Field Survey of *Glycyrrhiza* Plants in Central Asia (1). Characterization of *G. uralensis*, *G. glabra* and the Putative Intermediate Collected in Kazakhstan

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The characteristics of *Glycyrrhiza* plants from 12 collection sites in southeastern Kazakhstan were investigated. *G. uralensis* was observed at 9 of the sites from Almaty to Shu, and *G. glabra* was observed at 8 sites. At 4 sites near Shu, and 1 site near Almaty, *G. glabra* and *G. uralensis* grew together forming a mixed population, and intermediate-type plants between them were also observed at 3 sites. Although two nucleotide substitutions of the chloroplast *rbcL* gene were observed between *G. uralensis* and *G. glabra*, *rbcL* sequences of the intermediate-types were divided into *G. uralensis*-type (G-A type) and *G. glabra*-type (A-T type). HPLC analysis of the roots indicated that species-specific flavonoids, glabridin and glycycomarin, were detected in the roots of *G. glabra* and *G. uralensis*, respectively, but neither flavonoid was detected in underground parts of the intermediate-types. HPLC analysis of their leaves indicated a significant difference among *G. uralensis*, *G. glabra* and the intermediate-type plants. Both *G. glabra*-specific and *G. uralensis*-specific compounds were detected in the leaves of the intermediate-type, thus suggesting that the intermediate plants are hybrids of *G. glabra* and *G. uralensis*.

Key words licorice; *Glycyrrhiza uralensis*; *Glycyrrhiza glabra*; species-specific flavonoid

Licorice is one of the most important crude drugs in the world, and its major sweet constituent, glycyrrhizin (GL), is used in large quantities as a well-known natural sweetener and as a pharmaceutical.^{1,2)} Two *Glycyrrhiza* species, *G. glabra* and *G. uralensis*, are known as major GL-producing species, but their distribution in the world is different.²⁾ *G. glabra* is found in Spain, Italy, Turkey, the Caucasus, Central Asia and the western part of China, whereas the distribution area of *G. uralensis* is from Central Asia to China. In addition to these two species, *G. inflata*, another GL-producing species, is found in the western part of China.

Extensive chemical studies revealed that licorice roots contain many saponins and flavonoids, along with GL.^{2,3)} In addition, many species-specific flavonoids, such as glabridin (GB) for *G. glabra* and glycycomarin (GC) for *G. uralensis*, were detected in the underground parts of respective *Glycyrrhiza* species.^{3–7)} Variation in flavonoid contents in leaves of *G. glabra* was also reported.^{8,9)} These variations in roots and leaves are very useful to identify the species of plant materials, but their relationships between morphological^{10,11)} and chemical^{3–9)} characteristics have not yet been elucidated in detail.⁶⁾

Both *G. glabra* and *G. uralensis* are distributed in Central Asia as well as in the western region of China,^{2,10)} and both plants are capable of generating hybrids,¹²⁾ although their chemical characteristics were not reported. Thus, in order to compare the morphological and chemical characteristics of *Glycyrrhiza* plants, a field survey of these plants was performed in Kazakhstan, one of the countries of Central Asia.

MATERIALS AND METHODS

Plant Materials Underground parts, leaves and seeds of *Glycyrrhiza* plants used in the present study were collected in Kazakhstan in August, 2001, as shown in Fig. 1 and Table 1.

Chemicals Authentic samples of GL, GB and GC were obtained from Maruzen Pharmaceuticals, Japan. Isoquercitrin (IQ) and rutin (RT) were purchased from Extrasynthèse, France. Pinocembrin (PN) and licoflavanone (LF) were isolated from the leaves of *G. glabra*.¹³⁾

Amplification and Sequencing of *rbcL* Gene DNA was extracted from dry leaves by the cetyltrimethylammonium bromide (CTAB) DNA extraction method as reported previously.¹⁴⁾ The 240-bp DNA fragment covering the two nucleotide substitutions in the *rbcL* gene between *G. glabra* and *G. uralensis*⁶⁾ was amplified by the polymerase chain reaction (PCR) using the template DNA from leaves, Taq-DNA polymerase (Takara), *anti*-Taq high (Toyobo) and two primers, 5'-TTTATGCGTTGGAGAGACCG-3' and 5'-AAGTAGAC-CATTATCTCGGC-3'. After initial denaturation (2 min at 95 °C), 30 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C were performed on a thermocycler. The amplified fragment was treated with ExoSAP-IT (Amersham Pharmacia Biotech) to remove primers. The purified fragments were sequenced directly by the dideoxy chain termination method using a model 373A DNA sequencer (PE Biosystems).

