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High frequency plant regeneration from an amphiploid hybrid seed of *Glycine max* × *G. tomentella* through tissue culture and hybridity testing of the regenerants

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Abstract

Fifty-five plants from the amphiploid hybrid (2*n* = 118) of *Glycine max* (2*n* = 40) and *G. tomentella* (2*n* = 78) were regenerated from a single seed in tissue culture. The embryonic axis converted into a solid, green mass and gave rise to shoot buds on 6-benzyl aminopurine (BA)-supplemented medium. Shoots were eventually obtained on the same medium. Rooting occurred in 100% of the plants on Whites medium containing coumarin (9 mgl^{-1}). A total of 50 plants survived the hardening treatment. All of the tissue culture-raised plants were morphologically similar and flowered under greenhouse conditions. The hybridity of the regenerated plants was confirmed by using morphological and simple sequence repeat (SSR) markers. Attempts are being made to cross these plants with soybean to generate backcross-derived lines for further genetic studies.

Key words: Soybean, intersubgeneric hybrid, genetic stability, SSR marker

Introduction

The genus *Glycine* Willd. comprises two subgenera, *Glycine* and *Soja* (Moench) F. J. Herm. The subgenus *Glycine* contains soybean, *G. max* (L.) Merr., and wild annual ancestor, *G. soja* Sieb. & Zucc. Both species carry similar genomes (GG) and belong to the primary gene pool as described earlier [1]. The subgenus *Glycine* also contains 22 wild perennial species [2] which have a wide geographical distribution, and are morphologically, cytologically and genomically diverse [3, 4]. Wild perennial species of *Glycine* possess several useful agronomic traits [5-7], and it may be beneficial to introduce these traits into cultivated soybean through wide hybridization.

Due to an extremely low level of sexual compatibility and routine early pod abortion, only a few sterile intersubgeneric F_1 hybrids have been reported [8]. Amphidiploid F_1 hybrids were completely sterile. However, Singh *et al.* (1990) [9] produced, for the first time, backcross-derived (BC-derived) progenies from a synthetic amphiploid (2*n* = 118, genome GGDDEE) of *G. max* (2*n* = 40, genome GG) × *G. tomentella* Hayata (PI 483218) (2*n* = 78, genome DDEE). The objective was to obtain monosomic alien addition lines (MAALs) to study the introgression of genes from *G. tomentella* responsible for traits such as resistance to soybean rust (*Phakospora pachyrhizi* Sydow), soybean cyst nematode (*Heterodora glycines* Ichinoe) and tolerance to abiotic stresses [10]. The frequency of seed formation and recovery of plants through immature hybrid embryo rescue was very low and only four seeds were obtained from the amphiploid plants [9]. While backcrossing the amphiploid (2*n* = 118) with *G. max* cv. Clark-63 (2*n* = 40), only 15 BC_1 plants with a chromosome number of 2*n* = 76, instead of the expected chromosome number of 2*n* = 79, were obtained [9]. In order to develop additional BC-derived lines, the three remnant seeds of the amphidiploid hybrid were subjected to germination in a moist filter paper-lined Petri dish. Two of the three seeds did not germinate after storage at 10°C for about 10 years. The present investigation was undertaken to regenerate plants from the one remaining viable seed of a previously-generated amphiploid hybrid (2*n* = 118) of *G. max* cv. Altona (2*n* = 40) and *G. tomentella* PI 483218 (2*n* = 78). The goal was to multiply the amphiploid hybrid plants from the single seed through tissue culture and finally to employ the regenerants for use in backcrossing with *G. max* cv. Clark 63, so as to obtain BC-derived fertile lines for further genetic studies.

Grain legumes in general and *Glycine* species in particular, are recalcitrant to *in vitro* plant regeneration [11, 12]. Plant regeneration from hybrid embryos of soybean and wild perennials is also difficult [8]. The upsurge of both types of reports, *i.e.* occurrence and non-occurrence of variation in the tissue culture-raised plants [13], prompted us to confirm the hybridity of regenerated plants prior to their use for backcrossing. Therefore, assessment of morphological characteristics

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and analysis using simple sequence repeat (SSR) markers were also carried out to examine the hybridity of the tissue culture-raised plants.

