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PREMATURE DRYING AND GERMINATION IN WILD SOYBEAN SEEDS

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ABSTRACT: In the present study, we used the cultivated soybean, *Glycine max*, and its wild relatives *G. soja*, *G. tomentella*, and *G. tabacina* collected in Taiwan as the materials. We took immature, artificially dried, and naturally mature seeds and analyzed their seed moisture contents, germination rates, and the presence of seed maturation proteins. The results indicated that fresh immature wild soybean seeds acquired their germination ability after drying treatment, and that timing for the appearance of seed maturation proteins and the increase of germination ability coincided well.

KEYWORDS: germination ability, *Glycine* species, polyacrylamide gel electrophoresis, precocious drying, soybean seed maturation protein, Western blot.

INTRODUCTION

For most seeds, drying is the terminal event of seed developmnet. Studies have indicated that water loss or dessication played an important role for the maturation process in many monocot and dicot seeds, such as corn [*Zea mays*] (Spraque, 1936), wheat [*Triticum vulgare*] (Armstrong *et al.*, 1982), French bean [*Phaseolus vulgaris*] (Kermode and Bewley, 1985), and soybean [*Glycine max*] (Rosenberg and Rinne, 1986). The fresh immature seeds would not germinate on water when removed from the mother plant in a hydrated state, but would germinate after being dried. Thus, premature drying of immature seeds and subsequent rehydration of them resulted in a transition from embryonic development to germination and seedling growth, just as the naturally mature seeds did.

Changes in soybean seed metabolism at the level of protein biosynthesis were compared between fresh immature seeds, seeds undergoing precocious dehydration, as well as natually mature seeds (Rosenberg and Rinne, 1988; Rosenberg and Rinne, 1989). Results demonstrated that several unique polypeptides were synthesized *de novo* during precocious or natural seed maturation. These polypeptides were termed maturation proteins. In these studies, a striking correlation between the accumulation of these soybean seed maturation proteins and the capability of precociously dried soybean seeds or naturally matured seeds to exhibit normal germination was also reported.

The seed maturation proteins were also called as late embryogenesis abundant (Lea) proteins (Galau *et al.*, 1986). Other than transition to germination mode, the synthesis and

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accumulation of these proteins were also suggested to be correlated with desiccation tolerance (Dure *et al.*, 1989; Blackman *et al.*, 1991), seed dormancy (Ried and Walker-Simmons, 1990), and abscissic acid (ABA) content (Hughes and Galau, 1991). Many of these *Lea* genes have been cloned and sequenced (e.g. Dure *et al.*, 1989; Hsing *et al.*, 1992). Most of the studies suggested that Lea proteins acted as desiccation protectants, and protected the mature seeds or other tissues from damage caused by severe drying, an event that might very well be lethal (Dure, 1993; Blackman *et al.*, 1991). Only very few studies indicated that these proteins correlated with germination ability.

The cultivated soybean is both a major crop and an important experimental plant material for morphological, taxonomical, physiological and molecular biological studies. The genus *Glycine* Willd. consists cultivated soybean and several its wild relatives. Three wild *Glycine* species were found in Taiwan and the nearby islands, they are *G. soja*, *G. tomentella*, and *G. tabacina*.

In the present study, we used a cultivated soybean variety, Shi-shi, and three wild soybeans collected in Taiwan, including *G. soja* S001, *G. tomentella* To039, and *G. tabacina* Ta019 as the materials. All the wild soybean seeds were dormant because of being physical impermeable. They had black and thick seed coats that prevented water from reaching the embryos (Hymowitz and Singh, 1987). We used the dormant wild soybean seeds to determine whether the seed maturation proteins would also be present and whether a relationship would exist between seed dehydration, maturation protein and germination ability, as the cultivated soybean would.

MATERIALS AND METHODS

Plant materials

Seeds of Shi-shi were kindly provided by Kaoshiung District Agricultural Improvement Station, Taiwan. Seeds of the three wild soybean accessions used were collected by Drs. J. S. Hsieh and Y. C. Huang from Taiwan and the nearby islands. For *G. soja*, we used the accession S001 which was collected from Shimen, Taoyuan. For *G. tomentella*, we used the accession To039 which was collected from Tungho, Taitung. For *G. tabacina*, we used the accession Ta019 which was collected from Oonie, Penghu.