HPLC Analysis of Roots and Leaves Dried plant samples were powdered with a mortar and pestle, and then 200 mg of each powdered sample was extracted with 5 ml of 80% methanol at 60 °C for 2 h. An aliquot (10 µl) of the extract was analyzed by photodiode-array HPLC 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, 1 ml/min; column temp, 40 °C; detector, photodiode array SPD-M10A_{VP} system (Shimadzu). The quantities of constituents were determined on the basis of their peak area of UV absorption at 254 nm (GL), 280 nm (GB), 292 nm (PN, LF), 350 nm (GC, IQ, RT). Each constituent was identified by

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comparison of its retention time and UV spectrum with the respective authentic sample.

RESULTS

A Field Survey of *Glycyrrhiza* Plants from Almaty to Tashkent A field survey of *Glycyrrhiza* plants was carried out on the route from Almaty (Kazakhstan) to Tashkent (Uzbekistan) in 2001, and 24 *Glycyrrhiza* plants were collected at the 12 habitats shown in Fig. 1. Two species, *G. glabra* and *G. uralensis*, were discriminated on the basis of phenotypic changes of their fruits: the former species having straight pods and the latter falcated ones.¹⁰⁾ The morphological classification of *Glycyrrhiza* plants collected in Kazakhstan was first attempted based on phenotypic changes of

Fig. 1. Collection Sites of *Glycyrrhiza* Plants in Kazakhstan

A: Almaty; B: 15 km west of Almaty; C: 15 km east of Almaty; D: 2 km west of Samsy; E: 5 km west of Targan; F: 5 km northeast of Qurday; G: 5 km northwest of Qasyk; H: 5 km south of Blagoveshchenka; I: 20 km south of Shu; J: 5 km west of Aqyrtobe; K: 20 km southwest of Taraz; L: 5 km west of Zhaskeshu.

Table 1. Collection Sites of *Glycyrrhiza* Plants in the Field Survey in Kazakhstan

Date	Collection site	Plant No.	Species	Type of <i>rbcL</i> sequence
01.08.09	A	01A05	<i>G. uralensis</i>	n.d. ^{a)}
01.08.10	B	01A07	<i>G. uralensis</i>	n.d.
01.08.10	C	01A10	<i>G. uralensis</i>	G-A type
01.08.10	C	01A11	Intermediate	A-T type
01.08.10	C	01A12	<i>G. glabra</i>	A-T type
01.08.10	C	01A13	Intermediate	G-A type
01.08.11	D	01A14	<i>G. uralensis</i>	n.d.
01.08.11	E	01A15	<i>G. uralensis</i>	n.d.
01.08.11	F	01A16	<i>G. glabra</i>	n.d.
01.08.11	F	01A17	<i>G. uralensis</i>	n.d.
01.08.11	G	01A18	<i>G. uralensis</i>	n.d.
01.08.11	G	01A19	<i>G. glabra</i>	n.d.
01.08.11	G	01A20	<i>G. glabra</i>	n.d.
01.08.11	H	01A21	<i>G. glabra</i>	A-T type
01.08.11	H	01A22	<i>G. glabra</i>	A-T type
01.08.11	H	01A23	Intermediate	G-A type
01.08.11	H	01A24	Intermediate	A-T type
01.08.11	H	01A25	<i>G. uralensis</i>	G-A type
01.08.12	I	01A26	<i>G. uralensis</i>	G-A type
01.08.12	I	01A27	Intermediate	G-A type
01.08.12	I	01A28	<i>G. glabra</i>	A-T type
01.08.12	J	01A29	<i>G. glabra</i>	n.d.
01.08.12	K	01A30	<i>G. glabra</i>	n.d.
01.08.13	L	01A31	<i>G. glabra</i>	n.d.

a) Not determined.

the fruits. As shown in Table 1, *G. uralensis* plants were identified at 9 sites, and *G. glabra* at 8 sites. *G. uralensis* plants were exclusively observed at eastern collection sites A, B, D and E near Almaty, while only *G. glabra* plants were found at western collection sites J, K and L. At sites F, G, H and I near Bishkek, Kyrgyzstan, and site C near Almaty, *G. glabra* and *G. uralensis* grew together forming a mixed population. Putative intermediate-type plants between the two were also observed at sites C, H and I. Figure 2 shows the fruits of the three types of *Glycyrrhiza* plants collected in Kazakhstan. The fruit of the intermediate-type plant (01A24) was slightly falcated unlike those of *G. glabra* (01A30) and *G. uralensis* (01A07), suggesting that this plant is an intermediate form between the two species.

