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ORIGINAL PAPER

Intersubgeneric hybridization between *Glycine max* and *G. tomentella*: production of F_1, amphidiploid, BC_1, BC_2, BC_3, and fertile soybean plants

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Abstract

Key message **This paper describes methods for unlocking genetic treasure from wild perennial *Glycine* species of Australia for soybean improvement.**

Abstract The genetic resources of the ca. 26 species of the genus *Glycine* subgenus *Glycine* have not been exploited to broaden the genetic base of soybean (*Glycine max*; $2n = 40$). The objectives of this study were to develop methods for producing F_1, amphidiploid, BC_1, BC_2, BC_3, and fertile soybean plants from crosses of soybean and the genus *Glycine* subgenus *Glycine* species, in order to utilize the subgenus *Glycine* germplasm in soybean breeding. Soybean cultivars were hybridized with six accessions of 78-chromosome *G. tomentella* as well as one accession each of 40-chromosome *G. tomentella*, *G. argyrea* and *G. latifolia*. They were chosen because they exhibit resistance to soybean rust. We were successful in producing fertile soybean from soybean cv. 'Dwight' and 78-chromosome *G. tomentella* accession PI 441001, while other hybrids were discontinued either at F_1 or amphidiploid stage. The F_1 seeds aborted prior to reaching maturity, so developing seeds from 19 to 21 day old pods were cultured aseptically in various media formulations. Seed maturation and multiple embryo generation media were developed. F_1 plants with shoots and roots ($2n = 59$) were transplanted to pots in greenhouse. Amphidiploid ($2n = 118$) plants were backcrossed to 'Dwight'. BC_1 ($2n = 79$) plants were propagated through in vitro and 43 mature BC_2F_1 seeds were harvested. Fifteen surviving BC_2F_1 plants were morphologically distinct, sterile, and had chromosome numbers ranging $2n = 56$–59. Chromosome numbers of the BC_3F_1 plants ranged $2n = 40$–49. Derived fertile soybeans were first planted in the field in 2008 and are being evaluated for yield, resistance to pathogens and pests and tolerance to salt through material transfer agreement.

Abbreviations

PI	Plant introduction
BC	Backcross
GA_3	Gibberellic acid
2, 4-D	2, 4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
IAA	Indole-3-acetic acid
NAA	1-Naphthaleneaceticacid
MAALs	Monosomic alien addition lines
DAALs	Disomic alien addition lines

Introduction

The genetic diversity of wild perennial *Glycine* Willd. species has not been exploited in domesticated soybean [*Glycine max* (L.) Merr.; $2n = 40$] breeding. These species are extremely diverse morphologically, cytologically, and genomically and grow in diverse climatic and soil conditions and have a wide geographical distribution in Australia and surrounding islands (Chung and Singh 2008). Screening of accessions has demonstrated that wild perennial *Glycine* species harbor genes for resistance to soybean rust (*Phakopsora pachyrhizi* Sydow) (Burdon and Marshall 1981; Burdon 1988; Schoen et al. 1992; Hartman et al. 1992), soybean cyst nematode (*Heterodera glycines*

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Ichinohe) (Riggs et al. 1998; Bauer et al. 2007), soybean brown spot (*Septoria glycines* Hemmi.) (Lim and Hymowitz 1987), *alfalfa mosaic virus* (Horlock et al. 1997), *bean pod mottle virus* (Zheng et al. 2005), and white mold [*Sclerotinia sclerotiorum* (Lib.) de Bary] (Chang 2014) and tolerance to 2, 4-D (Hart et al. 1991), and chloride (Pantalone et al. 1997). These traits can be transferred to the soybean if a wide cross methodology is developed.

Considering the gene pool concept developed by Harlan and de Wet (1971), *G. max* and its wild annual progenitor *G. soja* Sieb. and Zucc. ($2n = 40$) are included in the primary gene pool because both species are cross compatible and produce viable fertile F_1 plants and taxonomically are included in the subgenus *Soja* (Moench) F.J.Hermann. *Glycine max* does not have secondary gene pool. If we follow the definition of tertiary gene pool (hybrids between primary gene pool and tertiary gene pool are anomalous, lethal or completely sterile and gene transfer is either not possible with known techniques or else rather extreme or radical measures are required) of Harlan and de Wet (1971), 26 species of the subgenus *Glycine* can be placed into the tertiary gene pool of the soybeans because all intersubgeneric F_1 plants obtained, so far, were through immature seed rescue method and were completely sterile (Broué et al. 1982; Newell and Hymowitz 1982; Grant et al. 1986; Singh et al. 1987; Newell et al. 1987; Chung and Kim 1990, 1991; Bodanese-Zanettini et al. 1996.)

Of the 26 wild perennial *Glycine* species, *G. tomentella* Hayata is a very unique species because it consists of four cytotypes ($2n = 38, 40, 78, 80$), has a wide geographical distribution with $2n = 38$, 40, 78 and 80-chromosome types in Australia (Queensland, Northern Territory, and Western Australia). Accessions with $2n = 40$, 78, and 80-chromosome complements have been collected in Papua New Guinea and 80-chromosome accession from the Philippines, Timor, and Taiwan (http://www.ars-grin.gov/cgi-bin/npgs/html/site_holding.pl?SOY). Since the publication of Palmer and Hadley (1968) and Ladizinsky et al. (1979), there were a few attempts that produced intersubgeneric F_1 hybrids ($2n = 59$, 60) between *G. max* and 78 and 80-chromosome *G. tomentella* (Newell and Hymowitz 1982; Newell et al. 1987; Bodanese-Zanettini et al. 1996). Amphidiploid ($2n = 118$, 120) plants were sterile and they failed to produce BC_1 plants.

Singh et al. (1990) obtained the amphidiploid ($2n = 118$) and BC_1 progeny from a *G. max* cv. 'Altona' × *G. tomentella* ($2n = 78$; PI 483218) F_1 plant ($2n = 59$) which had been produced by Newell and Hymowitz (1982). Soybean cv. 'Clark 63' was selected as a recurrent parent because it flowered profusely and was a good pollinizer in the greenhouse. 'Altona' which is in maturity group 00 did not produce sufficient flowers. The BC_1 plants contained $2n = 76$ chromosomes; three fewer chromosomes than were expected. Singh et al. (1993) produced three BC_2F_1 plants with $2n = 55$, 56, and 58 chromosomes. Backcrossing to 'Clark 63' produced four progeny from plant H622-1 ($2n = 58$), 11 progeny from plant H628-1 ($2n = 56$) and one progeny from plant H638-1 ($2n = 55$). In BC_4, they isolated plants with $2n = 40$–64 chromosomes. Based on morphological modifications produced by one extra PI 483218 chromosome added to 'Clark 63' chromosome complement ($2n = 41$), Singh et al. (1998) identified 22 monosomic alien addition lines MAALs. These materials did not cover 39 PI 483218 chromosomes because the BC_1 lacked three chromosomes and the five BC_3 plants were derived from only three BC_2 plants. Patzoldt et al. (2007) screened 423 of the lines, produced by Singh et al. (1998), for resistance to soybean rust, and found that all lines were susceptible, while PI 483218 and the amphidiploid clones were resistant. Brucker (2004) did not find resistance to soybean cyst nematode (SCN) isolates HG Type 0 or HG Type 2.5.7 in BC_4-derived lines but PI 483218 and amphidiploid clones were resistant.