The cultivated or wild soybean plants were grown to maturity in the greenhouse. Pods were harvested at mid-development (about 35 days after flowering, DAF), and seeds were precociously matured by air-drying the intact pods (pod-dried) for 4 days (Hsing *et al.*, 1990; Hsing and Wu, 1992). Fresh, pod-dried seeds, or mature seeds were immediately frozen in liquid N_2 after the harvesting or the drying treatments and stored at -70°C prior to protein extraction.

Determination of seed moisture content and germination rate

The seed fresh weight, dry weight and moisture content were based on weight determined before and after oven-drying seed samples at 100 °C for 48 hr. The seed moisture content was calculated as (fresh weight - dry weight)/ fresh weight.

Following harvest at fresh, pod-dried, or mature stage, seeds were cut using a blade before surface sterilizing in a solution containing 1% NaClO. Germination test was done

at 27℃ in darkness. Triplicate samples of 20 seeds were imbibed in sterile filter papers. Germination rates were evaluated at the end of the fifth day.

Seed protein preparations

Seeds were homogenized at 4℃ with mortar and pestle in ice-cold grinding buffer consisting of 63 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 10 mM β-mercaptoethanol and 1 mM PMSF (phenylmethyl-sulphonyl fluoride). Following homogenization, an equivalent volume of Laemmli protein solubilization buffer (Laemmli, 1971) was added and the total slurry transferred to a microfuge tube and incubated at 100℃ for 10 min. Proteins were separated by one-dimensional 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue R-250.

Western blot immuno-detection

Western blotting was performed as described by Towbin *et al.* (1979). Three kinds of primary antisera were used. The anti-130 kD soybean seed maturation protein serum was prepared by immunizing mice with 130 kD proteins (Hsing *et al.*, unpublished data). The anti-GmPM1/9 soybean seed matuation protein serum was prepared by immunizing rabbit with purified fusion GmPM1 protien (Hsing *et al.*, unpublished data). The anti-carrot embryonic protein DC8 antibody was kindly provided by Dr. Z. R. Sung, University of California in Berkeley, USA. For the secondary antiserum, goat anti-mouse or goat anti-rabbit IgG conjugated to alkaline phosphatase (AP) was used, and nitroblue-tetrazolium was used as the chromogenic substrate.

RESULTS AND DISCUSSION

Seed protein analysis

There are two major storage proteins in cultivated soybean seeds. One is conglycin, also known as legumin or 11 S storage protein, which consists of acidic and basic subunits (Kitamura *et al.*, 1976). Another is glycinin, also known as vicilin or 7S storage protein, which is a glycoprotein composed of three major subunits, α, α' and β (Thanh and Shibasake, 1977). Together glycinin and conglycinin proteins constitute approximately 70% of the total seed protein at maturity. Fig. 1 illustrates the total protein analysis of the four *Glycine* species. The positions of α and β subunits of conglycinin and the basic and the acidic glycinin of cultivated species are indicated. This protein profile indicated there was no band that absent in fresh seeds but present in pod-dried or mature seeds for all species tested.

Three antibodies were used to detect the soybean seed maturation proteins in these seeds. The anti-GmPM1/9 antibody recognized two protein bands in cultivated varieties, with apparant molecular weight of 20 and 22 kD. For GmPM2 Western blot, anti-DC8 antibody was used. DC8 was a carrot embryonic-specific protein (Franz *et al.*, 1989), their deduced protein sequences shared high homology with soybean GmPM2 protein (Hsing *et al.*, 1992). This antibody recognized another two soybean seed protein bands in cultivated varieties, with apparant molecular weight of 52 and 60 kD. The anti-130 kD antibody recognized only one protein band in cultivated variations. The results of Western blot are

