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# Molecular Biology of Triterpenoid Biosynthesis in *Glycyrrhiza* Plants

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## ABSTRACT

Licorice (roots and stolons of *Glycyrrhiza* plants, Leguminosae) is a well-known natural sweetener, and its sweet constituent is an oleanane-type triterpene saponin, glycyrrhizin. Cultured cells of *G. glabra* do not produce glycyrrhizin, but produce two structurally different triterpenoid constituents, betulinic acid and soyasaponins. Both glycyrrhizin and soyasaponins are oleanane-type triterpenes, and glycyrrhizin is localized exclusively in the woody parts of thickened roots and stolons, whereas soyasaponins are localized mainly in seeds and rootlets of *G. glabra*. On the other hand, betulinic acid, a lupane-type triterpene, is localized in the cork layer of thickened roots and stolons. To elucidate the regulation of the triterpenoid biosyntheses in *G. glabra*, cDNAs of three oxidosqualene cylaces, cycloartenol synthase, β-amyrin synthase and lupeol synthase, which are situated at the branching point of triterpenoid biosynthesis, were cloned and characterized. mRNA levels of the three oxidosuqualene cyclase were differentally regulated in cultured cells and intact plants of *G. glabra*. Exogenously applied methyl jasmonate (MeJA) stimulated soyasaponin biosynthesis in cultured cells, and mRNA levels of squalene synthase and β-amyrin synthase were up-regulated by MeJA. In contrast, mRNA levels of squalene synthase and β-amyrin synthase were down-regulated by yeast extract, which induced flavonoid biosynthesis in leguminous plants. These results suggested that the transcription of other enzymes involved in the late steps of saponin biosynthesis might be up-regulated by MeJA in the cultured licorice cells.

**Keywords:** betulinic acid, biosynthesis, *Glycyrrhiza glabra*, glycyrrhizin, methyl jasmonate, oxidosqualene cyclase, saponin, soyasaponin, triterpene

**Abbreviations:** **bAS**, β-amyrin synthase; **CAS**, cycloartenol synthase; **GC**, gass chromatography; **HPLC**, high performance liquid chromatography; **LUS**, lupeol synthase; **MeJA**, methyl jasmonate; **OSC**, oxidosqualene cyclase; **SQS**, squalene synthase

## CONTENTS



## INTRODUCTION

Licorice (roots and stolons of *Glycyrrhiza glabra*, Leguminosae) is one of the most important crude drugs from ancient times (Gibson 1978; Shibata 2000). Its sweet constituent, glycyrrhizin, is an oleanane-type triterpene glucuronide, and is used in large quantities as a well-known natural sweetener and as a pharmaceutical (Shibata 2000). Chemical constituents of licorice have been extensively studied to isolate not only glycyrrhizin but also many triterpenoids and flavonoids (Nomura and Fukai 1998). Although structural elucidation of triterpenoids has been extensively studied (Mahato *et al*. 1988), our understanding about the regulation of their biosynthesis is quite limited (Chappell 1995; Haralampidis *et al*. 2002). Since diverse triterpenoids have been isolated from *G. glabra*, this plant would be one of the model plants to elucidate the regulation of triterpenoid biosynthesis in higher plants. In this review, regulation of triterpenoid biosynthesis in the intact plant and cultured cells of *G. glabra* will be discussed.

## TRITERPENOIDS IN LICORICE

### Triterpenoids produced by cultured licorice cells

Glycyrrhizin, a sweet oleanane-type triterpene saponin in licorice roots, was not detected by HPLC analysis in any callus and cell suspension culture of *G. glabra* (Hayashi *et al*. 1988). However, structurally different triterpenoid constituents, soyasaponins I and II (Hayashi *et al*. 1990) and betulinic acid (Hayashi *et al*. 1988), were isolated from the cultured licorice cells. Soyasaponins I and II were also oleanane-type triterpene saponins, like glycyrrhizin, and were isolated from various leguminous plants, such as soybean