Next, the shapes of leaves of *G. uralensis*, *G. glabra* and the intermediate-type plants collected at collection site C were compared, as shown in Fig. 3. Leaflets of plant 01A10 were ovate in shape, typical of those of *G. uralensis*, whereas those of plant 01A12 were linear-oblong, characteristic of *G. glabra*. Leaves of plants 01A11 and 01A13 were intermediate between these two species. These two intermediate-type plants could be identified as *G. glabra* from the shape of their legumes¹⁰⁾ but were different from those of typical *G. glabra* collected at the other sites in Kazakhstan.

It is noteworthy that a mixed population of *G. uralensis*, *G. glabra* and intermediate-type plants was observed at collection site C near Almaty, whereas only *G. uralensis* was observed at the other sites A, B, D and E near Almaty. It is sug-

Fig. 2. Comparison of Fruits of *G. uralensis* (01A07), the Intermediate Plant (01A24) and *G. glabra* (01A30) Collected in Kazakhstan

Fig. 3. Comparison of Leaves of *Glycyrrhiza* Plants from Collection Site C

gested that *G. glabra* and the intermediate-type plants were accidentally introduced into this habitat. In contrast, the mixed population of *G. glabra* and *G. uralensis* was very common at sites F, G, H, and I. At site H, intermediate-type plants were the most common, followed by *G. uralensis*. Although typical *G. glabra* were rare at this habitat, two typical *G. glabra* plants, having echinulate fruits (01A21) or fruits without echines (01A22), were collected.

It is also noteworthy that the germination rate of seeds from the intermediate plant (01A27) collected at site I was high (70%), whereas those from other intermediates at collection sites C and H were low. It is of interest to elucidate the characteristics of the descendants of these intermediate-type plants, which might be important sources for breeding of *Glycyrrhiza* plants.

Comparison of Chloroplast *rbcL* Sequence In our previous work⁶⁾ we determined the nucleotide sequences of a chloroplast gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*) of *Glycyrrhiza* plants

to elucidate their phylogenetic relationship. Two nucleotide substitutions (in 1324 nucleotides) were observed between the *rbcL* gene sequences of *G. glabra* (A-T type) and *G. uralensis* (G-A type). Thus, the *rbcL* sequence determination might be useful for characterization of the intermediate-type plants collected in Kazakhstan.

DNA was isolated from dry leaves of *Glycyrrhiza* plants collected at sites C, H and I, where the intermediate-type plants were collected together with *G. glabra* and *G. uralensis*. The 240-bp DNA fragment covering the two nucleotide substitutions in the *rbcL* gene was amplified by PCR and sequenced. As shown in Table 1, the *rbcL* genes for *G. glabra* plants, having linear-oblong leaflets and straight fruits, from the three collection sites were identified as the A-T type sequence (*G. glabra*-type), and those of *G. uralensis* plants, having ovate leaflets and falcated fruits, were the G-A type sequence (*G. uralensis*-type). These are in accordance with our previous results.⁶⁾ Interestingly, *rbcL* genes of the intermediate-type plants were divided into *G. glabra*-type and *G.*

Table 2. Content of Glycyrrhizin (GL), Glabridin (GB) and Glycycomarin (GC) in Underground Parts of *Glycyrrhiza* Plants Collected in Kazakhstan

Site	Plant No.	Species	Root/Stolon	Diameter (mm)	Content (% of dry weight)		
					GL	GB	GC
B	01A07	<i>G. uralensis</i>	Root	23.0	3.55	n.d. ^{a)}	0.07
C	01A10	<i>G. uralensis</i>	Stolon	6.5	5.57	n.d.	0.20
C	01A11	Intermediate	Stolon	6.7	5.28	n.d.	n.d.
C	01A12	<i>G. glabra</i>	Stolon	6.9	2.14	0.28	n.d.
C	01A13	Intermediate	Stolon	8.0	3.69	n.d.	n.d.
H	01A24	Intermediate	Root	14.5	5.72	n.d.	n.d.
K	01A30	<i>G. glabra</i>	Stolon	5.4	2.16	0.34	n.d.

a) Not detected (including not identified by UV spectrum).

