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Each species of *Glycine* collected in Taiwan has a unique seed protein pattern

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Summary

Several annual and perennial species in the genus *Glycine* Willd., including *G. soja*, long-pod *G. tomentella*, short-pod *G. tomentella* and *G. tabacina*, collected in Taiwan and nearby islands were studied for variations of their seed proteins. SDS-PAGE and Western blotting were used to analyze the total proteins, the heat soluble proteins, six seed maturation proteins (GmPMs) and one seed storage protein. The various species had different patterns of seed heat soluble proteins. In addition, each species of *Glycine* collected in Taiwan exhibited unique seed maturation protein patterns. They had several cross-reactive polypeptides recognized by specific antibodies against GmPM1, GmPM2 and GmPM8, but only one polypeptide recognized by antibodies against GmPM4, GmPM5 and MP130. The long pod *G. tomentella*, which has been suggested as a new species and renamed as *G. dolichocarpa*, could be distinct from the short pod *G. tomentella* on the basis of the analysis of these biochemical markers. It is also indicated that these GmPM antibodies may be used to distinguish between and within other *Glycine* species.

Abbreviations: GmPM – *Glycine max* physiological mature; LEA – late embryogenesis abundant

Introduction

The cultivated soybean, *Glycine max* (L.) Merr., is a major crop and has received a considerable attention in the area of taxonomical studies. The genus *Glycine* Willd. consists of many species belonging to two subgenera, *Soja* and *Glycine* (Newell & Hymowitz, 1983). The subgenus *Soja* includes the diploid (2n=40) cultivated soybean, *G. max* (L.) Merr., and its wild relative, *G. soja* Sieb. and Zucc. Both *G. max* and *G. soja* are annual, and intercross freely (Palmer et al., 1987). The subgenus *Glycine* includes about 15 described species, all of which are perennial (Hymowitz & Singh, 1987). *G. soja* is widely distributed including Russia, Korea, Japan, mainland China and Taiwan (Hymowitz & Singh, 1987), with Taiwan being the southernmost part of these areas. The two wild perennial *Glycine* species, *G. tomentella* Hayata and *G. tabacina* Benth., which have been suggested to be the probable ancestors of *G. soja*, are found in Ryukyu islands, Taiwan, the Philippines, the South Pacific islands and Australia (Hymowitz et al., 1998), with Japan being the northernmost part of these areas. All other perennial *Glycine* species are found only in Australia (Tindale & Craven, 1988). Therefore, Taiwan and Japan are unusual because they have representatives of both subgenera. The collection and study of wild soybean and their relatives in Taiwan and adjacent islands thus provide information for a better understanding of the evolution and relationships between these two subgenera. Accordingly, we have analyzed the seed proteins of different *Glycine* species collected in Taiwan by SDS-PAGE followed by immunoblotting. These proteins included GmPM (*Glycine max* physiological mature) proteins, the seed storage proteins, and the soluble proteins after heat treatment. The general characteristics of these proteins are described in the following paragraphs.

Proteins synthesized during late maturation of seeds are called maturation proteins (MP) (Rosen-

Table 1. Characteristics of the GmPM proteins in *G. max* seeds

	Apparent MW (kDa)	Protein MW recognized by hybrid selected translation (kDa)	Protein characters	references
GmPM1	22	20, 22	LEA IV protein, 96.0% similarity with GmPM9	Chen et al., 1992
GmPM2	52	52, 60	LEA III protein	Hsing et al., 1992
GmPM4	70	70	Seed-specific biotinylated protein	Hsing et al., 1998
GmPM5	40	42	Released from seeds by hot-water treatment	Hsing et al., unpublished
GmPM8	48	48, 50	LEA III protein, 93.7% similarity with GmPM10	Hsing et al., 1995a
GmPM9	20	20, 22	LEA IV protein, 96.0% similarity with GmPM1	Hsing et al., 1995c
GmPM10	50	48, 50	LEA III protein, 93.7% similarity with GmPM8	Hsing et al., 1995a
MP130	130	n.d.*	(sequence not known)	Hsing & Wu, 1992

* not determined.

