Variation of Glycyrrhizin and Liquiritin Contents within a Population of 5-Year-Old Licorice (Glycyrrhiza uralensis) Plants Cultivated under the Same Conditions

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Cultivated licorice plants (Glycyrrhiza uralensis Fisch.) contain smaller amounts of the triterpene saponin glycyrrhizin than wild licorice plants. To resolve this problem and to breed strains with high-glycyrrhizin content we determined the glycyrrhizin content of 100 samples of G. uralensis that were propagated from seed and grown under the same conditions in the field for 5 years. There was a 10.2-fold variation in glycyrrhizin content among these plants, ranging from 0.46 to 4.67% (average 2.11±0.90%). There was also a wide variation in liquiritin content, ranging from 0.11 to 2.65% (average 1.00±0.49%). The glycyrrhizin content was positively correlated with that of liquiritin in the taproots (r=0.5525). Our results indicate that there are various genetic strains for glycyrrhizin and liquiritin synthesis within a population of plants propagated from seed. The selected high-glycyrrhizin and liquiritin strains will be useful for licorice production and studies on biosynthetic analysis of glycyrrhizin and liquiritin.

Key words Glycyrrhiza uralensis; glycyrrhizin; liquiritin; Glycyrrhizae Radix; Leguminosae; licorice

Glycyrrhiza Radix, the underground material derived from licorice, Glycyrrhiza uralensis Fisch. (Leguminosae) and some other Glycyrrhiza species, is extensively used as an herbal medicine in worldwide.1) Biological activities of licorice have been attributed to glycyrrhizin, liquiritin (Fig. 1), and other compounds. G. uralensis is a perennial herb that grows primarily in the semi-arid zones of Asia. Licorice products are derived from wild or cultivated G. uralensis from China or other Asian countries. In recent decades, however, over-harvest has gradually exhausted the natural resources in these regions, and several habitats are undergoing desertification. Thus, it is important to optimized cultivation conditions of G. uralensis to adequately substitute for the collection of wild resources. In particular, high quality and high glycyrrhizin content are desirable attributes of G. uralensis for medicinal use. According to the Japanese Pharmacopoeia, the glycyrrhizin content of Glycyrrhizae Radix for medicinal use must be at least 2.5%.2) World Health Organization guidelines specify that glycyrrhizin content should be at least 4% for Glycyrrhizae Radix.3) However, the glycyrrhizin content is lower (<2.5%) in cultivated plants of G. uralensis than that in wild ones.3) Therefore, there is a demand for improved cultivars with high glycyrrhizin contents. Generally, there is wide genetic variation among plants propagated from seed, but there is no clear information on the variation in glycyrrhizin and liquiritin contents among seed-generated G. uralensis.

In this study, we hypothesized that there are various genetic strains within a population of G. uralensis grown from seed, and that some individuals would show higher glycyrrhizin and liquiritin contents. Accordingly, these could be selected to obtain a high-glycyrrhizin and high-liquiritin strain.

Plant Materials and Cultivation Conditions Glycyrrhiza uralensis Fisch. plants were cultivated in a field for 5 years. A voucher specimen (Accession No. HK 15739-09) has been deposited in the Herbarium of the Division of Hokkaido, Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation, Japan (Hokkaido Division, NIBIO). Approximately 7000 seeds were sown on 7 June 2004 in the agricultural research field of the Hokkaido Division, NIBIO (Hokkaido, Japan, 44°21′N, 142°27′E). The average annual temperature at the field site is 6.1 °C (range, −28.6 to 33.3 °C; Japan Meteorological Agency) and the rainfall is 891 mm (2004—2008). The plants were grown at a density of 12.5 plants/m2 (row spacing, 80 cm; plant spacing, 10 cm). Prior to planting, manure (20000 kg/ha), calcium carbonate (1000 kg/ha), and fertilizer (NPK 56:56:56 kg/ha) were applied to the field. Additional fertilizers were applied as follows: NPK 12:88:60 kg/ha in August 2004; calcium carbonate 10000 kg/ha and NPK 84:84:84 kg/ha in May 2005; NPK 18:132:90 kg/ha in August 2005; calcium carbonate 10000 kg/ha and NPK 84:84:84 kg/ha in May 2006; NPK 18:132:90 kg/ha in August 2006; and calcium carbonate 10000 kg/ha and NPK 126:126:126 kg/ha in May 2007. The underground parts of 600 plants were unintentionally dug up on 27 August 2008.
The HPLC system consisted of an LC-2000 Plus system for analysis. The taproot pieces were dried (50 °C) and ground to a fine powder (<150 μm). Quantitative analysis by HPLC of glycyrrhizin was performed according to the procedure in the Japanese Pharmacopoeia\(^3\) with minor modifications. Dried samples (250 mg) were extracted in 50% EtOH (12.5 ml) with reciprocal shaking (150 rpm) for 15 min, and then ultrasonicated for 10 min. After centrifugation at 2500 g for 10 min, the supernatant was collected. This was repeated twice and extracts were made up to a final volume of 25 ml. A volume of 20 μl was used for HPLC analysis. The HPLC system consisted of an LC-2000 Plus system (JASCO, Tokyo, Japan), a TSKgel ODS-80T\(_S\) QA column (5 μm, 150×4.6 mm, TOSOH, Tokyo, Japan), and a MightySil guard column RP-18 GP 5-4.6 (Kanto Kagaku, Tokyo, Japan) with column temperature of 30 °C. A solvent system consisting of 2% (v/v) acetic acid:CH\(_3\)CN (60:40) was used at a flow rate of 0.7 ml/min. Elution of compounds was monitored by measuring absorbance at 257 nm. A liquiritin standard was purchased from Kishida Chemical Co. (Osaka, Japan).

