Phylogenetic Relationship of Six *Glycyrrhiza* Species Based on *rbcL* Sequences and Chemical Constituents¹⁾

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The nucleotide sequences of ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbcL*) of *Glycyrrhiza glabra*, *G. uralensis*, *G. inflata*, *G. echinata*, *G. macedonica* and *G. pallidiflora* have been determined to construct their phylogenetic tree. Based on these sequences, the six *Glycyrrhiza* species were divided into two groups: three, *G. glabra*, *G. uralensis*, and *G. inflata*, which produce glycyrrhizin as a major saponin, and the others, *G. echinata*, *G. macedonica* and *G. pallidiflora*, which produce macedonoside C as a major saponin. Among the three glycyrrhizin-producing species, only two nucleotide substitutions were observed between the *rbcL* sequences of *G. glabra* and *G. uralensis*, and the sequence of *G. uralensis* was identical to that of *G. inflata*, indicating that *G. uralensis* and *G. inflata* are closely related. Among the three macedonoside C-producing species, only one nucleotide substitution was observed between those of *G. echinata* and *G. macedonica*, indicating that these two species are also closely related.

Key words Leguminosae; *Glycyrrhiza*; *rbcL*; glycyrrhizin; macedonoside C; phylogenetic tree

Licorice, roots and stolons of the *Glycyrrhiza* plants, *G. glabra* L., *G. uralensis* FISCH. and *G. inflata* BATAL., contain a large amount of glycyrrhizin (GL), which is a well recognized natural sweetener and pharmaceutical.²⁾ Some *Glycyrrhiza* species, such as *G. echinata* L., *G. macedonica* BOISS. et ORPH and *G. pallidiflora* MAXIM., however, do not contain GL.³⁾ It is of interest to elucidate the phylogenetic relationship among these species from a viewpoint of the evolution of GL biosynthesis. Recently, nucleotide sequences of a chloroplast gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*) were used to produce phylogenetic trees of higher plants,⁴⁾ and comprehensive phylogenetic studies of legume *rbcL* sequences were also reported.^{5,6)} In a preliminary communication,¹⁾ we determined the *rbcL* sequences of three GL-producing and two GL-non-producing *Glycyrrhiza* species. In this paper, the phylogenetic relationship based on their chemical constituents and *rbcL* sequences of six *Glycyrrhiza* species will be discussed.

MATERIALS AND METHODS

Plant Materials Seeds of *G. glabra* and *G. echinata* (collected in Turkey) were obtained from the medicinal plant garden of Kyoto University. Seeds of *G. uralensis* were obtained from the medicinal plant garden of Chiba University. Seeds of *G. pallidiflora* and *G. macedonica* were obtained from National Institute of Health Sciences, Japan, and a plant of *G. inflata* was obtained from Maruzen Pharmaceuticals Co., Ltd. All these species were cultivated in the medicinal plant garden of Niigata College of Pharmacy, and their leaves and roots were harvested for analysis.

Amplification and Sequencing of *rbcL* Gene Total DNA was extracted from the fresh leaves by the cetyltrimethylammonium bromide (CTAB) DNA extraction method as reported previously.⁷⁾ The 1374-bp DNA fragment covering most of the *rbcL* sequences was amplified by the

polymerase chain reaction (PCR) using total DNA, Taq-DNA polymerase (Takara, Japan), and two primers, 5'-ATGTCAC-CACAAACAGAACTAAAGC-3' and 5'-AGCAGCAGC-TAATTCAGGACTCCA-3'. After initial denaturation (2 min at 95 °C), 25 cycles of 40 s at 95 °C, 40 s at 55 °C and 2 min at 72 °C were performed on a thermocycler. The amplified fragment was purified by 1% agarose gel electrophoresis and GeneClean II kit (BIO 101 Inc., U.S.A.). The purified fragments were sequenced directly by the dideoxy chain termination method using an ALFred DNA sequencer (Pharmacia). Nine internal primers, located at every 300–400 bp of both strands, were used for sequencing.

HPLC Analysis of Roots and Leaves Freeze-dried plant samples were ground with a mortar and pestle, and then 40 mg of each powdered sample was extracted with 1 ml of 80% methanol at 60 °C for 6 h. An aliquot (20 µl) of the extract was analyzed by photodiode-array HPLC as reported previously.⁸⁾ Conditions of HPLC were as follows: column, Capcellpak C18 AG-120A (5 µm, 4.6 mm i.d. × 250 mm, Shiseido, Japan); solvent, MeCN–H₂O (1% AcOH) gradient of 15% MeCN to 25% MeCN in 15 min, to 70% MeCN in another 35 min, then to 100% in 10 min; flow rate, 0.9 ml/min; column temp, 40 °C; detector, Shimadzu photodiode array SPD-6A system (Shimadzu, Japan). Quantities of constituents were determined on the basis of the peak area of UV absorption at 254 nm. Each constituent was identified by comparison of its retention time and UV spectrum with those of authentic sample.