Materials and methods

Origin of material: The origin of amphiploid hybrid seed was as follows: *G. max* cv. Altona ($2n$ = 40, genome GG) × *G. tomentella* accession No. PI 483218 ($2n$ = 78, genome DDEE) → F_1 seeds ($2n$ = 59, genome GDE) → Colchicine treatment (CT) → F_1 seeds ($2n$ = 118, genome GGDDEE) → one seed germinated and produced F_2 ($2n$ = 118, genome GGDDEE) → four seeds obtained as F_3 ($2n$ = 118, genome GGDDEE) → only one seed ($2n$ = 118, genome GGDDEE) was available that was designated as H213-2aF_3 [9] and used for regeneration of plants through tissue culture during present investigation.

Plantlet regeneration: The single seed (H213-2aF_3) was surface-sterilized with 90% alcohol for 1 min followed by 3 rinses with sterile distilled water. The seed was soaked in 20% commercial bleach, Chlorox (6% sodium hypochlorite) containing one drop of Tween-20 for 20 min and rinsed with sterile distilled water for 3 times under the laminar flow hood. The seed was cultured on nutrient initial medium (IM) and maintained for 10 weeks. Thereafter, it was transferred to germination medium (GM) for 8 weeks. Later, the cotyledons were removed and embryonic axis was transferred to multiplication medium (MM) for shoot regeneration. The details of composition of the media used at different stages of the protocol are given in Table 1.

From initiation of culture to shoot regeneration, the seed/cultures were transferred to respective fresh media after every 2 weeks. Once the shoot buds developed, the original culture was subcultured to fresh MM medium; and each subcultu'ring is referred to as one subculture cycle. To induce rooting, developed shoots (2-3 cm) with 3-5 leaves were transferred on rooting medium (RM).

Hardening treatment: Rooted shoots were subjected to hardening treatment in Magenta boxes (GA7 vessel, Magenta Co.) containing sterile vermiculite and peat moss (3:1) saturated with Hoaglands solution [14] and maintained under cool white florescence illumination (Philips, 34 watt) of *ca.* 45 mol m^{-2} s^{-1}(16-h photoperiod, 25 ± 2°C). After 6-8 days, the lids of boxes were slightly removed and subsequently after 2 weeks, the plantlets were exposed to environment by completely removing the lids of Magenta boxes. The plantlets were watered twice a week. After 2-3 weeks of hardening, the plants were transplanted to clay pots (20 cm diameter) containing soil : peat : torpedo sand (1:1:1) and allowed to grow in the greenhouse at the Department of Crop Science, University of Illinois, Urbana, under 16-h photoperiod at 25 ± 2°C with 1197 mol m^{-2} s^{-1} photosynthetic active radiation.

Table 1. Composition of media used for plant regeneration in amphiploid hybrid (*G. max* × *G. tomentella*)

Chemical constituent (mg l^{-1})	Initial medium (IM)	Germination medium (GM)	Multiplication medium (MM)	Rooting medium (RM)
KNO_3	2,500	2,500	2,000	80
$(NH_4)_2SO_4$	134	134	-	-
NH_4NO_3	-	-	1,000	
$NaH_2PO_4.H_2O$	150	150	100	-
KH_2PO_4	-	-	300	-
$MgSO_4.7H_2O$	250	250	375	-
$MgSO_4.H_2O$	-	-	-	576
Na_2SO_4	-	-	-	200
$CaCl_2.2H_2O$	150	150	600	-
$Ca(NO_3)_2$	-	-	-	43
$2H_3BO_3$	3.0	3.0	3.0	1.5
$MnSO_4.H_2O$	10.0	10.0	10.0	7.0
$ZnSO_4.7H_2O$	2.0	2.0	2.0	3.0
KI	0.75	0.75	0.75	0.75
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.1
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.01
$CoCl_2.6H_2O$	0.025	0.025	0.025	-
KCl	-	-	-	65.0
EDTA	-	-	26.1	-
Na_2EDTA	37.3	-	-	-
Fe.NaEDTA	-	73.4	-	73.4
$FeSO_4.7H_2O$	27.8	-	24.9	-
Nicotinic acid	1.0	1.0	1.0	1.0
Pyridoxine.HCl	1.0	1.0	2.0	1.0
Thiamine.HCl	10.0	10.0	10.0	1.0
Glycine	2.0	2.0	2.0	4.0
Ascorbic acid	100	-	-	-
L-glutamine	7,300	-	-	-
Coumarin	-	-	-	9.0
IAA	0.2	0.3	-	1.0
NAA	2.0	-	-	-
Kinetin	1.28	0.75	-	-
BA	0.5	-	0.25	-
Sucrose	100,000	30,000	25,000	10,000
Agar	8,000	8,000	6,000	8,000
pH	5.8	5.6	5.6	6.0