### RESULTS

**Glycyrrhizin Content**

Glycyrrhizin contents of the 100 samples of *G. uralensis* that were grown under the same conditions for 5 years are shown in Fig. 3A. There was a wide variation in glycyrrhizin content; the highest was 4.67% (plant No. 79), 10.2-fold higher than the lowest (0.46% in No. 21). The average glycyrrhizin content was 2.11 ± 0.90%. Of the 100 samples of *G. uralensis*, five had glycyrrhizin contents of 0—1%, 47 had 1—2%, 32 had 2—3%, 9 had 3—4%, and 7 plants had 4—5%.

**Root Weight**

The fresh root weight (whole root, Fig. 2) of each of the 100 samples of *G. uralensis* was recorded not to dry within 24 h after digging up (data not shown). There were considerable differences in the whole root weights of the plants. The average whole root fresh weight was 163.6 ± 61.0 g, with a 5.9-fold difference between the highest (431.6 g, No. 15) and the lowest (73.0 g, No. 53). There was no clear relationship between glycyrrhizin content and whole root weight \(r^2=0.0082, \text{Pearson's correlation coefficient}\). The taproot diameter of each of the 100 samples of *G. uralensis* was also recorded (data not shown). Taproot diameter ranged from 18.1 mm (No. 53) to 36.0 mm (No. 15), with an average of 26.5 ± 3.9 mm. There was a positive correlation between whole root weight and taproot diameter \(r^2=0.6532\). However, there was no clear correlation between glycyrrhizin content and taproot diameter \(r^2=0.0205\).

**Liquiritin Content**

The liquiritin contents of the taproots are shown in Fig. 3B. There was a wide variation in liquiritin content among the 100 samples of *G. uralensis*. The highest liquiritin content was 2.65% (No. 15), 24.1-fold higher than the lowest (0.11% in No. 55). The average liquiritin content was 1.00 ± 0.49%. Of the 100 samples of *G. uralensis*, 56 had liquiritin contents of 0—1%, 41 had 1—2%, and three had 2—3%. Liquiritin content was not correlated with whole root fresh weight \(r^2=0.0042\) or taproot diameter \(r^2=0.0303\).

**Relationship between Glycyrrhizin Content and Liquiritin Content**

The glycyrrhizin content was positively correlated with that of liquiritin in the taproots (Fig. 4, \(r^2=0.5525\)).

### DISCUSSION

In this study, we observed wide variations in glycyrrhizin and liquiritin contents among 100 samples of 5-year-old cultivated *Glycyrrhiza uralensis*. It is thought that the environmental conditions for cultivation, genetic characteristics of each plant influence the differences in secondary metabolites production. We think that the differences of each content of glycyrrhizin and liquiritin in this study is chiefly caused by a difference of the biosynthesis ability at each gene level. Glycyrrhizin is generated with the triterpenoid biosynthetic pathway. In *Glycyrrhiza* plants, the biosynthesis of glycyrrhizin involves the initial cyclization of 2,3-oxidosqualene to a triterpene, β-amyrin, followed by a series of oxidative reactions and glucuronylations. Genes for
two of the key enzymes in this pathway were discovered in *G. glabra*: *GgSQS1* encodes squalene synthase, which catalyzes the synthesis of 2,3-oxidosqualene from squalene, and *GgbAS1* encodes β-amyrin synthase, which produces β-amyrin from 2,3-oxidosqualene.6,7) The *CYP88D6* gene, which was isolated from *G. uralensis*, encodes a cytochrome P450 monooxygenase that catalyzes the two-step oxidation of β-amyrin at C-11 to produce 11-oxo-β-amyrin.8) Recently, Sudo *et al.* analyzed 56857 expressed sequence tags of *G. uralensis*.9) On the other hand, liquiritin is generated with the flavonoid synthetic pathway. Studies on the roles of cytochrome P450s in the production of flavonoids (to which class liquiritin belongs) are also progressed in *Glycyrrhiza* plants.10) The glycyrrhizin content was positively correlated with that of liquiritin in the present study, even though glycyrrhizin and liquiritin are synthesized via different biosynthetic pathways. It is assumed that the each biosynthetic pathway of the triterpenoid and flavonoid takes part in parallel or the upstream part in these two biosynthetic pathways takes part in the biosynthesis regulations of glycyrrhizin and liquiritin. However, the detail reason for the positive correlation between glycyrrhizin and liquiritin content is unclear now. These phenomena in this study will be further clarified by genetic analysis of secondary metabolites synthesis including glycyrrhizin and liquiritin. We expect that the present results will contribute to such biosynthetic studies.

We are propagating the selected high contents of glycyrrhizin and liquiritin plants by stolon cuttings (Fig. 2) and *in vitro* micro-propagation using tissue culture.11) Thus, it may be possible to produce high quality strains of *G. uralensis* with high glycyrrhizin and high liquiritin contents. The positive correlation between glycyrrhizin and liquiritin content is important information for selection or breeding of high glycyrrhizin and high liquiritin strains. There has been no report on the cultivation of *Glycyrrhiza* plants with only high content of glycyrrhizin. Yamamoto and Tani4) reported glycyrrhizin contents of 1.52±0.57% in taproots of cultivated 4-year-old *G. uralensis*. This level is lower than that required for medicinal use. The result may be due to the contamination with low glycyrrhizin individuals. By using cloned plants of the selected strains in our study, it will be possible to clarify the effects of environmental factors such as cultivation period, fertilizer, temperature, etc. on glycyrrhizin and liquiritin production.

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