Table 3. Contents of Rutin (RT), Isoquercitrin (IQ), Pinocembrin (PN), Licoflavanone (LF) and Two Unidentified Compounds (1, 2) in the Leaves of *Glycyrrhiza* Plants Collected in Kazakhstan

Species	Site	Plant No.	Content (% of dry weight)				Relative ratio of peak area ^{a)}	
			RT	IQ	PN	LF	1	2
<i>G. uralensis</i>	A	01A05	0.11	0.19	n.d. ^{b)}	n.d.	100	55
	B	01A07	0.09	0.37	n.d.	n.d.	38	14
	C	01A10	0.06	0.13	n.d.	n.d.	56	41
	D	01A14	0.13	0.52	n.d.	n.d.	43	18
	E	01A15	0.10	0.37	n.d.	n.d.	68	29
	F	01A17	0.12	0.45	n.d.	n.d.	44	16
	G	01A18	0.12	0.46	n.d.	n.d.	204	110
	H	01A25	0.14	0.43	n.d.	n.d.	112	117
	I	01A26	0.10	0.23	n.d.	n.d.	185	114
Intermediate-Plants	C	01A11	0.40	0.05	0.24	0.28	8	1
	C	01A13	0.39	0.05	0.04	0.07	33	37
	H	01A23	0.55	0.07	0.15	0.22	11	9
	H	01A24	0.58	0.08	0.15	0.18	19	20
	I	01A27	0.26	0.04	0.18	0.29	17	18
<i>G. glabra</i>	C	01A12	0.34	0.05	2.30	0.50	n.d.	n.d.
	F	01A16	0.44	0.03	1.93	0.59	n.d.	n.d.
	G	01A19	0.34	0.02	1.64	0.24	n.d.	n.d.
	G	01A20	0.35	0.05	1.01	0.26	n.d.	n.d.
	H	01A21	0.60	0.05	1.19	0.27	n.d.	n.d.
	H	01A22	0.66	0.04	2.41	0.20	n.d.	n.d.
	I	01A28	0.51	0.03	1.22	0.17	n.d.	n.d.
	J	01A29	0.33	0.02	1.46	0.17	n.d.	n.d.
	K	01A30	0.75	0.04	0.82	0.26	n.d.	n.d.
	L	01A31	0.46	0.03	1.43	0.28	n.d.	n.d.

a) 100%=peak 1 in plant 01A05 from collection site A, detection: UV 292 nm. b) Not detected (including not identified by UV spectrum).

uralensis-type. These results, coupled with the fact that chloroplast genes including *rbcL* gene arise from their female ancestors, suggest that the *G. glabra*-type (01A11 and 01A24) and the *G. uralensis*-type (01A13, 01A23 and 01A27) of the intermediate-type plants are derived from the female ancestors of *G. glabra* and *G. uralensis*, respectively.

HPLC Analysis of GL and Species-Specific Flavonoids in Underground Parts Since a sweet saponin, GL, and species-specific flavonoids, GB for *G. glabra* and GC for *G. uralensis*, were important index compounds for the underground parts of licorice, photodiode-array HPLC analysis was performed to determine the contents of GL, GB and GC in the roots and stolons collected in the present survey. Table 2 shows these contents in the roots and stolons collected in habitats B, C, H, and K; the GL contents were found to vary from 2.14 to 5.72% of dry weight, depending on the sample. On the other hand, the species-specific flavonoids, GB and GC, were detected in the underground parts of *G. glabra* and *G. uralensis*, respectively, but neither flavonoid was detected in the underground parts of the intermediate-type plants (01A11, 01A13, and 01A24).

HPLC Analysis of Flavonoids in Leaves Variations in the flavonoid content were reported in the leaves of *Glycyrrhiza* plants,^{3,6,8,9} and these differences in flavonoid composition might serve as a good marker to determine the species and strain of the plants. Thus, a photodiode-array HPLC analysis was performed to compare the HPLC profile of the leaves collected in this survey. As shown in Table 3, the HPLC profiles of leaf extracts from the 24 plants were divided into three groups: *G. uralensis*-type, *G. glabra*-type and their intermediate-type. Figure 4 shows the structures of two flavonol glycosides (RT and IQ) and two flavanones (PN and LF), which were identified by comparison of their HPLC behavior and UV spectra with those of the authentic samples. Typical HPLC profiles for *G. uralensis*, *G. glabra* and the intermediate plants are shown in Fig. 5.

