Hybrid Embryo Formation in an Intersubgeneric Cross of Soybean
(\textit{Glycin max Merill}) with a Wild Relative (\textit{G. tomentella Hayata})

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Percentage of successful pod setting in the intersubgeneric hybridization between \textit{G. max}(2 n=40, maternal) and \textit{G. tomentella}(2 n=80, paternal) was estimated to be 2.5 to 7.6\%. Germinability of the \textit{G. tomentella} pollens of \textit{G. max} stigmas, elongation of the pollen tube in the style and arrival of the pollen-tube tips at the ovules were detected by SEM and fluorescence microscopy. Aneuploidy (2 n=64) of the hybrid embryo cells and peroxidase zymogram pattern specific to the hybrid-derived calli were observed by light microscopy (smear method) and polyacrylamide gel electrophoresis, respectively. Moreover, histogeal observation of the hybrid embryo was carried out to investigate the extent of the hybrid embryo growth. It was concluded that morphologically normal growth may continue at least until heart or a more advanced developmental stage, but markedly retarded growth accompanies it and eventually it ceases growing and death of the hybrid embryo results. Though, we couldn't get intact hybrid plant, all of the facts revealed proved formation of the genuine hybrid embryos between \textit{G. max} and \textit{G. tomentella}.

KEY WORDS: \textit{Glycin max Merill, G. tomentella, soybean, species hybridization, hybrid embryo formation.}

\textbf{Introduction}

Wild soybean (\textit{Glycine soja Sieb. and Zucc.}) has sometimes been used as a gene source in soybean breeding programs in the world (Karasa\w{a}wa 1936, Weber 1950, Tang and Chen 1959, Kaizuma 1975). However, the other wild perennial \textit{Glycine} species has never been dealt with in the same manner. Principal reason of that lies in the general belief prevailed until recent time that successful interspecific hybridizations might be very difficult. As soybean growing area has expanded worldwide, breeding objectives of the soybean cultivars are inevitably diversified depending on cultivation conditions of the area. A role of the wild perennial \textit{Glycine} species as a new gene source is now greatly expected.

For example, resistant genes to soybean rust, an important disease in tropics and subtropics, have been surveyed among some wild perennial \textit{Glycine} species by Burdon and Marshall (1981 a and b). Interspecific hybridizations will more often be utilized in further soybean breeding programs.

The genus \textit{Glycine Willd.} is divided into three subgenera consisting of nine species (Hermann 1962, Verdecourt 1966 and 1970, Hymowitz and Newell 1981). The subgenus \textit{Soja F. J. Herm.} contains two species: \textit{G. soja Sieb. and Zucc.} (a wild annual known as progenitor of the cultigen) and \textit{G. max Merill} (the cultigen). The habitat of \textit{G. soja} ranges from far-east Asia as far as subtropical China proper. Fertile F\textsubscript{1} hybrids between the two species can be easily obtained although some degree of the meiotic irregularities such as chromosome bridges and segments were observed by Ahmad et al. (1977 and 1979).
The subgenus *Glycine Verdcourt* includes six species: *G. clandestina* Wendl. and its var. *sericea* Benth., *G. tabacina* Benth., *G. tomentella* Hayata., *G. latrobeana* Benth., *G. canescens* F. J. Herm. and *G. falcata* Benth. They distribute over wide area as South-east Asia, South pacific Islands (Micronesia) and Australia. Further, Newell and Hymowitz (1980) set up a new taxson named *G. latifolia* Newell and Hymowitz in this subgenus. According to them this species is a derivative from *G. tomentella* complex. Hybridization experiments among the subgenus *Glycine* were extensively carried out by Broue et al. (1979), Putievsky and Broue (1979), Broue et al. (1982) and Newell and Hymowitz (1983). As a result, Varing degree of the cross compatibility was noticed in a number of the cross combinations. The cross of *G. canescens* with *G. clandestina* deserves special attention because of easy procurement of the F1 plants with 80 to 100% pod setting (Putievsky and Broue 1979). Studies of phylogeny among the subgenus *Glycine* have begun with the studaes cited above.

The subgenus *Bracteata Verdcourt* is constituted with only one species named *G. wightii* Verdcourt. However, this species was recently excluded from the genus *Glycine* and revised as *Neonotonia wightii* Lacky (Lacky 1977). Distribution area of the species is mapped from East Africa through India.