The objectives of this study were: (1) to hybridize *G. max* cultivars with 40- and 78-chromosome *G. tomentella*, *G. argyrea* Tindale, and *G. latifolia* (Benth.) Newell and Hymowitz accessions, (2) to develop methodologies to rescue immature seeds to obtain F_1 plants, (3) to produce amphidiploid, BC_1, BC_2, BC_3, and fertile plants, and (4) to isolate and identify plants with $2n = 40$ (disomic), 41 (MAALs), and 42 (disomic alien addition lines, DAALs) from *G. max* cv. 'Dwight' ($2n = 40$; genome G_1G_1) with *G. tomentella* ($2n = 78$; genome D_3D_3EE) accession PI 441001. We will compare the effect of 'Dwight' and PI 441001 cytoplasm on plant morphology of F_1, amphidiploid, BC_1, BC_2, and BC_3 fertile plants with $2n = 40$, 41, 42 chromosomes.

Materials and methods

Materials

Table 1 lists *G. tomentella*, *G. argyrea* and *G. latifolia* accessions, $2n$ numbers and collection sites used in hybridization with *G. max* cultivars. *Glycine tomentella* accessions PI 441001, PI 441008, PI 583970, PI 505258, PI 378708, PI 563892 contain $2n = 78$ and were from Australia (Fig. 1). One accession of *G. argyrea* (PI 505151; $2n = 40$) and *G. latifolia* (PI 559298; $2n = 40$) were also included in this study. One accession of *G. tomentella* (PI 446998) with $2n = 40$ collected from Port Moresby, Papua New Guinea was included (Fig. 1). Hartman et al. (1992) reported that PI 441008, PI 446998 and PI 505151 accessions were resistant to soybean rust (*P. pachyrhizi*) and PI 441001 accession was moderately resistant. Schoen et al. (1992) reported that PI 441001 and PI 378708 were resistant to soybean rust. PI 441001, PI 441008, PI 505258 and PI 563892 were expected to have genes for

Table 1 *Glycine tomentella, G. argyrea, G. latifolia* accessions, plant introduction (PI), 2*n* chromosomes and collection sites used in the hybridization with *G. max* cultivars

Species	PI number[a]	2*n*	Collection site	Latitude	Longitude
G. tomentella	441001	78	Collected from Brampton Island Airstrip (apron of turnaround at end of runway on small embankment; habitat, sandy soil), Queensland, Australia	20°49′0″S	149°17′0″E
G. tomentella	441008	78	Collected from Station Creek, Red Rock; (habitat: coastal sand dunes) New South Wales, Australia	29°56′0″S	153°13′0″E
G. tomentella	583970	78	Collected from Carnarvon Gorge, path to ampitheatre, Queensland, Australia	25°3′0″S	148°12′0″E
G. tomentella	505258	78	Collected from Alma Beach, Horseshoe Bay, Magnetic Island, Queensland, Australia	19°10′0″S	146°51′0″E
G. tomentella	378708	78	Collected from Eskdale, Queensland, Australia	27°9′0″S	152°15′0″E
G. tomentella	563892	78	Collected from Brunswick Heads; (habitat, growing on sand at back of beach) New South Wales, Australia	28°32′0″S	153°33′0″E
G. tomentella	446998	40	Collected from Port Moresby, Papua New Guinea	8°30′0″S	147°147′0″E
G. argyrea	505151	40	Collected from Rainbow Beach, Cooloola National Park, Queensland, Australia	25°54′8″S	153°5′30″E
G. latifolia	559298	40	Collected from Freshfield bore, 21 km SW of Capella, Queensland, Australia	23°16′0″S	148°6′0″E

[a] Plant introduction (http://www.ars-grin.gov/cgi-bin/npgs/html/site.pl?SOY)

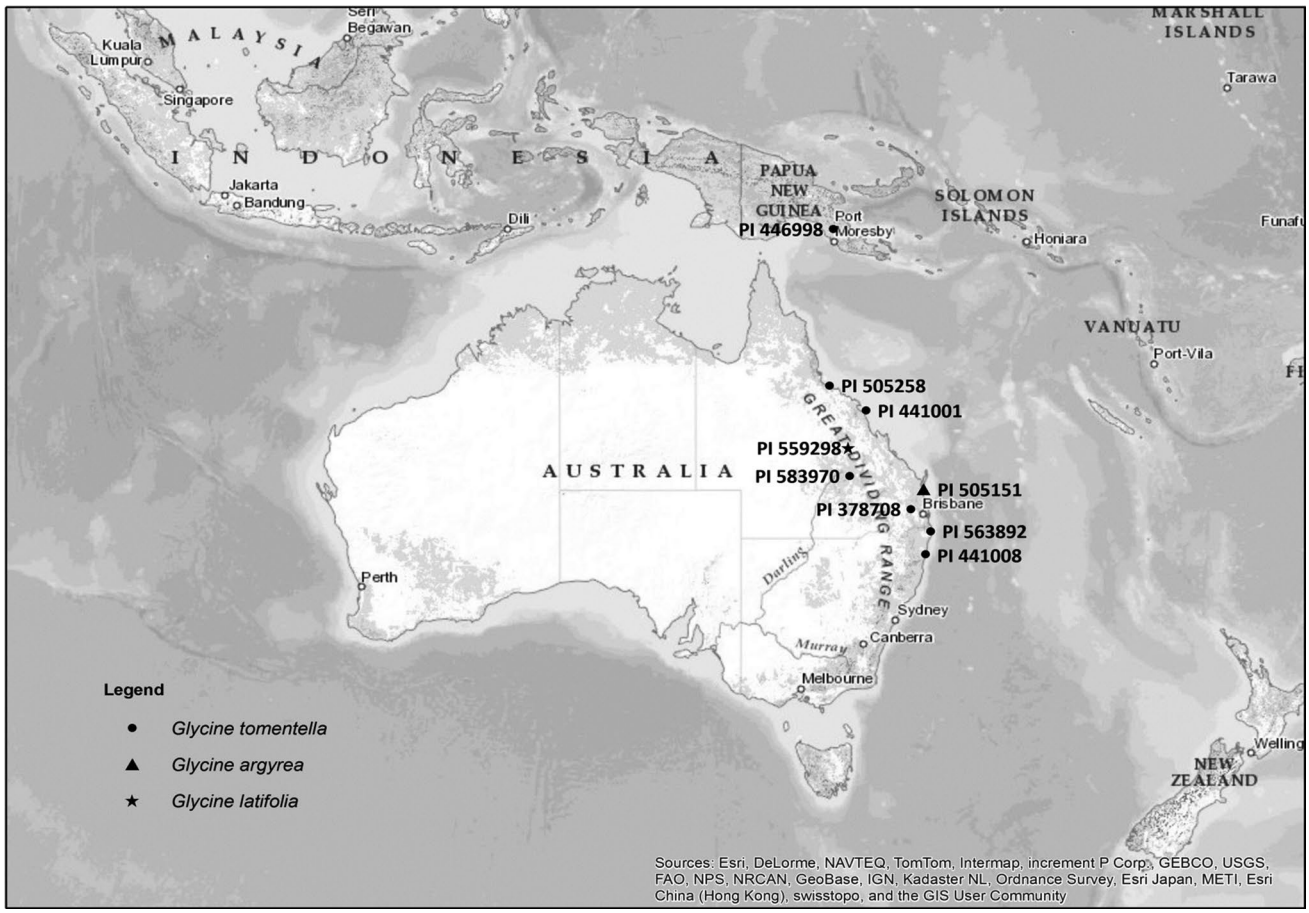

Fig. 1 A map of Australia including Papua New Guinea showing physical locations of accessions used in this study

salt tolerance because they were collected either from an island or from beaches (Table 1). PI 583970 was included in crossing program because it was resistant to *bean pod mottle virus*, however, extensive screening revealed that it was susceptible (Zheng et al. 2005). PI 559298 is resistant to white mold (*S. sclerotiorum*; Chang 2014). Table 1 lists soybean cultivars ('Clark 63', 'Dwight', 'IA 2052', 'IA 3010', 'Ina', 'Macon', 'Pella', and 'Williams 82') hybridized with the wild