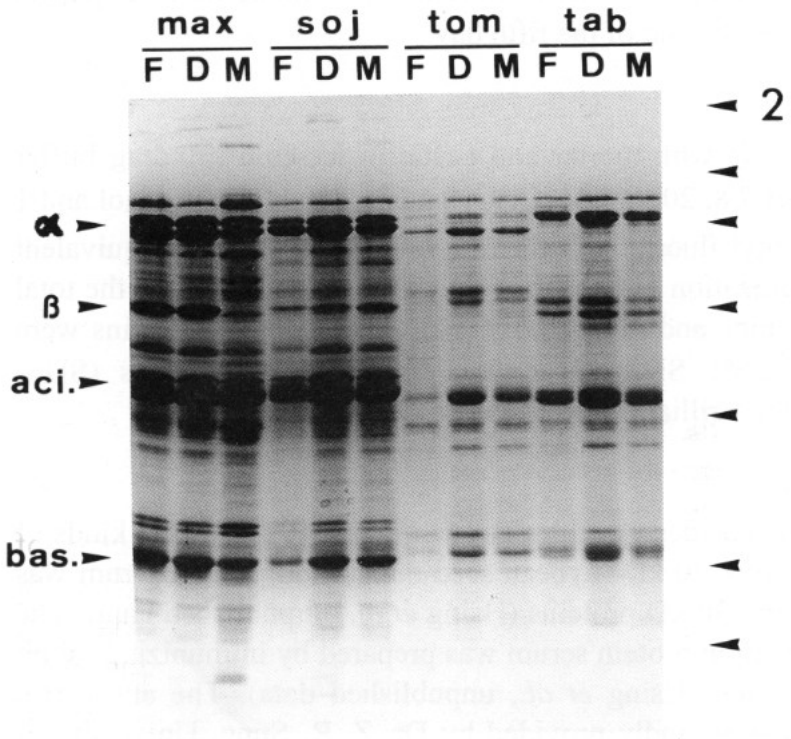

Fig. 1. SDS-PAGE of protein extracts of the cultivated and wild soybean seeds. Electrophoresis in a 12.5% slab gel was carried out in the Laemmli system and stained for protein with Coomassie blue R. The α and β –conglycinin as well as the acidic (aci.) and basic (bas.) polypeptides of glycinin are indicated. Protein were prepared from fresh immature (F), pod-dried (D), and natural mature (M) seeds of *G. max* Shi-shi (max), *G. soja* S001 (soj), *G.* tometella To039 (tom), and *G. tabacina* Ta019 (tab). Molecular weight standard are shown in kD.

showed in Figures 2-4.

Fig. 2 indicated that for all four *Glycine* species screened, fresh immature seed did not have the GmPM1 cross-reactive polypeptides, but the 4 days pod-dried and the mature seeds did. Figures 3 and 4 indicated the same phenomena for GmPM2 and 130 kD cross-reactive polypeptides, respectively. The developmental stage between fresh immature and pod-dried seeds was only 4 days apart. The seed maturation proteins tested were absent in fresh immature seeds, but were present in pod-dried or mature seeds.

The cultivated soybean seeds synthesized *de novo* several maturation proteins during drying treatment or the late maturation stage. These proteins were distinct from the known storage proteins in terms of protein molecular masses or expression patterns, etc. Their cDNA clones had been cloned, characterized, and sequenced in our lab (Hsing *et al.*, 1990; Hsing and Wu, 1992; Hsing *et al.*, 1992; Chen *et al.*, 1992; Lee *et al.*, 1992). These cDNA clones were designated pGmPM 1 through 10. GmPM stands for *Glycine max* physiological maturation. Sequence comparison studies indicated that these GmPM proteins had homology with several Lea proteins in other plant species, which would also be induced

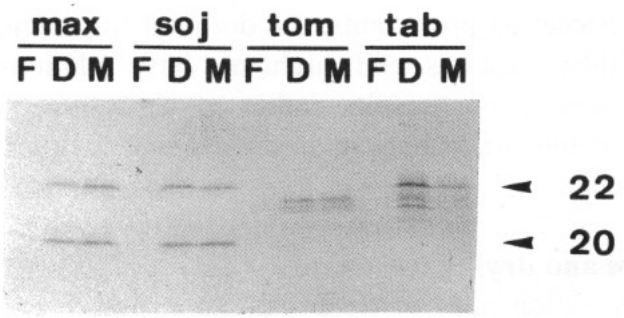

Fig. 2. Immunostaining of soybean seed maturation protein GmPM1/9 cross-reactive polypeptides. SDS-PAGE was carried out as in Fig. 1, and the separated polypeptides transferred to nitrocellulose paper. The Western blot was then reacted with rabbit anti-GmPM1 antiserum as the primary antibody. The letter descriptions are the same as listed in Fig. 1. Molecular weight standard are shown in kD.