RESULTS AND DISCUSSION

Comparison of Chemical Constituents in the Roots and Leaves of Six *Glycyrrhiza* Species As stated, the six *Glycyrrhiza* plants, i.e. *G. glabra*, *G. uralensis*, *G. inflata*, *G. echinata*, *G. macedonica* and *G. pallidiflora*, were cultivated in the medicinal plant garden of Niigata College of Pharmacy, and the roots and leaves were harvested for analysis of

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Fig. 1. HPLC Profiles of Methanol Extracts of the Roots of Six *Glycyrrhiza* Species
GB, glabridin; GC, glycy coumarin; GL, glycyrrhizin; LA, licochalcone A; MC, macedonoside C.

Table 1. Contents of Glycyrrhizin (GL), Macedonoside C (MC), Glabridin (GB), Glycycoumarin (GC) and Licochalcone A (LA) in Roots of *Glycyrrhiza* Plants

Species	Diameter (mm)	Contents (% of dry weight) of				
		GL	MC	GB	GC	LA
<i>G. glabra</i>	10.5	1.26	n.d.	0.11	n.d.	n.d.
<i>G. uralensis</i>	9.2	3.27	n.d.	n.d.	0.20	n.d.
<i>G. inflata</i>	6.4	2.49	n.d.	n.d.	n.d.	0.64
<i>G. echinata</i>	7.3	n.d.	1.19	n.d.	n.d.	n.d.
<i>G. macedonica</i>	6.7	n.d.	2.58	n.d.	n.d.	n.d.
<i>G. pallidiflora</i>	9.1	n.d.	2.33	n.d.	n.d.	n.d.

Fig. 2. Structures of Saponins and Flavonoids in the Roots of *Glycyrrhiza* Plants

chemical constituents and *rbcL* sequence. Figure 1 shows HPLC profiles of methanol extracts of the thickening roots of the six species and Table 1 shows the contents of two saponins, glycyrrhizin (GL) and macedonoside C (MC), and three flavonoids, glabridin (GB), glycycoumarin (GC) and licochalcone A (LC), known to be species-specific for *G. glabra*, *G. uralensis* and *G. inflata*, respectively, in the thickening roots.^{9,10} The structures of these compounds are shown in Fig. 2. In the thickening roots of *G. glabra*, *G. uralensis*

and *G. inflata*, GL was detected in common, and GB, GC and LA were specifically detected in the thickening roots of these three species, respectively. In the remaining three species, *G. echinata*, *G. macedonica* and *G. pallidiflora*, the HPLC profiles were closely similar, and MC, which was isolated for the first time from *G. macedonica*,³⁾ was detected instead of GL as the main saponin.

Next, HPLC analysis was made of methanol extracts of the leaves of the six *Glycyrrhiza* species and the HPLC profiles

Fig. 3. HPLC Profiles of Methanol Extracts of the Leaves of the Six *Glycyrrhiza* Species

AS, astragalin; CA, cacticin; IQ, isoquercitrin; KA, kaempferol-3-*O*-rhamnosylgalactoside; NA, narcissin; NI, nicotiflorin; RU, rutin; SC, schaftoside; VI, vicenin 2.

are shown in Fig. 3. The structures of flavonoid glycosides identified in this experiment are shown in Fig. 4. In the leaves of the three GL-producing species, which contained isoquercitrin (IQ) and astragalin (AS) as major flavonoid glycosides, the patterns of flavonoid glycoside constituents were similar, whereas in those of the three MC-producing species, the species-specific distribution of flavonoid glycosides was observed. Although the profiles of flavonoid glycosides were similar in the leaves of *G. echinata* and *G. macedonica*, the leaves of *G. echinata* contained narcissin (NA) instead of the AS present in *G. macedonica*. Both *G. echinata* and *G. macedonica* contained rutin (RU) as a major flavonoid glycoside, whereas the major flavonoid glycoside in *G. pallidiflora* was IQ. The HPLC profile of flavonoid glycoside in the leaves of *G. pallidiflora*, a MC-producing species, was different from those of the other two MC-producing species, *G. echinata* and *G. macedonica*: in *G. pallidiflora*, cacticin (CA) was detected as a species-specific flavonoid glycoside. These HPLC profiles of the leaves of the three MC-producing species were identical to those of previous reports.^{3,11)} Species-specific peaks were also observed at retention times of from 30 to 60 min in the HPLC profiles of all six species, respectively. Some of them have been identified as free