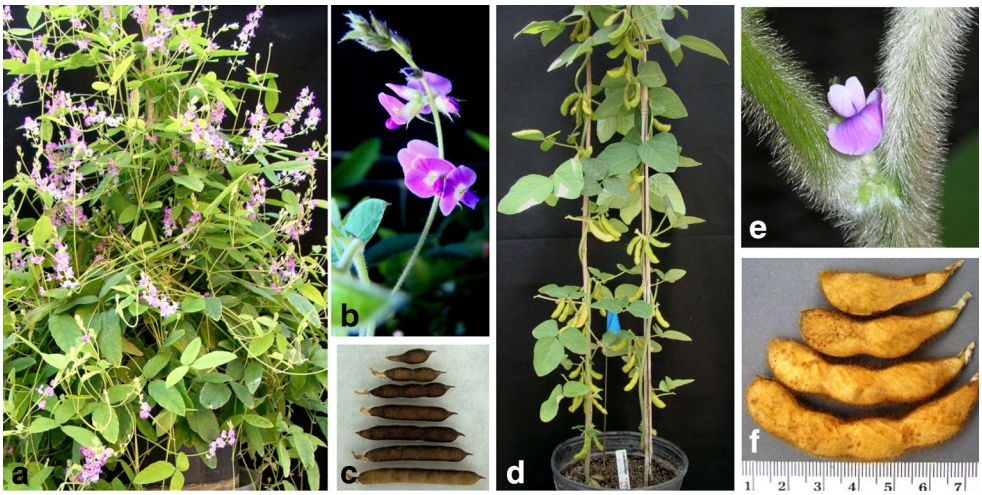

Fig. 2 Plants of *Glycine tomentella* PI 441001 and *G. max* cv. 'Dwight' growing in the greenhouse. **a** *Glycine tomentella*, PI 441001, at flowering stage showing twinning trait; **b** one long raceme of PI 441001 showing large purple flowers; **c** seven mature pods of PI 441001 showing *black* pod color and seed set ranges from one (*top*) to seven (*bottom*); **d** Two *Glycine max* cv. 'Dwight' plant growing in one pot at near maturity stage; **e** a branch of Dwight at flowering stage showing one *purple* flower; **f** Four mature pods of 'Dwight' with *tan* color showing one seeded (*top*) to four seeded pods (*bottom*) (color figure online)

perennial *Glycine* species accessions. Seeds of soybean cultivars, *G. tomentella*, *G. argyrea*, and *G. latifolia* accessions were obtained from the USDA Soybean Germplasm Collection (http://www.ars-grin.gov/cgi-bin/npgs/html/site.pl?SOY). In the greenhouse, wild perennial *Glycine* accessions used in this study were viney, vigorous climbers and all produced purple flowers. The one exception was PI 441008 that was slow growing and a non-climber. Soybean cultivars 'Macon' and 'Williams 82' produced white flowers, whereas 'Clark 63', 'Dwight', 'Ina', 'IA 2052', 'IA 3010', and 'Pella' produced purple flowers. These cultivars were used in the crossing program because they were growing in the greenhouse; there was no particular reason in selecting these cultivars. Figure 2 shows morphological differences between *G. tomentella* PI 441001 (Fig. 2a–c) and *G. max* cv. 'Dwight' (Fig. 2d–f).

Methods

Seeds of all accessions of *G. tomentella*, *G. argyrea*, and *G. latifolia* were scarified (an incision opposite to hilum was made by using a razor blade), germinated in the Petri plates on a filter paper, and grown in pots in the greenhouse. Soybean cultivars were grown in pots and also in the gravel benches that were flooded with a nutrient solution five times a day. Prior to anthesis, calyx, corolla and anthers of soybean cultivars were removed and simultaneously fresh pollen from the newly opened flowers of *G. tomentella*, *G. argyrea*, and *G. latifolia* accessions was dusted onto the stigma. A growth-hormone solution containing 100 mg GA_3, 25 mg NAA, and 5 mg kinetin/L distilled water was sprayed onto the pollinated gynoecia 24 h post-pollination (Jena and Khush 1989), and this was continued once a day for 19–21 days (Singh 2010).

Pods were removed from the plants approximately 3 weeks post-pollination. Pods were surface-disinfected in a 3 % solution of sodium hypochlorite (commercial bleach) for 20–30 min in a sanitized laminar flow cabinet. Pods were washed two times in sterile double-distilled water to remove all of the sodium hypochlorite. Scalpel and forceps were sterilized in Keller Steri 250 heating block. A clean incision was made on pod opposite to the funicle. Immature seeds were removed and embedded in the medium. Initially, we experimented with the commercial Murashige and Skoog's (MS) basal salts (Sigma M5519) + B5 vitamins (Sigma G 1019) supplemented with 40 mg/L 2, 4-D and B5 commercial salts (Sigma G5893) + B5 vitamins supplemented with 40 mg/L 2, 4-D. Immature seeds either died or produced non-morphogenic calluses on these media. We developed novel seed maturation (SM) medium without 2, 4-D (Singh 2010).

For seed maturation, vitamins that have been found to be most important are ascorbic acid, L-glutamine, and casein hydrolysate. Growth hormones IAA, NAA, kinetin were added in SM medium. These chemicals were not included in C5 medium and were also absent in MS and B5 medium (Singh 2010). Immature yellow-green seeds were carefully removed from pods, as described above, and imbedded in this SM medium. Petri plates were sealed with a strip of Para–film (Bemis Flexible Packaging-Neenah, WI 54956) and were kept in an incubator with 13 h of low-intensity light (25–30 µmol photons/m^2/s) at 25 °C. Seeds were transferred to fresh SM medium every 3–4 weeks. This process was continued until cotyledons or embryogenic calluses emerged.

The embryogenic calluses were transferred to multiple shoot regeneration medium (C5), which was developed after attempts using nine other media recipes that were unsuccessful. Modification was for the concentration of IAA, and BAP. Embryos turned brown when TDZ (thidiazuron) was added to C5 medium. Shoots were transferred to fresh C5 medium at 3–4 weeks intervals (Singh 2010). Actively growing shoots were transferred to a rooting medium [2.22 g MS basal medium w/vitamins (http://www.phytotechlab.com) + 15 g sucrose (Sigma; Lot # SLBC23332 V) + 3 g Gelzan™ (Sigma; Lot # 03M0031 V,)/L, pH 5.8]. Approximately 50 % of the shoots formed healthy seedlings that were transplanted to pots in the greenhouse.

Amphidiploid ($2n = 118$) plants from the crosses of 'Dwight' × PI 441001, 'Macon' × PI 441001, 'Ina' × PI 441001 were produced by treating multiple, actively growing shoots (one to two leaf stage) in a filter sterilized solution of 0.2 % colchicine + 2 % dimethyl sulfoxide (DMSO) + 10 drops Tween® 20 + 10 mg GA_3/L for 4–6 h in the culture incubator. Shoots were subsequently washed twice in sterile distilled-water in a sanitized laminar flow cabinet, and were transferred to C5 medium. All amphidiploids were identified cytologically using root tip chromosome preparations (Singh 2003).