berg & Rinne, 1986) or late embryogenesis abundant (LEA) proteins (Galau et al., 1986), which are correlated with desiccation tolerance (Blackman et al., 1991), ABA content (Hughes & Galau, 1991), and transition to seedling growth (Rosenberg & Rinne, 1986). We have selected several cDNA clones encoding seed maturation proteins from a soybean (*G. max* cv. Shi-shi) pod-dried seed cDNA library by differential screening (Hsing et al., 1990; Hsing & Wu, 1992). These clones are designated as GmPM. According to the results of genomic Southern analysis of several *Glycine* species, most of the GmPM messages are encoded by single or low copy-number genes (Hsing et al., 1995b); therefore, it is likely that each GmPM antibody detects only one or a few protein bands. Antibodies were raised against several of these GmPM proteins, whose characterization are listed in Table 1.

There are two major storage proteins in cultivated soybean seeds. One is glycinin, which consists of acidic subunits and basic subunits (Kitamura et al., 1976). The other is conglycinin, which is a glycoprotein composed of three (α',α, and β) major subunits (Thanh & Shibasaki, 1977). These storage proteins are easily separated and recognized by SDS-PAGE and thus may be used as genotype markers.

Some seed proteins may remain in the supernatant after heat treatment of seed extracts. For instances, soybean seed heat soluble proteins are accumulated during desiccation period of seed development (Blackman et al., 1991). They can be obtained easily and represent only a small fraction of the total seed proteins. We have also analyzed these heat soluble proteins in different *Glycine* species.

A wide variety of approaches, including cytogenetics, morphology, isozyme, RFLP, RAPD and AFLP, have been used to define the systematic relationships among species. These approaches necessitate large amounts of samples and/or efforts. However, the approach using SDS-PAGE following by immunoblotting of seed proteins require only five seeds and two working days. The objectives of the current study were firstly to detect the above-mentioned proteins in *Glycine* species collected in Taiwan and secondly to test if they are polymorphic to reveal the systematic relationships among the *Glycine* species.

Materials and methods

Plant materials

Soybean (*Glycine max* cv. Shi-shi) seeds were kindly provided by the Kaohsiung Agricultural Experimental Station, Pintung, Taiwan. The plants were grown to maturity at the Experimental Farm, Institute of Botany, Academia Sinica, Taiwan.

Seeds of wild soybeans and their relatives were collected from Taiwan and the nearby islands by Drs J.S. Hsieh and Y.C. Huang, Department of Agronomy, National Taiwan University. The accession numbers and locations of the collection are shown in Table 2 and Figure 1. The known permanent plant introduction (PI) numbers are also indicated. These wild soybeans were grown to maturity in a greenhouse at the Institute of Botany, Academia Sinica.

Protein extraction and analysis

Total seed soluble proteins were extracted by homogenization of the seeds in an ice-cold grinding buffer consisting of 63 mM Tris-HCl (pH 7.8), 20

Table 2. Accessions of the wild soybean and their relatives used in this study and their places of collection

Species	Accession numbers		Locations of collection # in Figure 1	
	TW #	PI # (UI #)		
G. soja	S001		1	Shimen, Taoyuan, Taiwan
	S022		2	Chutung, Hsinchu, Taiwan
G. tomentella (short pod)	To029	393557	3	Maopitou, Pintung, Taiwan
	To037	563879	4	Haikou, Pintung, Taiwan
	To045	320547	5	Kingmen, Taiwan
	To046	339655	6	Chenkunlin, Taichung, Taiwan
	To047		7	Longluentan, Pintung, Taiwan
G. tomentella (long pod)	To038		8	Chialulan, Taitung, Taiwan
[or *G. dolichocarpa*]	To039		9	Tungho, Taitung, Taiwan
G. tabacina	Ta005		10	Sitaigubau, Penghu, Taiwan
	Ta010		11	Wangan, Penghu, Taiwan
	Ta074		5	Kingmen, Taiwan
G. latifolia	Lat	(373-7)		Australia
G. canescens	Ca003	(535-1)		Australia
	Ca007	440932 (0434)		Australia
G. tomentella	To057	441005		Australia
	To059	446959		Australia
	To053	330961		the Philippines
	To061	446988		New Guinea
	To054	339657		Australia
	To055	440998		Australia
	To062	446993		New Guinea
	To052	441000		Australia

mM $MgCl_2$, 10 mM 2-mercaptoethanol and 1 mM PMSF (phenylmethyl-sulphonyl fluoride). Following homogenization, an equal volume of Laemmli protein solubilization buffer (Laemmli, 1970) was added. The slurry was incubated at 100 °C for 10 min, and then subjected to SDS-PAGE. Electrophoresis (12.5% slab gel) was performed in the Laemmli system, and the gels were stained for proteins with Coomassie blue. About 100 μg proteins were loaded onto each lane.