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Invited Mini-Review

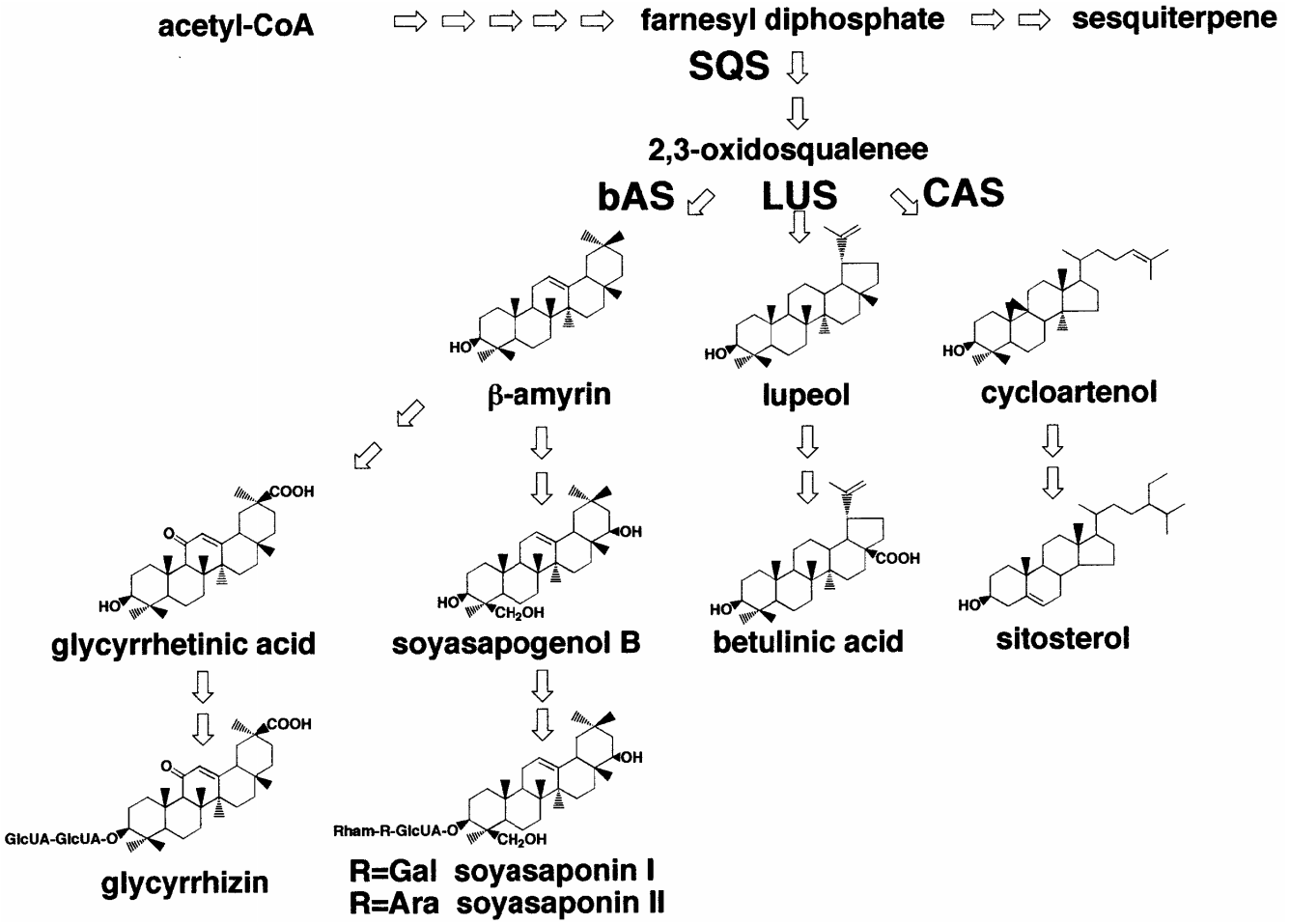


**Fig. 1 Biosynthetic pathways of triterpenes and saponins in *Glycyrrhiza glabra*.** Reproduced from **Hayashi *et al.*** (2004) *Biological and Pharmaceutical Bulletin* **27**, 1087, **Fig. 1**, with kind permission of The Pharmaceutical Society of Japan.