We used only amphidiploid plants of 'Dwight' (genome, G_1G_1; $2n = 40$) × PI 441001 (genome, D_3D_3EE; $2n = 78$) for producing backcross derived lines. 'Dwight' was used as a recurrent parent. Backcrossing was continued until self-fertile plants were produced. Backcross-derived plants were identified morphologically based on growth habit and leaf size and shape (compared with 'Dwight' and PI 441001) and cytologically (mitotic and meiotic chromosome count). Plants were also propagated through cuttings by treating with a commercial powder (Hormex Rooting Powder # 8, Brooker Chemical Corporation, Chatsworth, CA 91313). Mitotic and meiotic chromosomes were examined according to methods including fluorescence in situ hybridization (FISH) described by Singh (2003). Fertile plants with $2n = 40$, 41, 42, 43 derived from BC_3 to BC_6 generations were planted in the field during summer.

Results

Production of intersubgeneric F_1 hybrids between *Glycine max* and *G. tomentella*, *G. argyrea*, and *G. latifolia*

Crossability rate

Table 2 shows the crossability rates between soybean cultivars and 40- and 78-chromosome *G. tomentella* accessions and with one accession ($2n = 40$) of *G. argyrea* and *G. latifolia*. Growth hormone mixture improved pod retention (immature pod set) on the plants because in previous studies, pod set was not observed without growth hormone treatment (Singh and Hymowitz 1987; Singh et al. 1990). Pod development was normal, remained dark-green due to growth hormone treatment and produced pods with one to three seeds. The three developing seeds were clearly visible in a 21 days old pod of 'Dwight' × PI 441001(Fig. 3a). Pod set on crosses between soybean cultivars ('Dwight', 'Ina', 'IA2052', 'IA3010', 'Macon', 'Pella' and 'Williams 82') and PI 441001 ranged from 0 to 49 % (Table 2). Soybean cultivars 'Dwight', 'Ina', 'Macon', 'Williams 82' were pollinated by PI 441008. Pod set ranged from 3 to 17 % (Table 2). When 'Clark 63', 'Dwight', 'IA 3010', 'Ina', and 'Macon' were pollinated by 40-chromosome *G. tomentella*, PI 446998, pod set ranged from 0 to 31 %. Based on pod set and performance in culture differences, we selected only 'Dwight' to cross with PI 505258, 563892, 583970, and 378708. Pod set was observed in all of these hybrid combinations (Table 2).

PI 505151 [*G. argyrea* ($2n = 40$)] was used as a pollen parent in crosses with 'Clark 63', 'Dwight', 'IA 2052', 'IA 3010', 'Ina', 'Macon', 'Pella', and 'Williams 82'. Pod set ranged from 0 % ('IA 3010') to 30 % ('IA 2052') (Table 2). The lack of pod set with 'IA3010' was likely the result of a low number of attempted pollinations.

PI 559298 [*G. latifolia* ($2n = 40$)] was hybridized, as a pollen parent, with 'Dwight'. Of the 250 flower buds of 'Dwight' pollinated by PI 559298, 21 immature pods (8 % pod set) were harvested (Table 2). PI 559289 has high level of resistance to white mold (*S. sclerotiorum*) because this accession is less sensitive to oxalic acid (Chang 2014).

Immature seed rescue-production of F_1 plants

Many immature seeds were lost during the initial phase of this study before we were able to optimize the growth media. Immature green seeds turned yellowish or whitish within 1 week on media prepared from commercial grades of B5 salts + B5 vitamins, and MS salts + B5 vitamins + BAP; (1 mg/L) + 40 mg/L 2, 4-D. In these media, green embryos excised from immature seeds turned white within 24 h. Most of the seeds and embryos from cultivars 'Clark 63', 'IA2052', 'IA 3010', and 'Williams 82' crossed with PI 441001, 441008, 446998 and 505151 died. Addition of 2, 4-D to media induced friable calluses that failed to become morphogenic after they were moved to a medium without 2, 4-D + 1 mg/L BAP.

Intersubgeneric F_1 pods 19–21 days post-pollination were removed from the plants while they were still dark

Table 2 Crossability rates between *Glycine max* ($2n = 40$) × *G. tomentella* ($2n = 40, 78$), *G. argyrea* ($2n = 40$) and *G. latifolia* ($2n = 40$)

Parents		2*n*	PI number	Flowers pollinated	Pod set total	Pod set %
Soybeans	*Glycine* Species					
Clark 63	*G. tomentella*	78	441001	44	0	0
Dwight	*G. tomentella*	78	441001	166	50	30
IA 2052	*G. tomentella*	78	441001	22	2	9
IA 3010	*G. tomentella*	78	441001	92	4	4
Ina	*G. tomentella*	78	441001	165	24	16
Macon	*G. tomentella*	78	441001	179	87	49
Williams 82	*G. tomentella*	78	441001	47	17	36
Dwight	*G. tomentella*	78	441008	266	27	10
Ina	*G. tomentella*	78	441008	58	2	3
Macon	*G. tomentella*	78	441008	43	2	5
Williams 82	*G. tomentella*	78	441008	12	2	17
Clark 63	*G. tomentella*	40	446998	25	1	4
Dwight	*G. tomentella*	40	446998	13	4	31
IA3010	*G. tomentella*	40	446998	7	0	0
Ina	*G. tomentella*	40	446998	47	8	17
Macon	*G. tomentella*	40	446998	24	2	8
Dwight	*G. tomentella*	78	505258	12	5	42
Dwight	*G. tomentella*	78	563892	18	9	50
Dwight	*G. tomentella*	78	583970	58	7	12
Dwight	*G. tomentella*	78	378708	62	9	15
Clark 63	*G. argyrea*	40	505151	51	1	2
Dwight	*G. argyrea*	40	505151	241	54	22
IA 2052	*G. argyrea*	40	505151	181	55	30
IA 3010	*G. argyrea*	40	505151	15	0	0
Ina	*G. argyrea*	40	505151	334	86	26
Macon	*G. argyrea*	40	505151	266	46	17
Pella	*G. argyrea*	40	505151	24	6	25
Williams 82	*G. argyrea*	40	505151	478	38	8
Dwight	*G. latifolia*	40	559298	250	21	8

PI, plant introduction

green (Fig. 3a). Immature seeds were aseptically removed and embedded into SM medium. Seeds remained green in SM medium for 3–4 weeks (Fig. 3b). Seed coats turned black after three to five transfers (3–5 months) but the cotyledons remained green and healthy (Fig. 3c) and occasionally cotyledons emerged from the black seed coats (Fig. 3d). At this time, germinating seeds were transferred onto C5 medium (Singh 2010). Multiple embryogenic buds appeared after one to two more transfers on C5 medium (Fig. 3e). Multiple shoots were produced from single seeds on C5 medium (Fig. 3f). Developing shoots were transferred to the rooting medium, where a small seedling was developed (Fig. 3g). Morphogenic calluses were observed in crosses with 'Dwight', 'Ina' and 'Macon' by PI 441001. Immature seeds produced calluses, but did not produce morphogenic embryos in crosses with 'Williams 82', 'IA2052', 'IA3010', and 'Pella' by PI 441001.

