Phylogenetic Relationship of *Glycyrrhiza* Plants Based on *rbcL* Sequences

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The nucleotide sequences of the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbcL*) of *Glycyrrhiza glabra*, *G. uralensis*, *G. inflata*, *G. echinata*, and *G. pallidiflora* have been determined to construct the phylogenetic tree. In the phylogenetic tree based on the *rbcL* sequences, the five *Glycyrrhiza* species were divided into two groups: the three glycyrrhizin-producing species *G. glabra*, *G. uralensis*, and *G. inflata*; and the two glycyrrhizin-nonproducing species *G. echinata* and *G. pallidiflora*. Among the three glycyrrhizin-producing species, only two nucleotide substitutions were observed between the *rbcL* sequence of *G. glabra* and *G. uralensis*, and the sequence of *G. uralensis* was identical to that of *G. inflata*, indicating that the three glycyrrhizin-producing species are closely related.

Key Words Leguminosae; *Glycyrrhiza*; *rbcL*; sequencing; glycyrrhizin; phylogenetic tree

The roots and stolons of *Glycyrrhiza* plants, e.g., *G. glabra* L., *G. uralensis* FISCH. and *G. inflata* BATAL., contain large amounts of glycyrrhizin, which is a well-known natural sweetener and pharmaceutical. On the other hand, some *Glycyrrhiza* plants, such as *G. echinata* L. and *G. pallidiflora* MAXIM., do not contain glycyrrhizin. It is of interest to elucidate the phylogenetic relationship among these *Glycyrrhiza* plants from the viewpoint of the evolution of glycyrrhizin 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.²⁾ Comprehensive phylogenetic studies of legume *rbcL* sequences were also reported.^{3,4)} Although the *rbcL* sequence of *G. glabra* was reported,³⁾ this sequence was revealed to be confused.⁴⁾ In the present study, the *rbcL* sequences of three glycyrrhizin-producing and two glycyrrhizin-nonproducing *Glycyrrhiza* species were determined, and their phylogenetic relationships based on the *rbcL* sequences are discussed.

We analyzed the *rbcL* sequences of *G. glabra*, *G. uralensis*, *G. inflata*, *G. echinata*, and *G. pallidiflora* cultivated in the medicinal plant garden of the Niigata College of Pharmacy. Total DNA was extracted from the fresh leaves as reported previously,⁵⁾ and the 1374-bp DNA fragment covering most of the *rbcL* sequences was amplified by the polymerase chain reaction using total DNA, Taq-polymerase, and two primers, 5'-ATGTCACCACAAACAGAACTAAAGC-3' and 5'-AGCAGCAGCTAATTCAGGACTCCA-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 a GENECLEAN II kit (BIO 101 Inc.), and 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.

Table 1 shows the pairwise genetic distances based on the *rbcL* sequences. The *rbcL* sequence of *G. uralensis* was identical to that of *G. inflata*, and only two nucleotide substitutions were observed between *G. glabra* and *G. uralensis*. Surprisingly, all *Glycyrrhiza rbcL* sequences in the present study were different from that of the *G. glabra rbcL* (accession No. Z70171) reported by Häss and Wink.³⁾ Doyle *et al.*⁴⁾ reported that the *rbcL* sequence of *Abrus precatorius* L. (accession No. U74224) was very similar to that of the *G. glabra* (accession No. Z70171) reported by Häss and Wink,³⁾ and different from that of the *A. precatorius* (accession No. Z70167) reported by Häss and Wink.³⁾ These results might be attributed to a simple switch of the *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. Since our sequence of *G. glabra* is very similar to the *rbcL* sequences of other *Glycyrrhiza* species, the original plant for accession No. Z70167 is suggested to be different from *G. glabra*, but somewhat related to the *Glycyrrhiza* plants.

A phylogenetic tree (Fig. 1) was constructed from the nucleotide sequences of the *rbcL* gene using the UPGMA

Table 1. Pairwise Genetic Distances between the Five *Glycyrrhiza* Species and *Abrus precatorius* Based on 1324 Nucleotides (without Primers) of the *rbcL* Gene. Below diagonal: numbers of nucleotide substitutions; above diagonal: proportion of nucleotide positions differing between taxa (1.0=100%)

Species	(accession Nos.)	1	2	3	4	5	6	7	8
1 <i>G. glabra</i>	(AB012125)	—	0.002	0.002	0.006	0.009	0.064	0.031	0.066
2 <i>G. uralensis</i>	(AB012126)	2	—	0.000	0.005	0.008	0.066	0.029	0.068
3 <i>G. inflata</i>	(AB012127)	2	0	—	0.005	0.008	0.066	0.029	0.068
4 <i>G. echinata</i>	(AB012128)	8	6	6	—	0.003	0.066	0.026	0.068
5 <i>G. pallidiflora</i>	(AB012129)	12	10	10	4	—	0.065	0.027	0.067
6 ?(<i>G. glabra</i>)	(Z70171) ³⁾	85	87	87	87	86	—	0.042	0.004
7 ?(<i>A. precatorius</i>)	(Z70167) ³⁾	41	39	39	35	36	56	—	0.044
8 <i>A. precatorius</i>	(U74224) ^{*,4)}	87	89	89	89	88	5	58	—

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

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Fig. 1. Phylogenetic Tree Constructed from the Nucleotide Sequences of *rbcL* Genes. The phylogenetic tree was constructed using the UPGMA method of Nei⁶⁾ (GENETYX software, Software Development, Japan). The database accession numbers are shown on the right.

method of Nei⁶⁾ (GENETYX software, Software Development). The five *Glycyrrhiza* plants were divided into two groups: the three glycyrrhizin-producing species *G. glabra*, *G. uralensis*, and *G. inflata*; and the two glycyrrhizin-nonproducing species, *G. echinata* and *G. pallidiflora*. These results coincide with the phylogenetic tree suggested by RFLP and RAPD analyses of *Glycyrrhiza* plants.⁷⁾ Although the *rbcL* sequences are very similar among the glycyrrhizin-producing species, the *rbcL* sequence would be a good marker to identify the glycyrrhizin-nonproducing *Glycyrrhiza* plants. Many other *Glycyrrhiza* species are known in the worldwide, and experiments are underway to determine the *rbcL* sequence of other *Glycyrrhiza* species.

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