(Kitagawa *et al*. 1974) and pea (Yokota *et al*. 1982). Both glycyrrhizin and soyasaponins share a common biosynthetic intermediate, β-amyrin (**Fig. 1**). The cultured licorice cells convert β-amyrin into soyasapogenol B, the aglycone of soyasaponins, via oxidations at the C-22 and C-24 positions, and the specific oxidations of β-amyrin at C-11 and C-30 leading to glycyrrhetinic acid, the aglycone of glycyrrhizin, are blocked in the cultured cells. Betulinic acid is a lupane-type triterpene and is distributed widely in plant kingdom. Betulinic acid is synthesized from lupeol, a common biosynthetic intermediate for lupane-type triterpenes, via oxidations of C-28 (**Fig. 1**). Betulinic acid was also isolated from licorice roots (Saitoh and Shibata 1969; Hattori *et al*. 1986).

The contents of soyasaponins and betulinic acid in various culture strains of *G. glabra* were determined by GC analysis (**Table 1**). The contents of soyasaponins and betulinic acid widely varied with culture strains, and there was no significant correlation between the contents of soyasaponins and betulinic acid (Hayashi *et al*. 1990).

### Distribution of triterpenoids in *Glycyrrhiza glabra*

As shown in **Table 1**, the contents of glycyrrhizin, soyasaponins and betulinic acid in various organs of *G. glabra* were determined by GC analysis (Hayashi *et al*. 1988, 1993, 2004). Glycyrrhizin was found chiefly in the woody parts of thickening root, but not in the aerial parts, rootlets or root nodules. Soyasaponins were detected in all parts of the plants examined, and the soyasaponin contents were much higher in the seeds, rootlets and root nodules (Hayashi *et al*. 1993, 2004). It is noteworthy that an inverse relationship between the soyasaponin content and glycyrrhizin content was observed during the development of main roots after germination. On the other hand, betulinic acid was localized in the rootlets, root nodules and the cork layer of thickening roots (Hayashi *et al*. 1988, 2004). Since soyasaponins and betulinic acid were produced in the rootlet, root nodules and cultured cells, the triterpenoid metabolism of the cultured licorice cells is suggested to be similar to that of the rootlet and root nodules.

## MOLECULAR BIOLOGY OF TRITERPENOID BIOSYNTHESIS IN LICORICE

### Oxidosqualene Synthase cDNAs of *Glycyrrhiza glabra*

Oxidosqualene cyclases (OSCs) catalyze the cyclization of 2,3-oxidosqualene, a common intermediate of both triterpene and sterol biosyntheses (Abe *et al*. 1993, Haralampidis *et al*. 2002). To elucidate the regulation of the triterpenoid biosyntheses in *G. glabra*, cDNAs of the three OSCs, β-amyrin synthase (bAS), lupeol synthase (LUS) and cycloartenol synthase (CAS), which are situated at the branching step for biosynthesis of oleanane-type triterpene saponins (glycyrrhizin and soyasaponins), lupane-type triterpene (betulinic acid) and phytosterols, respectively (**Fig. 1**), were cloned and characterized (Hayashi *et al*. 2000, 2001, 2004). These three OSCs cloned from *G. glabra* were monofunctional triterpene synthases, each of which produces a sole major triterpene product, although multifunctional triterpene synthase, producing multi-triterpene-products, was cloned from other leguminous plants (Morita *et al*. 2000; Iturbe-Ormaetxe *et al*. 2003). In addition, two cDNAs for squalene synthase (SQS), another enzyme common to both triterpene and sterol biosyntheses, were cloned from the cultured licorice cells (Hayashi *et al*. 1999). Molecular cloning of these