As mentioned, Knowledge on the intersubgeneric hybridization of the genus *Glycine* is still limited. Substantial contribution to the intersubgeneric hybridization was made for the first time by Ladzinsky et al. (1979). They suggested possibility of the successful hybridization between *G. max* and *G. tomentella* through observation of the pod growth initiation after the cross-pollination. On the other hand Hood and Allen (1980) reported occurrence of ca. 11% pod setting after the cross of *G. max* with *G. falcata*. Recently, regeneration of the F1 plants between *G. max* and *G. tomentella* with ovule culture has been published by Newell and Hymowitz (1982). However, details of the intersubgeneric hybridization remain to be elucidated in future study.

The most important objective of the present study was to demonstrate the hybrid embryo formation between *G. max* (maternal) and *G. tomentella* (paternal) with as much evidence as possible. In particular, point was placed on clarification of the growth extent of the hybrid embryo on maternal plants.

### Materials and Methods

1. Hybridization experiment

   Various cultivars of *G. max* and some strains of *G. tomentella* belonged of two groupes different in chromosome number, i.e., 2n=40 group (PI 319-696 and Inverelle) and 2n=80 group (PI 245-332 and Eskdale) were hybridized as maternal and paternal parent, respectively during three crop seasons from 1980 to 1982. Pod growth after the cross-pollination was observed every other day until initiation of yellowing of pod, in a total number of 523 crosses made in 1980.

   If a particular pod continued its growth at least for ten days after the pollination, it was regarded as a pod succeeded in fertilization. Success rate of the cross was calculated as: the number of the pods sustaining thier growth for more than ten days after the cross-pollination/the number of the flower organs cross pollinated.
2. Pollen germination on stigma

(1) Observation by Scanning Electron Microscopy (SEM)

Germinability of *G. tomentella* pollens of *G. max* stigmas was observed by SEM. First, anthers of *G. tomentella* (three strains: Eskdale, Lindeman and PI 245-332) and *G. max* (three cultivars: Raiden, Kum-du and Akasaya 1) were gathered from flower buds ca. two days before blooming. Secondly, *G. max* pistils were sampled 10 hours after pollinating of *G. tomentella* pollens. These samples were fixed for two hours in 2% glutaraldehyde at 4°C and dehydrated in a series of ethanol solutions: 50, 70, 95, and 100% (2 times) in turn for 15 min, each. Substitution of the ethanol to n-amylacetate was performed immersing the dehydrated samples for one or two days in n-amylacetate. Crystal point drying with liquidified CO₂ was then applied to the substituted samples. Lastly, the anthers the walls of which were partially broken and the stigmas to which the exotic pollens were artificially attached were thinly coated with gold vapour after completion of the crytical point drying. The pollen morphology and the presence or absence of exotic pollen germination were investigated by a scanning electron microscope, Hitachi 450.

(2) Observation of Fluorescence microscopy

*G. tomentella* pollen tube elongation into *G. max* styles were observed by fluorescence microscopy. *G. max* styles with ovaries were harvested in 24 hours after the cross pollination by *G. tomentella* pollens. The fluorescence microscopy by Martin's method, i.e., detecting fluorescence light ejected from complex of a pollen-tube constituent (callose) and a fluorescent dye (aniline blue), was applied to them (Martin 1958).

3. Demonstration of hybrid embryo formation

(1) Somatic Chromosome number

Somatic chromosome number was counted in the cells of embryos resulting from the cross between *G. max* (2n=40) and *G. tomentella* (2n=80). A total number of 180 immature seeds were used as maternals. These immature seeds resulting from the successful crosses were fixed in acetic acid:ethanol (1:1) after the pretreatment of 0.02% colchicine immersion for 24 hours at 4°C. Then the ovules or embryos were excised from seeds under a low magnitude microscope and double-stained with Feulgen and acetocarmín. Smeread preparation was microscopically investigated for counting chromosome number.

(2) Peroxidase zymograms by polyacrylamide gel electrophoresis (PEGE)

The hybrid embryos between *G. max* (cultivar, Tenshin Daiseito and Akasaya 1) and *G. tomentella* (strain, Eskdale) were transplanted on callus-inducing media 41 and 45 days after the cross pollination. Initially, they were cultured for two months on the modified Gamborg’s B5 basal media, supplemented with Kinetin (0.5 mg/l), (NH₄)₂SO₄ (1340 mg/l), casein hydrolysate (500 mg/l) and sucrose (8%). Then they were subcultured for 10 months on the Murashige and Skoog's basal media added by kinetin (0.5 mg/l) and 2,4-D (2 mg/l). Vigorously growing calli derived from selfed *G. max* embryos (approximately at the same growth stage as that of the hybrid embryos) were used as control.