Immature crossed seeds of 'Dwight' × PI 441008 produced weak F_1 seedlings through embryogenesis. Five regenerants were transferred to vermiculite, but died before the second trifoliolate leaf (V2 stage) was produced. Embryogenic callus was not produced in 'Macon', 'Ina', and 'Williams 82' by PI 441008 and cultures were discarded. However, by using a bridge cross, 'Dwight' × F_1 (PI 441001 × PI 441008), viable and vigorous F_1 hybrid plants were recovered with flowers without anthers. Viable F_1 plants were obtained from the cross of 'Dwight' × PI 583970 and 'Dwight' × PI 378708. Other crosses involving *G. tomentella* accessions, PI 505258 and 563892 with soybean cultivar 'Dwight' produced immature seeds and friable calluses but embryogenic calluses were not recovered.

We were unable to rescue seeds to produce intersubgeneric F_1 plants derived from *G. tomentella* (PI 446998;

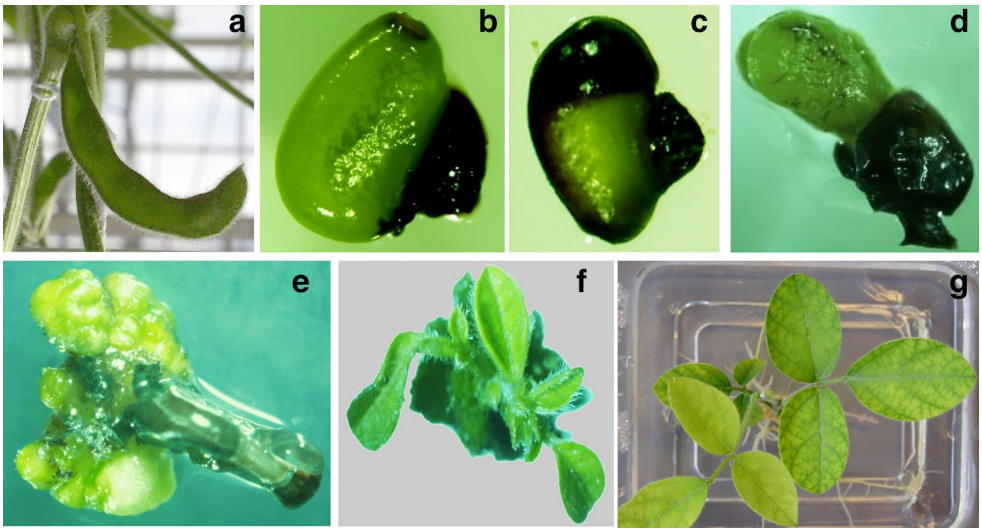

Fig. 3 Immature seed rescue of *Glycine max* cv. 'Dwight' ($2n = 40$) × *G. tomentella* PI 441001 ($2n = 78$). **a** One 21-day old hybrid pod showing three developing small seeds, **b** one immature seed in seed maturation (SM) medium after one week, **c** seed coat turns *black* but cotyledons are *green* and healthy, **d** cotyledons emerging from a seed with *black* seed coat, **e** multiple embryos, **f** one developing shoot with leaves, **g** a small seedling with roots and shoot in a rooting medium (color figure online)

$2n = 40$) and *G. argyrea* (PI 505151), despite the development of pods (Table 2). Seeds of these crosses were cultured at the time when we were formulating the media compositions. These seeds were cultured on a media with 40 mg/L, 2, 4-D. Immature seeds turned into friable calluses and embryogenic shoots were not produced. Multiple shoots are produced between 'Dwight' × *G. latifolia* (PI 559298) and shoots are being maintained in an incubator; F_1 plants have not been transferred to greenhouse.

Identification of F_1 hybrids and amphidiploids from Glycine max × G. tomentella crosses

Morphologically, F_1 plants resembled *G. tomentella* (PI 441001; D_3D_3EE) more than the soybean parents 'Dwight', 'Ina', and 'Macon' (G_1G_1). The initial growth of F_1 plants in the greenhouse was slow (Fig. 4a) but vigor recovered after roots were established. Figure 4a shows two seedlings; one shows normal growth while second seedling (arrow) is small and growing slowly. Both seedlings originated from the same clump of multiple shoot. The F_1 plants were perennial like *G. tomentella* and flowered profusely, but were sterile. Cytologically, F_1 plants contained expected $2n = 59$ (G_1D_3E) chromosomes. 'Dwight' and PI 441001 chromosomes were indistinguishable by Feulgen staining because all chromosomes were metacentric (Fig. 4b). At meiotic metaphase I, 50 microsporocytes were examined. An average chromosome association was 9 bivalents (II) + 41univalents (I). Rod-shaped bivalents were loosely associated (Fig. 4c; arrow), and ring-shaped bivalents were also observed (Fig. 4c). Trivalent and quadrivalent configurations were not seen. Movement of univalents to their respective poles was precocious.

Colchicine-induced amphidiploid ($2n = 118$) plants from culture were morphologically dwarf and stunted (Fig. 5a). Occasionally, plants produced one to two seeded mature pods (Fig. 5b) and seeds (Fig. 5c). Cuttings from the colchicine–induced amphidiploid plants were morphologically vigorous and produced normal leaves and flowers and were perennial. Amphidiploid plants contained $2n = 118$ chromosomes (Fig. 5d). The F_1 hybrid and amphidiploid plants were maintained by cuttings. Amphidiploid plants from 'Macon' × PI 441001 and 'Ina' × PI 441001 were morphologically similar to the amphidiploid plants of 'Dwight' × PI 441001 and produced mature pods and seeds.

Meiosis was studied in the amphidiploid of 'Dwight' × PI 441001. At diakinesis, a majority of microsporocytes showed 59 II (Fig. 5e). Detailed studies of meiosis of amphidiploid plants were not conducted because flower buds were cleistogamous (Fig. 5a). Meiosis of amphidiploid of 'Macon' × PI 441001 and 'Ina' × PI 441001 were not studied because backcrossing was discontinued for these amphidiploids. We directed our efforts to producing fertile backcross-derived plants from 'Dwight' × PI 441001 because 'Dwight' produced a large number of flowers with good pollen in greenhouse compared to 'Macon' and 'Ina'.

The F_1 plants from 'Dwight' × F_1 (PI 441001 × PI 441008) were initially weak and slow in vegetative growth but eventually recovered and grew vigorously. Leaves were