Fig. 4. Structures of Flavonoid Glycosides in the Leaves of *Glycyrrhiza* Plants

Table 2. Pairwise Genetic Distances between the Six *Glycyrrhiza* Species and *Abrus precatorius* Based on 1324 Nucleotides (without Primers) of the *rbcL* Gene

Species	(Accession no.)	1	2	3	4	5	6	7	8	9
1 <i>G. glabra</i>	(AB012125)	—	0.002	0.002	0.006	0.007	0.009	0.064	0.031	0.066
2 <i>G. uralensis</i>	(AB012126)	2	—	0.000	0.005	0.005	0.008	0.066	0.029	0.068
3 <i>G. inflata</i>	(AB012127)	2	0	—	0.005	0.005	0.008	0.066	0.029	0.068
4 <i>G. echinata</i>	(AB012128)	8	6	6	—	0.001	0.003	0.066	0.026	0.068
5 <i>G. macedonica</i>	(AB032424)	9	7	7	1	—	0.004	0.066	0.027	0.069
6 <i>G. pallidiflora</i>	(AB012129)	12	10	10	4	5	—	0.065	0.027	0.067
7 ?(<i>G. glabra</i>)	(Z70171)	85	87	87	87	88	86	—	0.042	0.004
8 ?(<i>A. precatorius</i>)	(Z70167)	41	39	39	35	36	36	56	—	0.044
9 <i>A. precatorius</i>	(U74224)*	87	89	89	89	90	88	5	58	—

* 1310 nucleotides were used for comparison with the other sequences.

Below diagonal: number of nucleotide substitutions; above diagonal: proportion of nucleotide positions differing between taxa (1.0=100%).

Fig. 5. Phylogenetic Tree Constructed from the Nucleotide Sequences of *rbcL* Genes of the Six *Glycyrrhiza* Species by the UPGMA method of Nei. The phylogenetic tree was constructed by Genetex software, Software Development, Japan. The database accession numbers are shown on the right.

flavonoids and isoflavonoids, and the variation of these constituents was also reported in *G. glabra*.^{11,12)}

Phylogenetic Relationship of Six *Glycyrrhiza* Species Based on *rbcL* Sequences Total DNA was extracted from the fresh leaves of the six *Glycyrrhiza* species as reported previously,⁷⁾ and the 1374-bp DNA fragment covering most of the *rbcL* sequence was amplified by PCR and sequenced.¹³⁾ Surprisingly, *rbcL* sequences of all six species determined in the present study were different from that of *G. glabra* (accession No. Z70171) reported by Häss and Wink.⁵⁾ Doyle *et al.*⁶⁾ reported that the *rbcL* sequence of *Abrus precatorius* L. (accession No. U74224) was very similar to that of *G. glabra* (accession No. Z70171) by Häss and Wink,⁵⁾ and different from that of *A. precatorius* (accession No. Z70167) by Häss and Wink.⁵⁾ These results are attributed to a simple switch of *G. glabra* and *A. precatorius* sequences. However, the sequence of accession No. Z70167 is also different from that of *G. glabra* obtained in the present study, which is very similar to *rbcL* sequences of the other five *Glycyrrhiza* species. This suggests that the original plant for accession No. Z70167 is not *G. glabra*, but is somewhat related to the *Glycyrrhiza* species. Table 2 shows the pairwise genetic distances based on the *rbcL* sequences. A phylogenetic tree (Fig. 5) was constructed from the nucleotide sequences of the *rbcL* gene using the unweighted pair-group method using arithmetic averages (UPGMA method) of Nei¹⁴⁾ (Genetex software, Software Development, Japan). The six *Glycyrrhiza* species were divided into two groups: the three GL-producing species, *G. glabra*, *G. uralensis*, and *G.*

inflata, and the three MC-producing species, *G. echinata*, *G. macedonica* and *G. pallidiflora*. The result coincides with a phylogenetic tree suggested by RFLP analyses of four *Glycyrrhiza* species, i.e. *G. glabra*, *G. uralensis*, *G. echinata* and *G. pallidiflora*.¹⁵⁾ It is noteworthy that the *rbcL* sequence of *G. uralensis* was identical to that of *G. inflata*, indicating that *G. uralensis* and *G. inflata* are closely related, and that only two nucleotide substitutions were observed between *G. glabra* and *G. uralensis*. The *rbcL* sequences were also similar among the GL-non-producing species, which contain MC in the roots. Only one nucleotide substitution was observed between *G. echinata* and *G. macedonica*, and their HPLC profiles of the thickening roots and leaves were also very similar. These results indicate that the two species are closely related. The present study indicates that the phylogenetic relationship deduced from their *rbcL* sequence is in accordance with the similarity in their chemical constituents. The *rbcL* sequence is a good marker to identify the GL-non-producing *Glycyrrhiza* species. It is difficult, however, to distinguish the various strains of GL-producing species by this sequence since it is very similar in all of them.

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