**Table 1** Content of glycyrrhizin, soyasaponins and betulinic acid in cultured cells and various organs of *Glycyrrhiza glabra*.

| | Content (% of dry weight ) of | | |
|---|---|---|---|
| | **Glycyrrhizin** | **Soyasaponins** | **Betulinic acid** |
| **Cultured strains** | | | |
| RNS-1A | n.d. | 0.06 | 0.24 |
| RNS-1B | n.d. | 0.28 | 0.33 |
| RNS-1C | n.d. | 1.1 | 0.004 |
| RNS-1D | n.d. | 0.25 | 0.001 |
| **Organ** | | | |
| Seed | n.d. | 0.35 | n.d. |
| Leaf | n.d. | 0.001 | n.d. |
| Stem | n.d. | 0.001 | n.d. |
| Thickened Root (ϕ 32 mm) | | | |
| Woody part | 2.2 | 0.004 | n.d. |
| Cork layer | 0.006 | 0.004 | 0.32 |
| Root (6 month old) | | | |
| Thickened root (ϕ 4 mm) | 0.23 | 0.28 | 0.14 |
| Rootlet (< ϕ 1 mm) | 0.002 | 0.82 | 0.10 |
| Root nodule | n.d. | 0.98 | 0.06 |

n.d.: not detected

genes provides useful tools for studying the regulation of triterpenoid biosyntheses in licorice.

## Regulation of triterpenoid biosynthesis in intact licorice plants

The three OSCs, bAS, LUS and CAS, are situated at a crucial branching step for the biosynthesis of soyasaponins, betulinic acid and phytosterols, respectively, in the cultured licorice cells. bAS also play an important role in the biosynthesis of glycyrrhizin in the thickened roots and stolons. Thus, the mRNA levels of three OSCs in the cultured cells and intact plant were compared by Northern blot analysis (Hayashi *et al*. 2003, 2004). High level of bAS mRNA was observed in the cultured cells, thickened main roots and root nodules. This was consistent with the high-level accumulation of soyasaponins in the cultured cells and root nodules, and that of glycyrrhizin in the thickened main roots. The LUS mRNA was detected in the cultured cells and root nodules, in which relatively high level of betulinic acid was detected. The mRNA level of CAS, an OSC responsible for sterol biosynthesis, was relatively constant in the cultured cells and various organs of *G. glabra*. These results indicate independent regulation of three OSCs. The levels of their mRNAs correlated with the accumulation of respective end products, suggesting that the transcription of OSCs is an important regulatory step for the triterpene biosynthesis.

The site of glycyrrhizin biosynthesis is localized in the thickened roots and stolons of *G. glabra* (Fuggersberger-Heinz and Franz 1984; Hayashi *et al*. 1998), and the seasonal variation of the incorporation of $^{14}$C-mevalonic acid into the glycyrrhizin fraction by the root segments was observed (Hayashi *et al*. 1998). The incorporation rate was high in May, June, and September, and low in August and winter. The seasonal variation of bAS mRNA level in the thickened roots was also observed (Hayashi *et al*. 2004). As shown in **Fig. 2**, the level of bAS mRNA in thickened roots was high in May, June, July and September, when the aerial parts were growing. The level of bAS mRNA was low in August, when many leaves abscised due to the high temperature and high humidity in Japan, and in winter when the aerial parts were inactive. These results indicate that the existence of the active aerial parts is necessary for the expression of bAS mRNA and glycyrrhizin biosynthesis in the thickened roots. The level of CAS mRNA was relatively constant even in winter, suggesting that CAS is a housekeeping enzyme. The level of LUS mRNA was not detectable in the thickened roots.