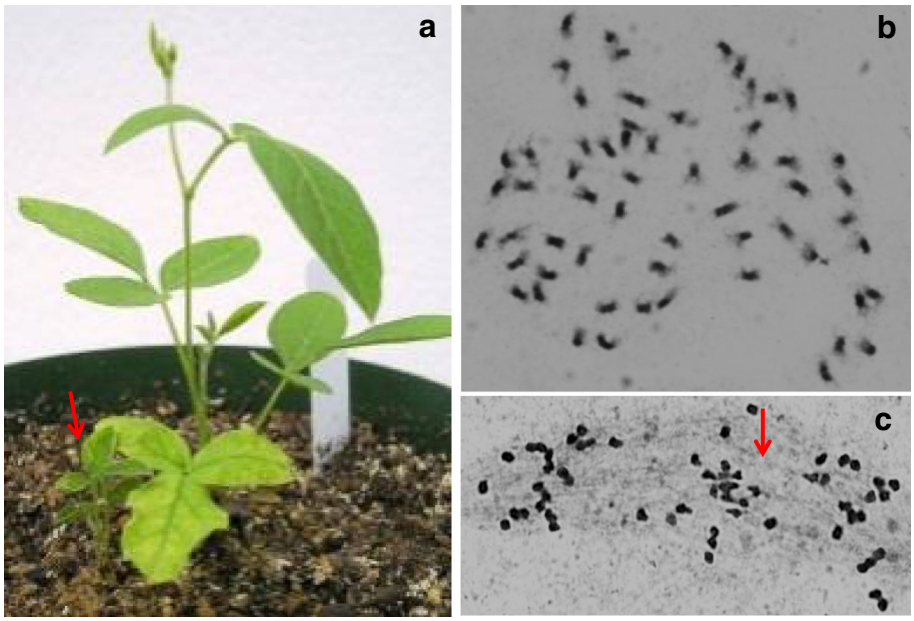

Fig. 4 Morphological and cytological identification of F_1 from *Glycine max* cv. 'Dwight' ($2n = 40$) × *G. tomentella* PI 441001 ($2n = 78$). **a** Two seedling growing in soil in a pot in greenhouse, growth of one seedling is slower (*arrow*) than the other seedling, **b** mitotic metaphase showing $2n = 59$ chromosomes; **c** meiotic metaphase I showing one ring bivalent, six rod bivalents (associated loosely; *arrow*) and univalents (most univalents are located at the poles but few are scattered in the cytoplasm (color figure online)

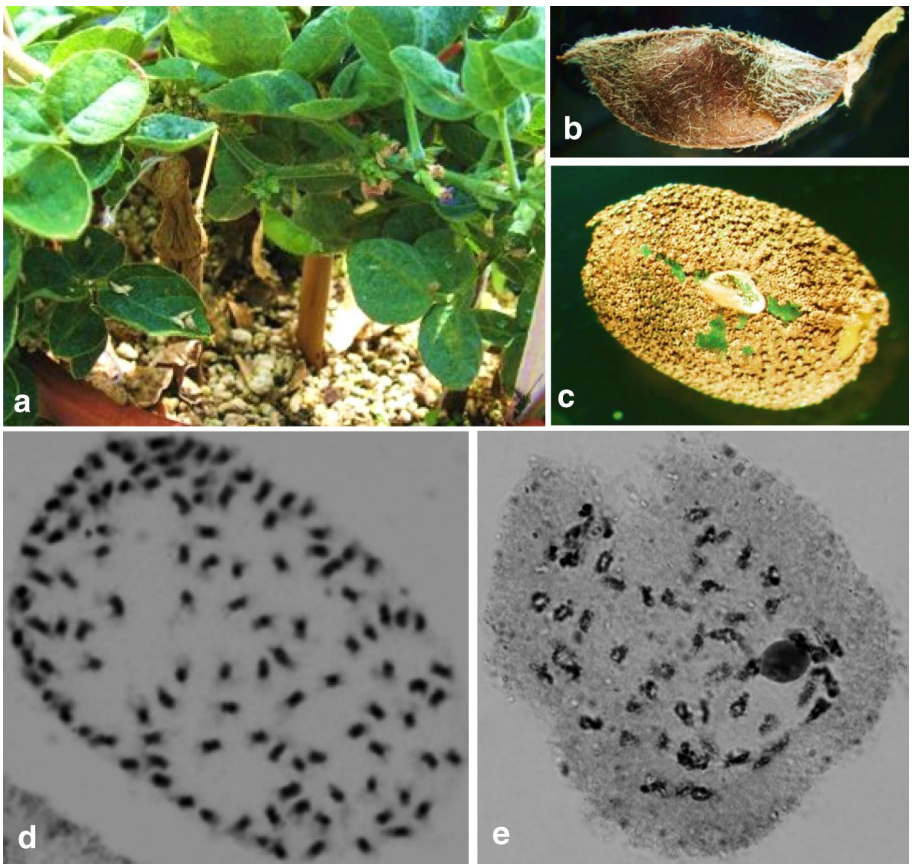

Fig. 5 Morphological and cytological identification of amphidiploid of *Glycine max* cv. 'Dwight' ($2n = 40$) × *G. tomentella* PI 441001($2n = 78$). **a** One amphidiploid plant growing in a pot in greenhouse showing stunted growth with one pod, **b** a mature pod with one seed, **c** a mature seed with seed coat structure like PI 441001, **d** mitotic metaphase showing $2n = 118$ chromosomes, all chromosomes are similar morphologically, **e** a microsporocyte of an amphidiploid plant showing mostly bivalent configuration at diakinesis (color figure online)

long, narrow and thick. Plants produced long racemes with large dark purple flowers without androecium. These morphological traits were different than those observed in 'Dwight' × PI 441001, 'Macon' × PI 441001, 'Ina' × PI 441001, or 'Dwight' × PI 378708 crosses.

Production and identification of BC_1 plants

Amphidiploid ($2n = 118$; $G_1G_1D_3D_3EE$) plants of 'Dwight' (G_1G_1) × PI 441001(D_3D_3EE) were hybridized with 'Dwight'. Of the 3072 amphidiploid flowers

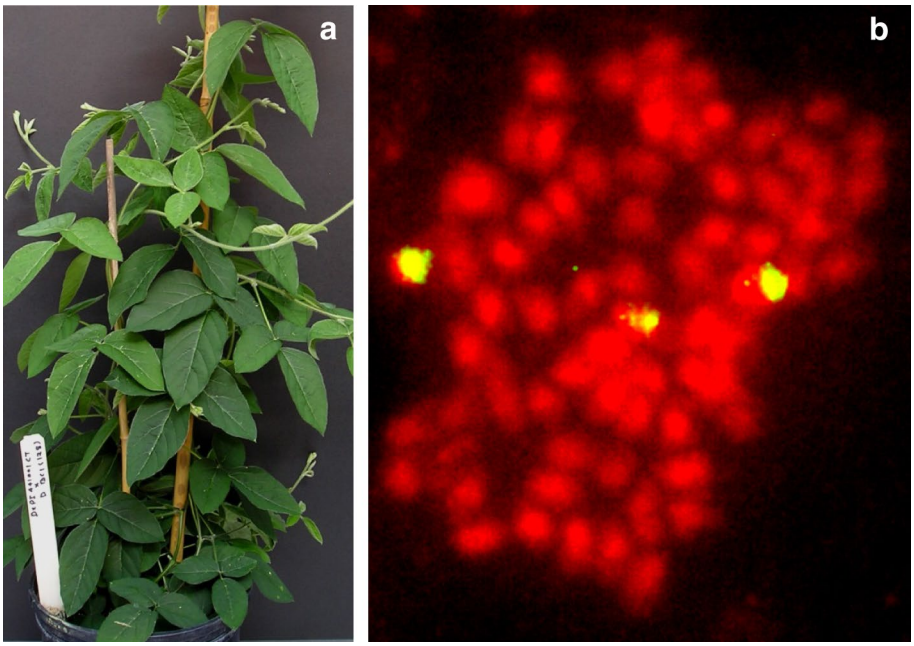

Fig. 6 Morphological and cytological identification of BC_1 plant of *Glycine max* cv. 'Dwight' ($2n = 40$) and *G. tomentella* PI 441001 ($2n = 78$). **a** BC_1 plants growing in a pot in greenhouse showing twining trait of PI 441001, **b** mitotic metaphase cell showing $2n = 79$ chromosomes with 3 signals representing 2 NOR (nucleolus organizer region) of 'Dwight' and 1NOR from PI 441001 (color figure online)

pollinated, 122 (4 %) pods were harvested 19–21 days post-pollination. These included vestigial pods without seeds. Forty-three immature seeds were harvested and cultured on SM medium. One of the immature seeds produced many embryogenic calluses while 42 seeds did not produce embryogenic shoots. Several BC_1 seedlings were produced from one morphogenic seed and seedlings were transferred to the greenhouse. All BC_1 plants were morphologically vigorous, viney like PI 441001, perennial (Fig. 6a), and contained the expected $2n = 79$ chromosome complement (i.e., 40 chromosomes from 'Dwight'; $G_1G_1 + 39$ chromosomes from PI 441001; D_3E). FISH revealed three signals representing three satellite chromosomes (Fig. 6b). *Glycine tomentella* ($2n = 78$) and *G. max* have one pair of satellite chromosomes (Singh et al. 2001). We have been maintaining BC_1 plants through cuttings.