Heat soluble proteins were extracted by homogenization of soybean seeds in an ice-cold grinding buffer consisting of 60 mM Tris-HCl (pH 7.8) and 500 mM NaCl. The slurry was transferred to a centrifuge tube and incubated at 100 °C for 10 min and then at 4 °C for 10 min. Nonsoluble proteins including those coagulated by heat were removed by centrifugation. Proteins in the supernatant were concentrated by acetone precipitation. Subsequently, the Laemmli protein solubilization buffer was added to the precipitate, and the slurry was incubated at 100 °C for 10 min, and then subjected to SDS-PAGE. Electrophoresis (12.5% slab gel) was performed in the Laemmli system, and the gels were stained for proteins with Coomassie blue. About 60 μg proteins were loaded onto each lane.

Preparation of antisera and immunoblotting

Eight specific soybean seed proteins were analyzed by SDS-PAGE followed by immunoblotting. The sera against glycinin, MP130 and GmPM5 were prepared by immunizing rabbits with each of these protein separated by SDS-PAGE. The gel stripes containing the proteins were excised, grounded and then used for injection. The sera against seed maturation proteins GmPM1, GmPM2 and GmPM8 were prepared by immunizing rabbits with purified recombinant proteins solubilized in 10 mM phosphate buffer (pH 7.0) and mixed with adjuvant before the injection. GmPM4 is a seed-specific biotin binding protein (Hsing et al., 1998), and alkaline phosphate-conjugated streptavidin (Boehringer Mannheim) was used for its detection.

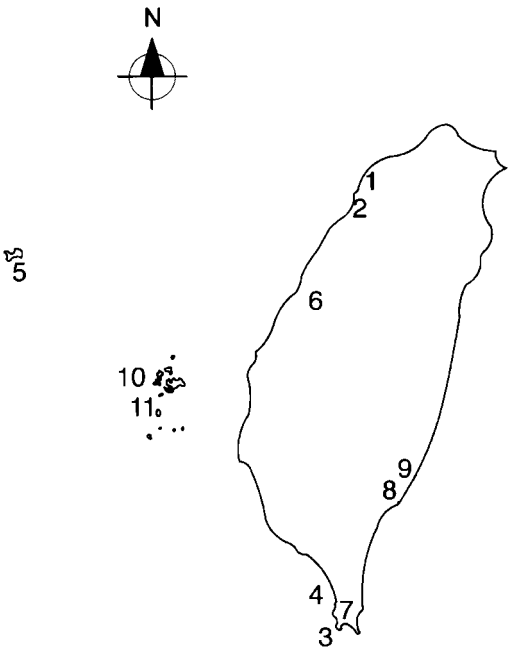

Figure 1. Distribution of *Glycine* accessions collected in Taiwan used in the present study. The indicated accessions are described in Table 2.

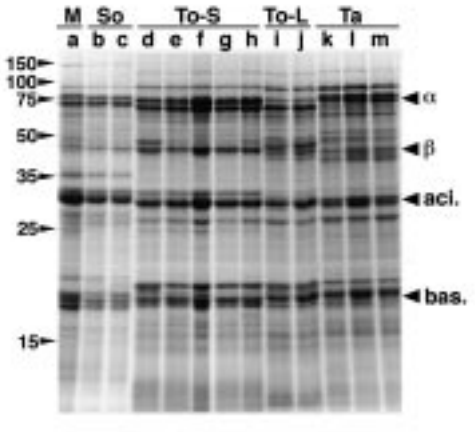

Figure 2. SDS-PAGE of total soluble seed proteins of various *Glycine* species. Proteins were prepared from (a) *G. max* Shi-shi; (b) *G. soja* S001, (c) S022; (d) *G. tomentella* (short pod) To029, (e) To037, (f) To045, (g) To046, (h) To047; (i) *G. tomentella* (long pod) To038, (j) To039;(k) *G. tabacina* Ta005, (l) Ta010, (m) Ta074. Arrows indicate molecular markers in kDa. The positions of α and β-conglycinin, and the acidic (aci.) and basic (bas.) polypeptides of glycinin, are indicated on the right.