IM = Modified B_5 medium [26]; GM = B_5 medium [26]; MM = Modified PC-L2 medium [27]; RM = Modified White's medium [23]

Culture conditions: The details of media used for regeneration of plantlets are given in Table 1. The pH of all media were adjusted with 0.1 N NaOH prior to the addition of agar-agar (Sigma) as specified in Table 1. Approximately 20 ml of medium was dispensed into culture tubes (25 × 150 mm, Borosil). The medium was autoclaved at 121°C temperature and 1.06 kg cm^{-1} for 20 min. Cultures were incubated at 25 ± 2°C

at a light intensity of *ca.* 45 mol m^{-2} s^{-1} provided by cool white flourescent lamps (Philips; 34 watt) at a photoperiod of 16-h in a Percival Model LVL incubator. All the chemicals used were of analytical grade (Sigma, Inc. St Louis, MO).

Morphological marker analysis: Morphological qualitative traits such as upper terminal leaflet shape, stipule shape, lower terminal leaflet shape, and calyx pubescence density were characterized. Quantitative traits such as upper terminal leaflet length and width (cm), and lower terminal leaflet length and width (cm) were measured as a mean of 10 measurements from five plants.

Simple sequence repeats (SSR) marker analysis: DNA from each of the 16 randomly selected plants was isolated [15]. A total of 29 primer pairs were tested to assess the polymorphism in soybean and *G. tomentella*. primer pairs for three soybean SSR loci (Sat_094, Satt 521 and Satt 584) were identified, which showed polymorphism between *G. max* cv. Altona and *G. tomentella* PI 483218 [16, 17]. These SSR primers were used to confirm the hybidity of regenerants in this study. PCR reactions were undertaken in 10 µl volumes containing 30-45 ng of template DNA, 1.5 pmole of each primer, 0.2 mM dNTPs (Pharmacia Biotech Inc., Piscataway, NJ), 1.5 mM $MgCl_2$, 1 × PCR buffer and 0.5 unit Taq polymerase (Gibco BRL Life Technologies Inc., Gaithersberg, MD). Temperature cycling was performed in an MJ Research PTC 100 Thermal Controller using the program of touchdown PCR [18] with slight modification. The amplification profile was set to run at 94°C for 3 min followed by 6 cycles of denaturing at 94°C for 30 sec. annealing from 55°C to 50°C with 1°C decreased by one cycle for 30 sec, and extending at 72° for 1 min. The final cycle (94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min) was repeated 35 times.

Amplification products were detected on 6% denaturing polyacrylamide gel electrophoresis and silver staining. Gels were made with 6% (w/v) acrylamide bis-acrylamide (19:1), 7 M ultra-pure urea, 40% (v/v) formamide, 1 × TBE buffer, 1% TEMED, and 10% ammonium persulfate. Following polymerization of the gel, wells were loaded with 5 µl of each amplification product mixed with 2.5 µl of loading buffer [98% formamide, 10 mM EDTA (pH 8.0), 0.075% Bromophenol Blue, 0.075% Xylene Cyanol FF]. Gels were run at 120 volt and constant power for 2 h. The silver staining protocol was used according to the instructions provided by BIORAD (Hercules, CA).