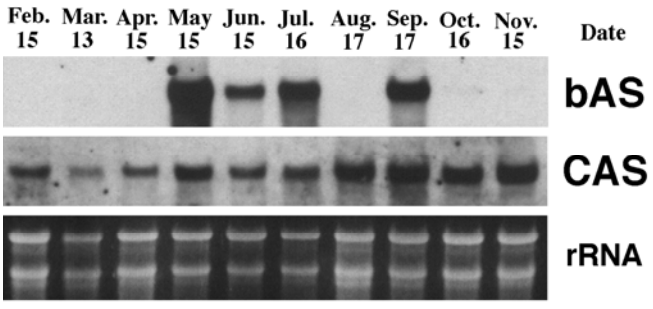


**Fig. 2 Seasonal variation of mRNA levels for bAS and CAS in the thickened main roots of *Glycyrrhiza glabra*.** Reproduced from **Hayashi *et al.*** (2004) *Biological and Pharmaceutical Bulletin* **27**, 1090, **Fig. 6**, with kind permission of The Pharmaceutical Society of Japan.

## Regulation of triterpene biosynthesis in cultured licorice cells

Since the mRNA levels of bAS and LUS were highly regulated in the intact plant of *G. glabra*, it is of interest to elucidate signaling molecules involved in the regulation of triterpene biosynthesis. Thus, the effects of various plant hormones on the mRNA levels of three OSCs in the cultured licorice cells were examined (Hayashi *et al*. 2003, 2004).

As shown in **Fig. 3**, the mRNA levels of three OSCs in the cultured licorice cells were differentially regulated by two plant hormones, methyl jasmonate (MeJA) and gibberellin $A_3$ ($GA_3$). Exogenously applied MeJA stimulated soyasaponin biosynthesis in the cultured licorice cells (Hayashi *et al*. 2003), and the mRNA level of bAS was up-regulated by MeJA, In addition, two mRNAs of SQS, an enzyme common to both triterpene and phytosterol biosyntheses, were also up-regulated by MeJA. Accumulations of bAS and SQS mRNAs were not transient but lasted for 7 days after exposure to MeJA (**Fig. 4**), resulting in a high level of accumulation (more than 2% of dry weight) of soyasaponins (Hayashi *et al*. 2003). By contrast, the mRNA level of LUS was down-regulated by MeJA, whereas the mRNA level of CAS was relatively constant. Furthermore, enzyme activity of UDP-glucuronic acid: soyasapogenol B glucuronyltransferase, an enzyme involved in a later step of soyasaponin biosynthesis, was also up-regulated by MeJA, suggesting that the transcription of other enzymes involved in the late steps of soyasaponin biosynthesis might be up-regulated by MeJA in the cultured licorice cells. On the other hand, exogenous $GA_3$ down-regulated the mRNA level of bAS but not those of LUS and CAS, suggesting that gibberellins may be negative regulators of the oleanane-type triterpene biosynthesis in *G. glabra* (Hayashi *et al*. 2004). Further experiments are underway to elucidate the effects of MeJA and $GA_3$ on biosynthesis of glycyrrhizin.

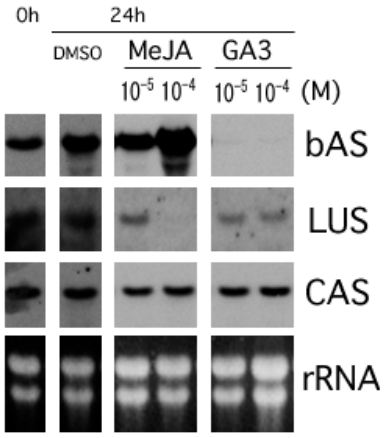


**Fig. 3 Effects of MeJA and $GA_3$ on mRNA levels for bAS, LUS and CAS in cultured licorice cells.** Reproduced from Hayashi *et al*. (2004) *Biological and Pharmaceutical Bulletin* **27**, 1091, **Fig. 7**, with kind permission of The Pharmaceu-tical Society of Japan.