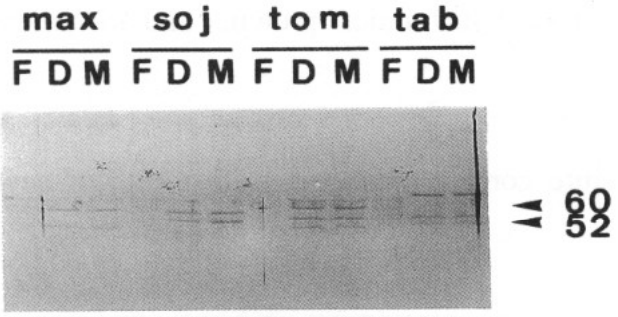

Fig. 3. Immunostaining of soybean seed maturation protein GmPM2 cross-reactive polypeptides. SDS-PAGE was carried out as in Fig. 1, and the separated polypeptides transferred to nitrocellulose paper. The Western blot was then reacted with anti-DC8 antiserum as the primary antibody. The letter descriptions are the same as listed in Fig. 1. Molecular weight standard are shown in kD.

Fig. 4. Immunostaining of 130 kD soybean seed maturaiton protein cross-reactive polypeptides. SDS-PAGE was carried out as in Fig. 1, and the separated polypeptides transferred to nitrocellulose paper. The Western blot was then reacted with anti-130 kD antiserum as the primary antibody. The letter descriptions are as the same as listed in Fig. 1. Molecualr weight standard are shown in kD.

by ABA or water stress treatment in leaves and so on. Since most studies supported the hypothesis that Lea protein acting as desiccation protectants, the dormant imper-meable wild soybean seeds might not contain these proteins, and might not have relation-ship between drying and germinaiton. However, our results indicated that there were maturation proteins present in dried or mature wild soybean seeds and that drying treatment induced the synthesis of these proteins.

Relationship between germination rate and drying treatment

The moisture contents and the germination rates of fresh immature, pod-dried, and mature *Glycine* seeds are shown in Table 1. All the fresh immature seeds contained high moisture content, i.e. 66 to 70%. However, none of these seeds were able to germinate. After being dried for 4 days, the pod-dried seeds contained less water, i.e. 31 to 45%. Most of these seeds were capable to undergo germination, and the germination rates were 88 to 100%. For the mature seeds, the moisture contents dropped to about 10%, and the germination rates were still high. Thus, there might be relationship existed between the decrease in moisture content and the ability of germination for the wild soybean seeds tested. Also the timing for appearance of seed maturation proteins and increment of germination ability coincided well.

Table 1. Seed developmental stage, moisture content, and germination rate of several *Glycine* species.

Species	Developmental stage	Water content (% of fresh wt.)	Germination (%)
Glycine max L. Merr.	F*	70	0
	D	43	100
	M	9	100
Glycine soja Sieb. & Zucc.	F	66	0
	D	45	96
	M	13	93
Glycine tomentella Hayata	F	66	0
	D	37	100
	M	12	98
Glycine tabacina (Labill.) Benth.	F	67	0
	D	31	88
	M	17	88

* F: fresh immature seeds
D: 4 days pod-dried seeds
M: mature seeds

In vivo labeling experiments indicated that there were more than ten maturation proteins synthesized *de novo* during drying treatment to the fresh immature soybean seeds (Hsing and Wu, 1992). We already identified ten cDNA clones encoding these proteins (Hsing, 1993). The reasons for mature seeds to contain so many maturation proteins were still a puzzle, but the biological functions for these maturation proteins might not be limited only to desiccasion protectants. Dormant impermeable wild soybean seeds were used in the present study and the results indicated once more the relationship between premature drying, maturation protein, and germinaiton. Soybean seed maturation proteins would be induced by drying. With the proteins present, seeds would then be able to germinate and grow once they imbided.

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野生大豆種子未成熟乾燥與發芽

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摘　　要

我們用栽培種大豆（*Glycine max*）及三種在台灣採集的野生種大豆(包括 *G. soja, G. tomentalla* 及 *G. tabacina*) 做爲材料，分析種子在未成熟、人工乾燥及自然成熟時的含水量與發芽率，並以西方墨點分析法檢測了三種種子成熟蛋白。實驗結果指出，新鮮未成熟的野生大豆種子在乾燥處理後即具有發芽能力，同時也有成熟蛋白的形成，自然成熟的種子亦同。

關鍵詞： 萌芽力， *Glycine* 種， 蛋白電泳膠片分析， 熟前乾燥， 西方墨點分析， 大豆種子成熟蛋白。

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