Immunoblotting after SDS-PAGE was performed as described by Towbin et al. (1979). The dilution factors used for each primary antibody were: GmPM1, 50,000; GmPM2, 80,000; GmPM4, 2,000; GmPM5, 20,000; GmPM8, 160,000; MP130, 20,000 and glycinin, 10,000. For the secondary antiserum, goat anti-rabbit IgG conjugated to alkaline phosphatase was used, and nitroblue-tetrazolium was used as the chromogenic substrate.

Results and discussion

Total proteins and heat soluble proteins in seeds of Glycine species

SDS-PAGE analysis of total soluble seed proteins showed no significant difference in the banding patterns of glycinin and conglycinin in *G. soja* species and cultivated species. However, the polypeptide sizes of these proteins varied slightly in *G. tomentella* and *G. tabacina* (Figure 2). Of the heat soluble protein profiles, there were prominent differences among those of *G. max*, *G. soja*, long-pod *G. tomentella*, short-pod *G. tomentella* and *G. tabacina* (Figure 3). There were several polypeptides present in *G. soja* but not

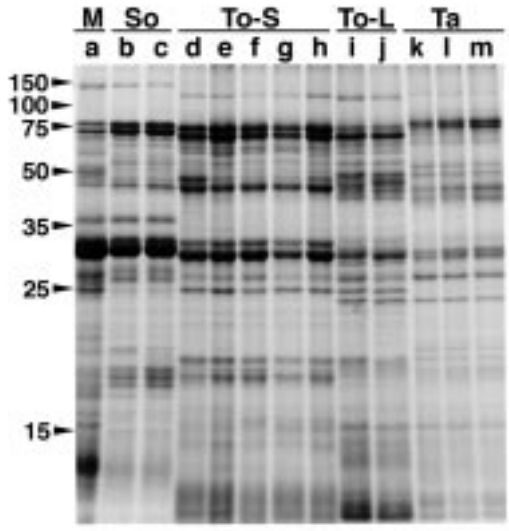

Figure 3. SDS-PAGE of seed heat soluble proteins of various *Glycine* species. The accessions and their labelings are the same as those in Figure 2.

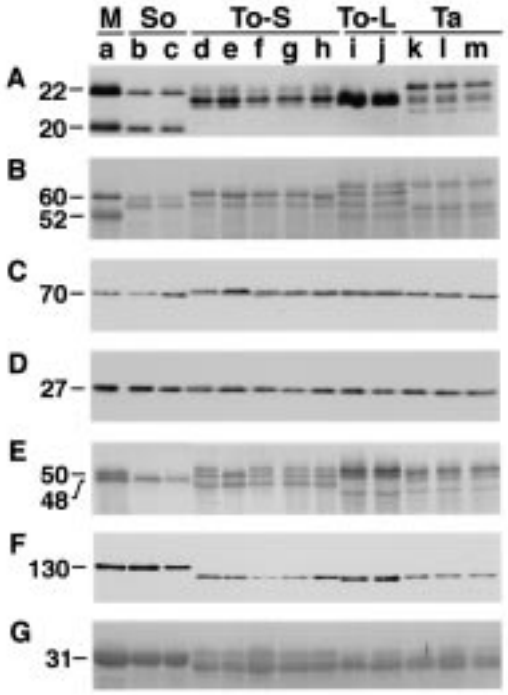

Figure 4. Immunostaining after SDS-PAGE of soybean seed proteins recognized by specific seed protein antibodies. The cross-reactive polypeptides recognized by antibodies against GmPM1 (Panel A), GmPM2 (Panel B), GmPM4 (Panel C), GmPM5 (Panel D), GmPM8 (Panel E), MP130 (Panel F) and glycinin (Panel G) are illustrated. The accessions and their labelings are the same as those in Figure 2. Numbers on the left indicate the apparent molecular weights of specific polypeptides.

in *G. max*. These polypeptides included those of 20 kDa, 30 kDa, and 50 - 75 kDa. The most obvious difference between the annual and perennial *Glycine* species was the presence of a 130-kDa protein in the former species and a 120-kDa protein in the latter species. The long-pod and short-pod *G. tomentella* gave very distinct patterns, especially in the proteins ranging of 25–35 kDa and 40–75 kDa. The three *G. tabacina* accessions exhibited a similar protein pattern among themselves, but this pattern was quite different from those of other species. Overall, the soybean seed heat soluble protein profile is a more useful parameter than the total seed protein profile for distinguishing the *Glycine* species.