Each callus about 300 mg in weight crushed in a motor and pestle with 0.5 ml distilled water. The supernatant after centrifugation was subjected to PEGE under Davis system at PH 8.3. The electrophoresed gel was soaked in the stain solution containing 3-amino 9—
ethylcarbasol (420 mg/l), \( \beta \)-naphtol (420 mg/l) and 1 mg of 35\% \( \text{H}_2\text{O}_2 \). Difference of the hybrid embryo-derived calli zymograms from the paternal ones (control) was visually examined.

(3) Hystological examination of the hybrid embryo formation

Two kind of the hybrid pods, i.e., the ones passed 3 to 7 days after the cross pollination and the others passed more than 40 days were fixed in acetic acid : ethanol (1 : 3) for 24 hours and dehydrated in a series of ethanol solutions : 70, 80, 90, and 95\% for 30 min, each. Then they were embedded in JB-4 resin and sliced in 5 \( \mu \)m thick with a microtome. Sliced pieces of the resin were left in water drop of a slide glass until dried and stained with HEIDENHEIN's iron haematoxylin. The hybrid embryo formation and its growth stage were microscopically determined.

Results

1. Success rate of the hybridization

A total number of the crosses between \( G. \text{max} \) (maternal) and \( G. \text{tomentella} \) (paternal) attained as many as 2,306 during three crop seasons from 1980 to 1982. The number of the crosses which continued pod growth for more than ten days after the cross pollination was only 58. The success rate of the cross was roughly estimated to be 2.5\%.

Detailed visual observation on pod growth was carried out in the 523 crosses made in 1980. Fig. 1 illustrates frequency distribution on the longevity of the flower organs cross-pollinated or the pods resulting from the cross pollination. Extent of the longevity was expressed by the number of days during which the flower organs cross-pollinated or the pods resulting from the cross-pollination remained green without and sign of yellowing.

![Fig. 1. Frequency distribution on longevity of the flower organs cross-pollinated or the pods resulting from cross-pollination between \( G. \text{max} \) (female) and \( G. \text{tomentella} \) (male). Arrow: the averaged longevity of the pods.](image-url)
For example, zero day of the longevity means that the flower organs cross-pollinated were dropped off from maternal plant (G. max) or turned yellow within a day after the pollination. The total number of the crosses in the zero day class was as many as 331 (Fig.1). The average longevity was calculated as 2.57 days. The total number of pods keeping green for more than ten days was equal to 40, indicating the success rate of the cross to be 7.6%. Prolonged growth over 30 days appeared in 6 pods. However, the hybrid seeds in these pods were much less developed than the selfed seeds (Fig.2). Therefore, the hybrid embryo development may be nearly completely stopped in early developmental stage or greatly retarded through whole stage.

2. Pollen germination on stigma

(1) SEM

Fig. 3 shows differential morphology of the matured G. max and G. tomentella pollens in pregermination condition. Network structure of the exine on the pollen surface was more markedly developed in G. tomentella than in G. max. Moreover, four germination furrows and hemispherical structures protruding from the furrows were much more clearly visualized on the pollen surface of G. tomentella than of G. max. These morphological characteristics were commonly observed among the strains used of each species. Consequently the respective pollens of both species were identified with no ambiguity by SEM.

Germination of G. tomentella pollens on the stigma was recognized by SEM as shown in Fig. 4.

(2) Fluorescence microscopy

G. tomentella pollen-tube elongation into G. max style was detected by the fluorescence microscopy as photographically shown in Fig. 5A. The pollen germination rate was fairly high. Some pollen-tubes apparently reached G. max ovules (Fig. 5B).

3. Hybrid embryo formation

(1) Somatic chromosome number

An embryo cell with somatic chromosome number 2n=64 was found in an embryo 19 days passed after the cross-pollination (Fig. 6). The chromosome number 2n=60 is theoretically expected in the hybrid embryo between G. max (2n=40) and G. tomentella (2n=80). Therefore, the embryo with 2n=64 chromosomes apparently originated from successful hybridization between those species. The embryo cells being at nuclear division stage were rarely observed because of the retarded embryo growth.