In the HPLC profile of all *G. uralensis* plants, IQ at 11.6 min was detected as the major flavonol glycoside, and two major unidentified peaks at 25.9 min and 35.3 min were observed. It is noteworthy that the HPLC profile of *G. uralensis* leaves collected in the present study was different from that of *G. uralensis* leaves reported in our previous paper,⁶ which used a *G. uralensis* plant from China, suggesting that the flavonoid composition was also different among the various strains of this species.

In the HPLC profile of all *G. glabra* plants, RT at 10.4 min was detected as the major flavonol glycoside, unlike the case of *G. uralensis* plants. In addition, two flavanones, PN at 36.9 min and LF at 41.5 min, were identified by comparison with the reference samples in their retention times and UV spectra. Although these flavanones were also detected in the leaves of *G. glabra* collected in Turkey, Italy and Spain,^{8,15,16} the major leaf flavonoid glycoside of *G. glabra* in these three countries was not RT but IQ, as observed in *G. uralensis* leaves in Kazakhstan. This difference might be a marker discriminating the origin of *G. glabra*. It is also noteworthy that no significant difference in the HPLC profiles was observed between the two types based on the absence (01A12, 01A16, 01A19, 01A22, 01A28, 01A29, 01A30) or presence (01A20, 01A21, 01A31) of echines on the fruits.

In contrast, the major flavonoid glycoside in the leaves of

Fig. 4. Structures of Flavonol Glycosides (RT, IQ) and Flavanones (PN, LF) in the Leaves of *Glycyrrhiza* Plants

Fig. 5. HPLC Profiles of Methanol Extracts of Leaves of *G. uralensis* (01A26), the Intermediate Plant (01A27) and *G. glabra* (01A28) at Collection Site I

Absorbance at 292 nm. RT, rutin; IQ, isoquercitrin; PN, pinocembrin; LF, licoflavanone, 1 and 2, unidentified.

the intermediate plants was RT, as in *G. glabra* leaves. Interestingly, however, PN and LF, two flavanones characteristic of *G. glabra* leaves, together with two unidentified ones, 1 and 2 in *G. uralensis* leaves, were detected in the leaves of all

intermediate plants, although differing in their content. This result also suggests that the intermediate plants are hybrids of *G. glabra* and *G. uralensis*.

DISCUSSION

The present study showed that *Glycyrrhiza* plants collected in Kazakhstan could be divided into three groups: *G. uralensis*-type, *G. glabra*-type and the intermediate-type, by comparison of their morphological characteristics and HPLC profiles of their underground parts and leaves. It is noteworthy that *G. uralensis*, *G. glabra* and the intermediate plants grew together forming a mixed population at the three collection sites C, H and I. Although the morphological characteristics and the HPLC profiles are similar among various intermediate-plants collected in this study, the chloroplast *rbcL* genes of the intermediate-plants were divided into *G. glabra*-type and *G. uralensis*-type, suggesting that the female ancestors of these types were *G. glabra* and *G. uralensis*, respectively. These results suggest that the intermediate plants are hybrids of *G. uralensis* and *G. glabra*, which form a mixed population in this region, although further studies are necessary to confirm this hypothesis.

It is interesting that the underground parts of the intermediate plants contain neither GB nor GC, which are known as species-specific flavonoids for *G. glabra* and *G. uralensis*, respectively. It was previously reported that *Glycyrrhiza* plants collected in northwestern China, which were identified as *G. glabra* from their morphological characteristics, do not contain *G. glabra*-specific GB.¹⁷⁾ At collection site H, the intermediate plants were the most common, and it was very difficult to find a typical *G. glabra*. Based on the similarity of the morphological characteristics between *G. glabra* and the intermediate plants, the latter would be classified as *G. glabra*. Thus, the *G. glabra* plants in the above-mentioned report¹⁷⁾ were suggested to be the intermediate-plants observed here. In contrast to the species-specific flavonoids of the roots, both *G. glabra*-specific and *G. uralensis*-specific constituents were detected in the HPLC experiments on the leaves of the intermediate plants, although many index compounds common to *G. uralensis* and the intermediate plants have not yet been identified. Characterization of these index compounds

from the leaves, which is important in determining the difference among the three types of *Glycyrrhiza* plants in Kazakhstan, is underway.

Acknowledgments This work was supported in part by a Grant-in-Aid for Scientific Research (No. 12576027) from the Ministry of Education, Science, Sports, Culture and Technology, Japan. The authors would like to thank Mr. Y. Tamura (Maruzen Pharmaceuticals) for providing authentic samples for GL, GB and GC.

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