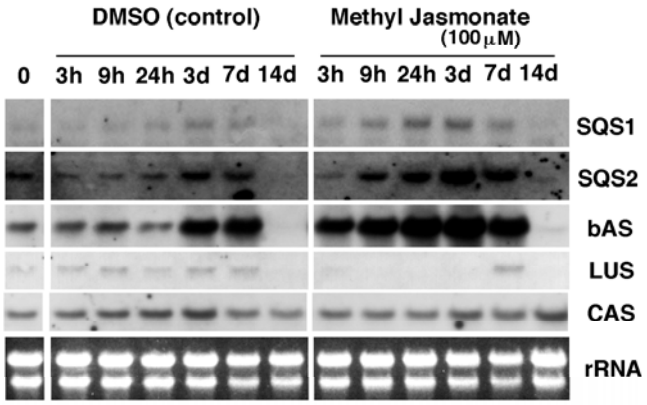


**Fig. 4 Time course of accumulation of SQS1, SQS2, bAS, LUS and CAS mRNAs in cultured licorice cells treated with MeJA. MeJA (100 mM) was added media on day 10 of culture.** Reproduced from Hayashi *et al*. (2003) *Plant and Cell Physiology* **44**, 408, **Fig. 5**, with kind permission of The Japanese Society of Plant Physiologists.

Yeast extract was shown to induce the flavonoid biosynthesis in cultured licorice cells (Ayabe *et al*. 1986; Nakamura *et al*. 1999), and mRNA level of polyketide reductase, an enzyme involved in 5-deoxyflavonoid biosynthesis in legumes, was transiently up-regulated by both yeast extract and MeJA (Hayashi *et al*. 2003). However, the mRNA levels of SQS and bAS were coordinately down-regulated by yeast extract in cultured licorice cells (Hayashi *et al*. 2003). The opposite effects of MeJA and yeast extract on the levels of bAS and SQS mRNA levels suggested that the signaling pathway leading to activation of soyasaponin biosynthesis is different from that of the flavonoid biosynthesis.

## CONCLUDING REMARKS

*G. glabra* produce diverse triterpenoids which are localized in the specific organs of the intact plants. Although the cultured licorice cells produce soyasaponins and betulinic acid, they produce no detectable amount of glycyrrhizin. To overcome this difficulty in producing glycyrrhizin by plant cell cultures, it is necessary to elucidate the regulation of triterpenoid biosynthesis of *G. glabra*. Molecular cloning of bAS and SQS genes involved in saponin biosynthesis revealed that the transcriptions of these genes are developmentally regulated in the different organs of *G. glabra*. Up-regulation of bAS and SQS mRNAs by MeJA awaken our expectation that the transcription of other enzymes involved in oxidations and glycosylations of β-amyrin leading to soyasaponins (or glycyrrhizin) might be up-regulated by MeJA in *G. glabra*. Identificatin of MeJA-inducing genes by microarray analysis in two model legumes, *Lotus japonicus* and *Medicago truncatula*, might be a powerful tool to identify genes involved in oxidation and glycosylation in saponin biosynthesis (Suzuki *et al*. 2002; Iturbe-Ormaetxe *et al*. 2003). Further experiments are underway to isolate these genes from *G. glabra*.

## ACKNOWLEDGEMENTS

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## JAPANESE ABSTRACT

生薬「甘草」はマメ科のカンゾウ属植物の地下部であり、その甘味成分はオレアナン型トリテルペン系サポニンのグリチルリチンである。カンゾウの幼植物から誘導した培養細胞はグリチルリチンを生産しなかったが、同じトリテルペン系化合物である大豆サポニンとベツリン酸を生産した。カンゾウ植物体におけるこれら３種の成分の局在部位はそれぞれ異なっていたことから、その生合成調節機構の解明を目的として、生合成の分岐点に位置する３種類のオキシドスクアレン閉環酵素遺伝子を解析した。これら酵素遺伝子のmRNA発現レベルは、それぞれの最終産物の生合成に対応して器官特異的、時期特異的に調節されていた。培養細胞にジャスモン酸メチルを投与すると、大豆サポニン生合成に関与するオキシドスクアレン閉環酵素の遺伝子発現が誘導された。今後、このジャスモン酸メチル誘導性を指標として、大豆サポニン生合成の後半に関与する未知の酵素遺伝子の同定が期待される。