Results and discussion

Plantlet regeneration: One seed of H213-2aF_3 responded on nutrient culture medium whereas the other two seeds of the same line did not germinate on the moist filter paper. However, the response of seed in culture was extremely slow. During the first 8 weeks from culture on IM, the seed showed only some turgidity but no sign of germination. The cultured seed was transferred to fresh medium every 2 weeks. After a total of 10 weeks on IM, the seed coat cracked. The germinating embryo was transferred to GM and maintained for 4 weeks on the same medium. During this period, expansion of the radicle was observed and the embryo remained green. At this stage the cotyledons were removed and embryonic axis was transferred to MM. After 4 weeks, two tiny linear leaves were observed that converted into rosette-shaped trifoliate leaves. These leafy shoots grew relatively fast on MM. After a total period of 8 weeks, the enlarged solid basal part produced three small shoot buds. The number of shoot buds increased with the transfer of subcultures onto fresh medium. After 20 weeks on MM, two shoots of *ca.* 2-3 cm length were developed alongwith some 4-6 shoot buds/subculture were observed (Fig. 1A). For multiplication of shoot buds or shoots, MM was supplemented with 6-benzyl aminopurine (BA) that supported shoot regeneration. The role of BA in regulating morphogenesis in *Glycine* species to induce shoot buds in *G. tomentella* [19], *G. canescens* [20], *G. clandestina* [21], has been documented. Singh *et al.* (1987) [22] also used BA to culture the hybrid embryo of *G. max* and *G. clandestina*.

Once the shoot buds were converted to small shoots (0.5 cm), the growth of the shoots was faster. Such shoots attained 2-3 cm length within 2 weeks. The shoots were excised from their base and cultured on RM for rooting. By incorporating the coumarin (9 mgl^{-1}) into White's medium [23] as modified by Hymowitz *et al.* (1986) [21], 100% of the shoots produced roots. Generally, 1-4 thick roots or rarely 5 roots regenerated within 10 days of culturing the shoots on RM (Fig. 1B). Within 2 weeks, secondary roots developed. The efficacy of coumarin in rooting of the hybrid plants of *G. max* × *G. clandestina* has also been reported earlier [22]. To get the first rooted plantlet from tissue culture, it took nearly one year from initiation of seed culture. The details of development of shoots/subculture cycle and survival of plants are given in Table 2. Recovery of hybrid plantlets is an extremely slow process in tissue culture. Not only with the hybrid embryos of soybean and wild perennials *i.e. G. max* × *G. tomentella* [6, 24, 25] but also with selfed embryo of *G. tomentella* [25], the recovery of plantlets in tissue culture tended to be very slow and with low regeneration capacity. Thus, *G. tomentella*, the male parent of the amphiploid hybrid, may be responsible for the extremely slow response in culture media.

Hardening of the plantlets: Plantlets were subjected to hardening; 50 out of 55 plants survived the hardening