(2) Peroxidase zymograms

Peroxidase zymogram comparison was made between the hybrid embryo-derived calli and the selfed embryo-derived ones. Nine different bands (a–i) were recognized as a whole on the PEGE examined. These zymogram patterns were photographically and diagramatically indicated in Fig. 7. Zymogramatical differences between calli of the parents and their
hybrids were found in the following three points: (1) occurrence of the g band unique to the hybrid, (2) massive accumulation of the f band the parent species lack or have only in small quantity, (3) weaken production of the h and i bands peculiar to *G. max* and *G. tomentella* (Eskdale), respectively. The zymogram pattern obtained from the different portion of the same callus showed no difference in replicated tests.

(3) Histological examination on hybrid embryo formation

The hybrid embryos at globular or a more advanced stage are given in Fig. 8A. Deve-
Fig. 4. *G. tomentella* pollen germination on *G. max* stigma in 10 hr after cross-pollination. A: *G. tomentella* pollen tube elongation (×1000), B: *G. tomentella* pollens germinated on *G. max* stigma (×400), PO: pollen, PT: pollen-tube, ST: stigma, SY: style. PA: papilla.

Fig. 5. Fluorescence microscopy of *G. tomentella* pollen germination on *G. max* stigma (A) and *G. tomentella* pollen-tube elongation into *G. max* ovule through its style (B). PT: Pollen-tube, OV: ovule.

...lopment of the endosperm cells around the hybrid embryos was normal in appearance. The hybrid embryo in Fig. 8B seemed to be at heart stage. However, the period after the cross-pollination has already attained 41 days in this case. Extremely retarded growth was considered to be under way. In some other cases (26 and 45 days embryos) morphologically deformed embryo development was microscopically disclosed. Partial destruction in the parenchmatous tissue cells surrounding the embryo (probably nucellus) was consistently indicated. Consequently, it was concluded that the hybrid embryo resulting from
the cross between G. max (maternal) and G. tomentella (paternal) shows normal growth at early stages at least up to heart or a little advanced stage. Subsequently a certain disorder in embryo growth takes place, leading to eventual cease or death of the hybrid embryo.

Discussion

The success rate of the cross, G. max × G. tomentella, recorded 37 out of 245 in LADIZINSKY et al. (1979) and 72 out of 484 in NEWELL and HYMOVITZ (1982). However, the authors result
Fig. 8 Hybrid embryo development 12 days after cross-pollination, 
×100 (A) and 41 days after cross-pollination, ×200 (B).
EM : embryo, EN : endosperm

was much lower, 2.5% (58 out of 2305) It was probably caused by unusual cold summer weather predominated in 1981 and 1982. Comparatively high value of the rate, 7.6% (40 out of 523), was obtained in 1980 (usual summer season). Consequently, the success rate of this species cross will fall in vicinity of 10%.

In the present study it was demonstrated by SEM and fluorescence microscopy that pollen germination on stigmas, pollen-tube elongation into styles and its arrival at ovules proceeds with no difficulty in this cross between G. max (maternal) and G. tomentella (paternal). In addition, aneuploidy (2n = 64) in the hybrid cells and markedly retarded embryo growth following the cross-pollination were microscopically recognized. Aneuploidy of the regenerated hybrid plants was detected as 2n = 59 in Newell and Hymowitz (1982) and Broue (1982). On the other hand, peroxidase zymograms of the hybrid embryo-driven calli were quite different from those of the paternal species. Similar results were shown for leaf indophenol oxidase isozymes by Broue et al. (1982). All of the facts indicated above prove formation of the genuine hybrid embryo between G. max and G. tomentella.

Recently F1 hybrid plants regenerated through ovule culture were reported by Newell and Hymowitz (1982). According to them these F1 plants showed intermediate morphological traits between G. max and G. tomentella in some characters. Further, it was accompanied with complete sterility and aneuploidy (2n = 59) in somatic chromosome number. Therefore, there is no room to doubt that G. max and G. tomentella are cross-compatible. This encourage us making phylogenic studies on the genus Glycine and introducing intersubgeneric hybridization to future soybean breeding programs. In these programs, G. tomentella might act not only directly as a new gene source for soybean improvement, but also indirectly as a bridge-cross plant to the hybridization of soybean with some other species within the subgenus Glycine. According to the papers cited above, G. tomentella is known to be successfully crossed with G. tabacina, G. canescens (Putievsky and Broue 1979) and with G. canescens (Broue et al. 1982). In fact, Broue
et al. (1982) succeeded in developing five sterile F1 individuals of a "G. tomentella × G. canescens" × G. max, by means of embryo or ovule culture. Although it is unknown whether G. max and G. canescens are cross-compatible or not, this is strong evidence for G. tomentella to work as a bridge-cross plant to the intersubgenic hybridization.