Production and identification of BC_2 plants

Mature pods with mature seeds (BC_2F_1) were harvested from BC_1 plants backcrossed to 'Dwight'. Forty-three BC_2 mature seeds harvested from 3006 BC_1 flowers pollinated. Thirty seeds germinated and seedlings were identified cytologically. Chromosome numbers in the plants were $2n = 55$ (1), $2n = 56$ (12), $2n = 57$ (6), $2n = 58$ (7), $2n = 59$ (1), $2n = 60$ (2), and $2n = 99$ (1). However, only 15 BC_2 plants survived past flowering (35 % survival rate). BC_2 plants that survived contained $2n = 56$ (9 plants), $2n = 57$ (2 plants), $2n = 58$ (3 plants) and $2n = 59$ chromosomes (1 plant) (Table 3).

All BC_2 plants were morphologically distinct, indicating that the extra PI 441001 chromosomes expressed characteristic morphological modifications, and all were self-sterile. A few BC_2F_1 plants were compared with 'Dwight' plants grown in the greenhouse. Plant 06H1-1 ($2n = 58$) grew like a small shrub, was vigorous, and flowered profusely (Fig. 7a). It produced 43 BC_3 seeds from 1063 flowers pollinated with 'Dwight'. Pods turned black (Fig. 7a left insert) contained one to two seeds which had greenish-brown seed coats (Fig. 7a right bottom insert). Plant 06H1-3 ($2n = 56$) grew slowly, was only a few centimeters tall, produced small, narrow leaves with short petioles, and flowered earlier than the recurrent parent. Most of the flowers were cleistogamous. It produced 43 seeds from 466 flowers pollinated. Pod color was black but all seeds were round, yellow, with dark black hilum; seed size was smaller than the recurrent parent. Plant 07H1-4 ($2n = 57$) was vigorous, leaves were short and rolled inwards, flowers were arranged on long racemes. This plant produced one seed from 350 pollinated flowers. Plant 07H1-5 ($2n = 56$) was slow growing with crinkled short leaves and produced few flowers; 13 seeds were produced from 238 flowers pollinated. Plant 07H1-7 ($2n = 56$) was slow growing, produced one stem and leaves were narrow and curved. This plant produced 21 brown seeds from black pods after 263 flowers pollinated.

Plant 07H1-14 ($2n = 58$) was a vigorous climber, with large, dark-green round leaves and produced black pods with 16 brown seeds from 1692 flowers pollinated. Seven plants survived (Table 3). FISH of prophase and metaphase cells showed two satellite chromosomes; both are from 'Dwight' (Fig. 8a, b). Plant 07H1-16 ($2n = 57$) was morphologically weak and produced few flowers but produced 22 greenish-brown seeds after 106 pollinations.

Table 3 Chromosome segregation in 15 BC_2F_1 plants derived from *Glycine max* cv. 'Dwight' ($2n = 40$) × *G. tomentella* PI 441001 ($2n = 78$)

BC_2 plants	$2n$	Cytological identification of BC_3 plants ($2n$)											Plants survived	Seeds died	Total seeds	No. pollination
		40	41	42	43	44	45	46	47	48	49	Total				
06H1-1	58	0	4	6	1	4	3	4	2	3	2	29	17	14	43	1063
06H1-3	56	0	1	2	2	1	4	3	2	3	1	19	6	24	43	466
07H1-4	57	0	0	0	0	1	0	0	0	0	0	1	1	0	1	350
07H1-5	56	0	0	1	0	4	0	0	4	1	0	10	2	3	13	238
07H1-7	56	1	0	4	3	1	2	0	0	1	0	12	10	9	21	263
07H1-14	58	0	0	1	3	1	0	0	0	2	0	7	7	9	16	1692
07H1-16	57	0	0	2	1	0	4	0	2	1	1	11	8	11	22	106
07H1-18	59	0	0	0	0	1	0	0	0	1	0	2	1	0	2	25
07H1-25	58	0	0	4	3	1	0	1	3	0	1	13	7	8	21	98
07H1-26	56	0	0	2	4	4	7	2	5	4	4	32	11	11	43	1941
07H1-27	56	0	0	1	1	4	2	0	2	2	0	12	11	4	16	949
07H1-38	56	0	1	2	1	1	2	2	5	2	0	16	6	17	33	1028
09H1-40	56	0	2	1	3	4	4	5	0	1	0	20	19	16	36	2553
13H1-41	56	0	0	0	0	1	0	0	0	0	0	1	1	4	5	No record
13H1-43	56	0	0	2	2	0	0	1	1	0	0	6	6	0	6	244
		1	8	28	24	28	28	18	26	21	9	191	113	130	321	11,016

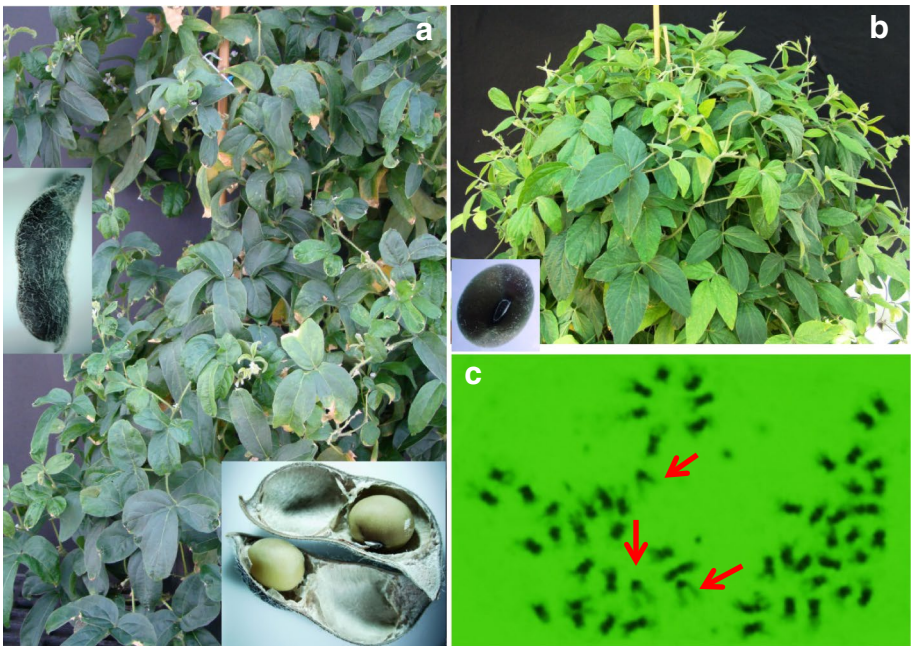

Fig. 7 Morphological features of 2 BC_2 plants and mitotic metaphase chromosomes of a BC_2 plant. **a** Plant 06H1-1 with $2n = 58$ showing shrub like growth habit, *insert left* is showing one 2 seeded mature black pod and *insert right* is showing a pod with *greenish-brown* seeds, **b** plant 07H1-26 showing *droopy branches*, narrow trifoliolate leaves and *black* seed (*insert left*), **c** mitotic metaphase cell of 07H1-26 with $2n = 56$ showing three (two from 'Dwight' + one from PI 441001) satellite chromosomes (*arrows*) (color figure online)

Plant 07H1-18 was weak, contained $2n = 59$ chromosomes and could not be maintained through cuttings. All flowers (25) were pollinated and two mature brownish colored small seeds were harvested before the plant died. Plant 07H1-25 contained $2n = 58$ chromosomes and produced black pods and brown seeds. A total of 98 flowers pollinated produced 21 seeds; seven plants survived from 13 seeds germinated.