Seed proteins recognized by antibodies

Seven antibodies prepared against distinct soybean seed proteins were used to detect their presence in the *Glycine* species. The results of Western blot analysis are shown in Figure 4. Two obviously different types of antibody reactivity were observed: 1. several polypeptides recognized by one antibody and 2. only one polypeptide specifically recognized by one antibody. The antibodies against GmPM1, GmPM2 and GmPM8 belong to the first type, and the other four antibodies belong to the second type. These two types of antibody reactivity coincided well with the results of hybrid-select translation (Table 1).

Anti-GmPM1 antibody recognized two identical polypeptides in *G. max* and *G. soja* seeds. It recognized two polypeptides in the five accessions of short-pod *G. tomentella*, one polypeptide in two long-pod *G. tementella*, and three polypeptides in three *G. tabacina*; the apparent molecular mass of these recognized polypeptides were quite different. Anti-GmPM2 antibody detected two polypeptides of different sizes in *G. max* and *G. soja*. It detected two polypeptides in short-pod *G. tomentella*, four polypeptides in long-pod *G. tomentella*, and three polypeptides in *G. tabacina*; again, these polypeptides were different in sizes. One of the accessions of short-pod *G. tomentella*, To047, gave a pattern which was slightly different from that of other short-pod *G. tomentella* (lane h, Figure 4B). Anti-GmPM8 antibody reacted with two polypeptides in *G. max* and only one polypeptide in *G. soja*. It reacted with three polypeptides in short-pod *G. tomentella*, long-pod *G. tomentella* and *G. tabacina*; these three polypeptides were different in sizes. One accession of the short-pod *G. tomentella*, To037, gave a pattern which was slightly different from that of other short-pod *G. tomentella* (lane e, Figure 4E). Hybrid select translation prepared from *G. max* seeds indicated that there was only one polypeptide of GmPM4 or GmPM5. Anti-GmPM4 or anti-GmPM5 each detected only one polypeptide of an identical size in all the accessions of *Glycine* tested. Also, there was one polypeptide detected by anti-MP130 in all *Glycine* species, but the size of the polypeptide in the perennial *Glycine* species was smaller, about 120 kDa. Anti-glycinin recognized one or two polypeptides of similar sizes in all the *Glycine* species tested (Figure 4G).

Based on Western blot analysis, several conclusions can be drawn (Figure 4). There are two types of soybean seed maturation proteins, one including

GmPM1, GmPM2 and GmPM8, and the other including GmPM4, GmPM5 and MP130. For each species, the former type contains several different cross-reactive polypeptides, and gives distinct patterns for each accession. There are one or two polypeptides in *G. max* and *G. soja* while are many polypeptides in *G. tomentella* and *G. tabacina*. These three GmPM (GmPM1, GmPM2 and GmPM8) proteins belong to group 3 or 4 LEA proteins (Chen et al., 1992; Hsing et al., 1992; Hsing et al., 1995a). The latter type gives only one cross-reactive polypeptide with an identical or slightly different molecular weight for each accession. None of these three GmPM (GmPM4, GmPM5 and MP130) proteins belongs to any known LEA protein. *G. max*, *G. soja*, short-pod *G. tomentella*, long-pod *G. tomentella* and *G. tabacina* each has a unique seed maturation protein pattern. These results indicate that the antibodies against soybean seed maturation proteins may provide a powerful tool for genetic studies. For instance, the antibody against GmPM2 may detect 52-kDa and 60-kDa polypeptides in *G. max* but detect 55-kDa and 60-kDa polypeptides in *G. soja*. F_1 hybrids resulted from a cross between these two species may thus be confirmed by using this antibody.