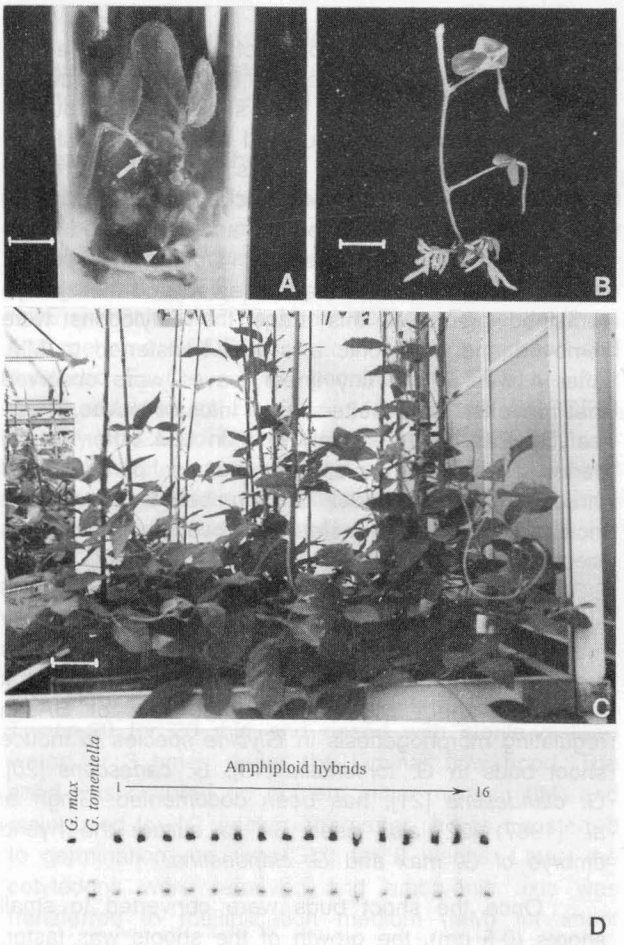

Fig. 1. (A) 25-week-old culture on MM showing new shoot development (arrow-head) and old shoot (arrow) ready for rooting (Bar = 2 cm). (B) 2-week-old shoot culture on RM showing rooting (Bar = 1.5 cm). (C) Tissue culture-raised amphiploid hybrid plants growing in the greenhouse (Bar = 0.2 cm). (D) SSR marker (Satt521) profile: First two lanes show marker from *G. max* cv. Altona and *G. tomentella* (PI 483218), respectively, and lanes 1 → 16 show presence of markers from both the parents in amphiploid hybrid plants.

treatment (Table 2). The plants with less than 2 cm height did not survive the hardening treatment even if they had a good root system. We observed that 2-3 cm long shoots with 3-5 leaves were most successful during the hardening process. All the hardened plants grew successfully in the greenhouse (Fig. 1C) and flowered within 3 months of transfer to the greenhouse.

Morphological marker analysis: The morphological traits of these plants and their parents were studied and the data are presented in Table 3. Various factors such as the *in vitro* procedure, stress in culture, growth substances and other culture conditions are known to induce the variations in tissue culture-raised plants [13]. On the basis of phenotypic characters studied, the data show that the hybrid clones morphologically exhibited the intermediate characters inherited from their parents

Table 2. Details of plantlet regeneration of amphiploid hybrids and their survival in greenhouse

Subculture cycle	No. of sub-cultures	No. of shoot buds/ sub-culture	No. of shoots subjected to rooting	No. of shoots rooted	No. of plantlets survived after hardening	No. of plants survived in greenhouse
I	1	4.0	2	2	2	2
II	10	6.3	8	8	8	8
III	10	9.4	11	9	9	9
IV	5*	7.3	8	8	8	8
V	10	6.6	10	10	10	10
VI	11	5.9	7	7	5	5
V	11	5.4	11	11	8	8

*Five subcultures of subculture cycle III were contaminated and discarded, thus, only 5 subcultures were retained in subculture cycle IV

Table 3. Morphological data of amphiploid (tissue culture-regenerants) and its parents

Morphological trait	*G. tomentella* PI 483218 ($2n = 78$)	Amphiploid ($2n = 118$)	G. max cv. Altona ($2n = 40$)
Upper terminal leaflet shape	Elliptical	Lanceolate	Elliptic-oval
Stipule shape	Ovate	Lanceolate	Lanceolate-deltoid
Upper terminal leaf length (mm)	36.2	69.6	78.6
Upper terminal leaf width (mm)	13.4	35.2	59.2
Lower terminal leaflet shape	Oval	Elliptical	Elliptic-oval
Calyx pubescence density	Medium	Dense	Dense

i.e. G. max cv Altona × *G. tomentella* PI 483218 (Table 3) and are similar to each other.

SSR marker analysis: SSR analysis was performed on 16 randomly selected plants regenerated through tissue culture to confirm their hybrid nature. Of the 29 SSRs tested, three (Satt584, Satt521 and Sat_094) showed polymorphism [16, 17]. All the tested regenerants showed presence of fragments from *G. max* cv. Altona and *G. tomentella* PI 483218, thus, confirming their hybrid status (Fig. 1D). The SSR markers used in this study proved to be efficient for identification of alien chromosome of *G. tomentella* in the background of *G. max*, thus, unambiguously confirming the hybridity of plants regenerated through tissue culture.

Thus, in the present study a total of 55 amphiploid hybrid plants were regenerated from one seed through tissue culture. After hardening, the plants grown in the

greenhouse flowered. We demonstrated that high frequency of plant regeneration is possible from even one single hybrid seed derived from a very difficult cross of *G. max* × *G. tomentella*. The tissue culture-raised hybrid plants, confirmed using morphological and SSR markers, are being used to backcross with *Glycine max* cv. Clark 63 as the male parent to study the introgression of genes introduced from *G. tomentella*.

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