On the other hand, many attempts were made for the direct cross of G. max with some other species within the subgenus Glycine (Ladizinsky et al. 1979, Hood and Allen 1980, Newell and Hymowitz 1982). Pod set was noticed between G. max and any one of the species such as G. canescens, G. clandestina, G. tabacina, and G. falcata. However, more reliable evidence for the cross-compatibility should be shown hereafter.

Growth extent of the hybrid embryos between G. max (maternal) and G. tomentella (paternal) has not previously reported. The conclusion from the present study was that the maximum growth extent without morphological abnormality is at best up to heart or early cotyledon stages. Deformed growth of the cotyledons and/or some other abnormalities appeared to occur at the subsequent stages. Therefore, hybrid plants regeneration may possibly attained by placing the hybrid embryos excised at comparatively early developmental stages on a certain appropriate media.

The authors attempted preliminary regeneration experiments through embryo culture after exploiting the modified Gamborg's B5 basal media supplemented with kinetin or benzylaminopurine (0.5 mg/l), (NH4)2SO4 (1340 mg/l), casein hydrolysate (250 mg/l) and sucrose (8%). However, no positive results were obtained probably because the hybrid embryos used were considerably aged (41 and 45 days after pollination). Newell and Hymowitz (1982) succeeded in regeneration of F1 plants from 2 to 5 weeks ovules. Broue et al. (1982) also used 11 to 33 days embryos or ovules and successfully induced regerated hybrid plants. In further experiments, younger embryos at least 40 days or less should be transplanted. The hybrid embryos used in the present study were at the initial stage of embryo abortion.

Acknowledgements

The authors would like to thank Mr. Tanimura, the electronmicroscope labo., Iwate University for technical assistance of SEM. Simultaneously acknowledgement should be expressed to Dr. S. Mikami, Tohoku Forest Station, Ministry of agriculture, Forestry and Fishery for his courtesy to the peroxydase PAGE experiment. The present study was supported in part by the Grant-in-Aid for Scientific Research (Project no.34025), the Ministry of Education, Science and Culture.

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**ダイズ** (Glycine max Merill) と近縁野生種 (G. tomentella Hayata) の亜属間雑種における雑種胚形成

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ダイズ属植物の種分化およびダイズの種間雑種育種に関する研究に資する目的で、ダイズを母としてG. tomentellaを父とするダイズ属亜属間雑種を試み、雑種胚が形成されるか否かを確認しようとした。

1980年から1982年にかけて合計2306花について雑種を行なった結果、58花のみが交配後10日以上母植物上で生育し、若葉の伸長ならびに不完全種子の発達を示し、この10日を雑種成功の基準にとれば成功率は2.5%であった。

一方、走査型電子顕微鏡で花粉の形状をしらべたところ、G. tomentellaは外膜の細目構造、発芽孔の形に特徴がありダイズのそれと容易に区別がつく。それを手がかりとしてダイズの柱頭上でG. tomentellaの花粉が発芽していることが確認された。また、アヘリン青による花粉管の観光染色を観光顕微鏡でしらべ、G. tomentellaの花粉管がダイズの花柱内を伸长し胚珠に到達していることも確認された。さらにダイズ（2n=40）×G. tomentella（2n=80）に由来する不熟胚をとり、たくさん処理法で染色体数を調べたところ、ある1つの胚で2nの異数体の存在が確認された。雑種胚および自殖胚に由来するカルスを用いてパーキンソン症のダイズ
プログラムを比較したところ，雑種胚カリス特有のパターンの存在が認められた。

雑種胚の組織標本を作り胚の発育程度を調べたが，その結果雑種胚はハート期またはそれよりやや進んだ時期までは外観上正常な発育を示すこと，しかしながら，その速度は著しく遅いこと（交配後41日経過してもなおハート期に留まっているものがあった），それ以降の発育ステージにおいては，おそらく子葉の異常発達や胚周辺組織の柔細胞の崩壊など著しい異常が生じて最終的には胚の退化・致死が起こること，などが結論された。

上述の諸事実はすべてダイズと*G. tomentella* が交雑可能であることを示している。交雑胚の一部については人工培地上での植物体再分化実験も試みたが，供試した胚の交配後経過日数が長かったためか植物体の再分化には成功しなかった。