened roots and stolons of *G. glabra*.

Since the biosyntheses of two triterpene saponins, soyasaponin and glycyrrhizin, are developmentally regulated, these compounds are presumed to have unknown important function in the intact plant. These triterpene saponins are likely to participate in the plant defense systems against microbial pathogens^{3,4)} or insects.⁵⁾ Soyasaponin I was also reported to be a phytochrome killer in pea.⁴⁶⁾ Chromosaponin I, a conjugate of soyasaponin I and 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one,^{43,47)} was shown to stimulate elongation of roots in a variety of plants.^{6,7)} Although the general role of soyasaponin is not yet established, soyasaponin plays an important role in leguminous plants. In contrast to soyasaponin, nothing is known about the physiological function of glycyrrhizin in the thickened roots of *G. glabra*. Since the glycyrrhizin content in the thickened roots is very high, glycyrrhizin may act as a preformed chemical defense substance against pathogens, as has been shown for avenacin, an oleanane-type saponin, in oat.⁴⁾

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