Are long and short podded G. tomentella *different species?*

The morphological characteristics, chromosome numbers, and interspecific crosses of several *Glycine* species collected in Taiwan were studied intensively by Tang and his colleagues (Tang & Chen, 1959; Tang & Lin, 1962; Tang & Tai, 1962). At the time of study, the long-pod *G. tomentella* was called *G. tomentosa*, and the short-pod *G. tomentella* was called *G. tomentella*. Even though both long and short podded *G. tomentella* were tetraploid (2n = 80), their morphological features were quite different.

In a taxonomic study of the Leguminosae in Taiwan, Ohashi et al. (1991) observed a distinct morphological form of *G. tomentella* and described it as a new species, *Glycine dolichocarpa* Tateishi et Ohashi. They pointed out that *G. dolichocarpa* was clearly distinguishable from *G. tomentella* on the basis of the pods, seeds, leaflets, flowers and hairiness of the stems and petioles (Ohashi et al., 1991; Tateishi & Ohashi, 1992). However, this taxonomic report has not been generally accepted. The genomic diversity of tetraploid *G. tomentella* has been studied by cytogenetic analysis, seed protein profiles, protease inhibitor activity band profiles, Western blot analysis of anti-Kunitz

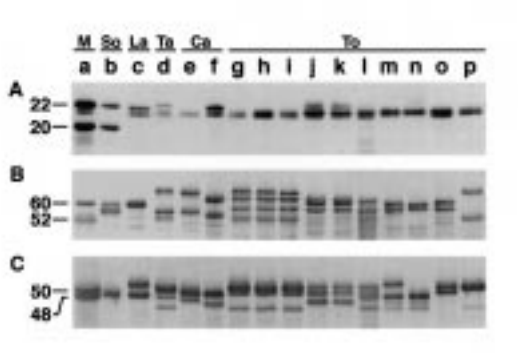

Figure 5. Immunostaining after SDS-PAGE of soybean seed proteins recognized by seed maturation protein antibodies. The cross-reactive polypeptides recognized by antibodies against GmPM1 (Panel A), GmPM2 (Panel B), and GmPM8 (Panel C) are illustrated. Proteins were prepared from (a) *G. max* Shi-shi; (b) *G. soja* S001; (c) *G. latifolia* Lat; (d) *G. tabacina* Ta010; (e) *G. canescens* Ca003; (f) *G. canescens* Ca007; (g) *G. tomentella* To039; (h) *G. tomentella* To057; (i) *G. tomentella* To059; (j) *G. tomentella* To047; (k) *G. tomentella* To053; (l) *G. tomentella* To061; (m) *G. tomentella* To054; (n) *G. tomentella* To055; (o) *G. tomentella* To062; (p) *G. tomentella* To052. Numbers on the left indicate the apparent molecular weights of specific polypeptides.

trypsin inhibitor, RFLP analysis and synthetic allotetraploids (Kollipara et al., 1994). On the basis of this research, *G. tomentella* was divided into seven groups. The short-pod *G. tomentella* collected in Taiwan was placed in group T4; the long-pod *G. tomentella* was not included in the above study.

In this paper, we have shown that there are differences in the seed heat soluble protein profiles and the antibody cross-reactive seed maturation protein patterns between short-pod and long-pod *G. tomentella* collected in Taiwan. By the analysis of nucleotide sequences of the internal transcribed spacer, we've also demonstrated that short-pod *G. tomentella* and long-pod *G. tomentella* indeed are different (Hsing et al., submitted). Therefore, we suggest that *G. dolichocarpa* (the long-pod *G. tomentella*) should be separated from *G. tomentella*, which generally have short pod.

Can GmPM antibodies be used to distinguish between and within other Glycine species?

In order to test if the immunoblot analysis may be used to assess the degree of genetic variability between and within different *Glycine* species, we employed the antibodies against GmPM1, GmPM2 and GmPM8. Several accessions (listed in Table 2) from *G. max*, *G. soja*, *G. latifolia*, *G. tabacina*, *G. canescens* and *G. tomentella* collected in Taiwan, the Philippines, New

Guinea, and Australia were used for the Western blot analyses (Figure 5). The results indicate that these three antibodies indeed can be used to distinguish between and within different *Glycine* species.

Acknowledgements

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