Plant 07H1-26 ($2n = 56$) was bushy, leaves were long, narrow, and droopy (Fig. 7b). This plant produced 43 seeds after 1941 flowers were pollinated. Pod color was black and seed color was dark brown to black (Fig. 7b left bottom insert). Mitotic metaphase chromosomes stained by Feulgen stain showed that plant 07H1-26 contained three nucleolus organizer chromosomes (Fig. 7c, arrows). Plant 07H1-27 ($2n = 56$) was bushy and vigorous with crinkled,

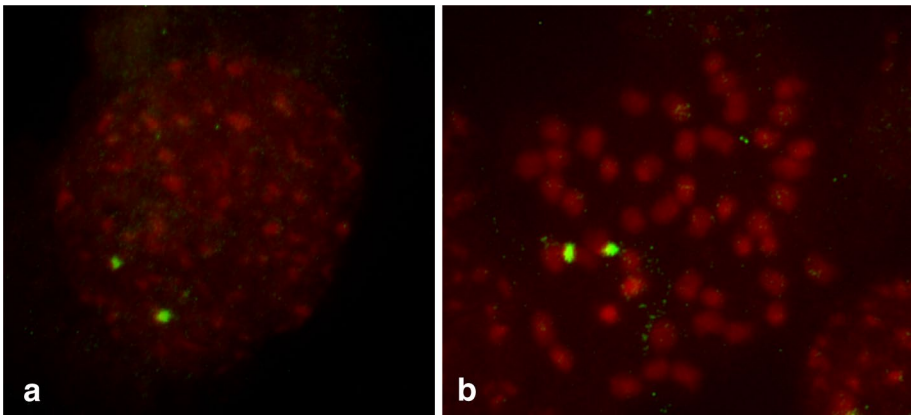

Fig. 8 Mitotic cells of a BC_2 plant 07H1-14 processed through fluorescence in situ hybridization. **a** Mitotic interphase cell showing 2 signals representing one pair of NOR chromosomes, **b** a mitotic metaphase cell showing 2 signals verifying the result of interphase cell (**a**) (color figure online)

dark green leaves. It produced 16 greenish-brown seeds from 949 flowers pollinated. Plant 07H1-38 ($2n = 56$) was derived from immature seed culture. This plant produced viney stem with dark-green-crinkle leaves, many cleistogamous flowers at each node. Pods were black and seeds were dark brown after backcrossing to 'Dwight'. This plant produced 33 seeds after 1028 flowers pollinated. Plant 09H1-40 ($2n = 56$) was initially slow in vegetative growth, stem was woody; leaves were yellowish green, curved inward, and flowered profusely. We pollinated 2553 flowers that produced 36 mature seeds. Pods were black but seed color was brown. Twenty seeds geminated and 19 plants survived (Table 3).

Three BC_2 plants, 13H1-41, 13H1-42 and 13H1-43, containing $2n = 56$ chromosomes were produced during late 2013. Plant 13H1-41 showed slow growth, crinkled, curved inwards leaves, and produced few flowers. This plant produced five seeds after backcrossing to 'Dwight'. Compared to 'Dwight' and BC_2 sibs, plant 13H1-42 was slow in vegetative growth, had small, dark-green, narrow leaves with short petioles. Flowers were cleistogamous. Three seeds were obtained by backcrossing to 'Dwight' and all seeds are being germinated in the culture. Plant 13H1-43 was a vigorous climber with normal leaves; 244 flowers were pollinated by 'Dwight' that produced six viable seeds (Table 3).

Seed coats of Dwight and PI 441001 expressed contrasting morphological differences observed under a dissecting microscope. The seed coat of 'Dwight' was smooth, round, and yellow, with dull luster and black hilum (Nickell et al. 1989; Fig. 9a). Seed of PI 441001 was flat and seed coat was black, rough, and reticulates (Fig. 9b). The amphidiploid contained complete genomes ($G_1G_1D_3D_3EE$) of 'Dwight' and PI 441001. Seeds expressed reticulate and rough seed coat and color was yellowish-brown (Fig. 9c). The intensity of the reticulate and rough structure on BC_2 seeds was less pronounced. The seed coat color was dark brown (Fig. 9d). The degree of reticulate and rough structure was not clearly visible in BC_3 seeds. The seed coat color was yellow (Fig. 9e), greenish-brown (Fig. 9f) or black (Fig. 9g).

Production and identification of BC_3F_1 plants

Of the 321 BC_3F_1 seeds obtained, 191 seeds germinated (60 %) and were cytologically identified but 113 plants (35 %) survived to maturity. Seventy-eight seedlings died from seedling to flowering stage (Table 3). Chromosome segregation among the progenies of 15 BC_2F_1 plants was $2n = 40$, one plant; $2n = 41$, eight plants; $2n = 42$, 28 plants; $2n = 43$, 24 plants; $2n = 44$, 28 plants; $2n = 45$, 28 plants; $2n = 46$, 18 plants; $2n = 47$, 26 plants; $2n = 48$, 21 plants; $2n = 49$, 9 plants.

Plants with $2n = 40$, and 41 chromosomes (MAALs) were self-fertile. Plants with $2n = 41$ chromosomes, although, contained the same chromosome constitution but were morphologically distinct. For example, plant 06H1-1 ($2n = 58$) produced 4 BC_3F_1plants with $2n = 41$ chromosomes and three plants (07H6-3, 07H6-21, 07H6-20) survived (Table 4). Figure 10 shows two 41-chromosome plants derived from 06H1-1. Plant 07H6-3 (Fig. 10a) was like 'Dwight' (Fig. 2d, e) but produced black pods and brown seeds. Plant 07H6-21 (Fig. 10b) was branching type and produced pods and seeds like 'Dwight' (Figs. 2f, 9a). The progenies of plant 06H1-3 ($2n = 56$) contained one plant (07H5-8) with $2n = 41$ chromosomes which was fertile and similar morphologically to 'Dwight' (Fig. 10c). These plants produced about 1000 seeds. A small portion of seeds was examined cytologically (Table 4) and remaining seeds were planted in the field without cytological examination.

Two 41-chromosome plants were isolated from 09H1-40. Plant 10H269-2 produced 50 BC_3F_2 (11H22) seeds and 40 plants reached to maturity. By contrast, 11H2-15 (12H6) produced 13 seeds. All three plants with $2n = 41$ chromosomes were morphologically similar but different than 'Dwight'.

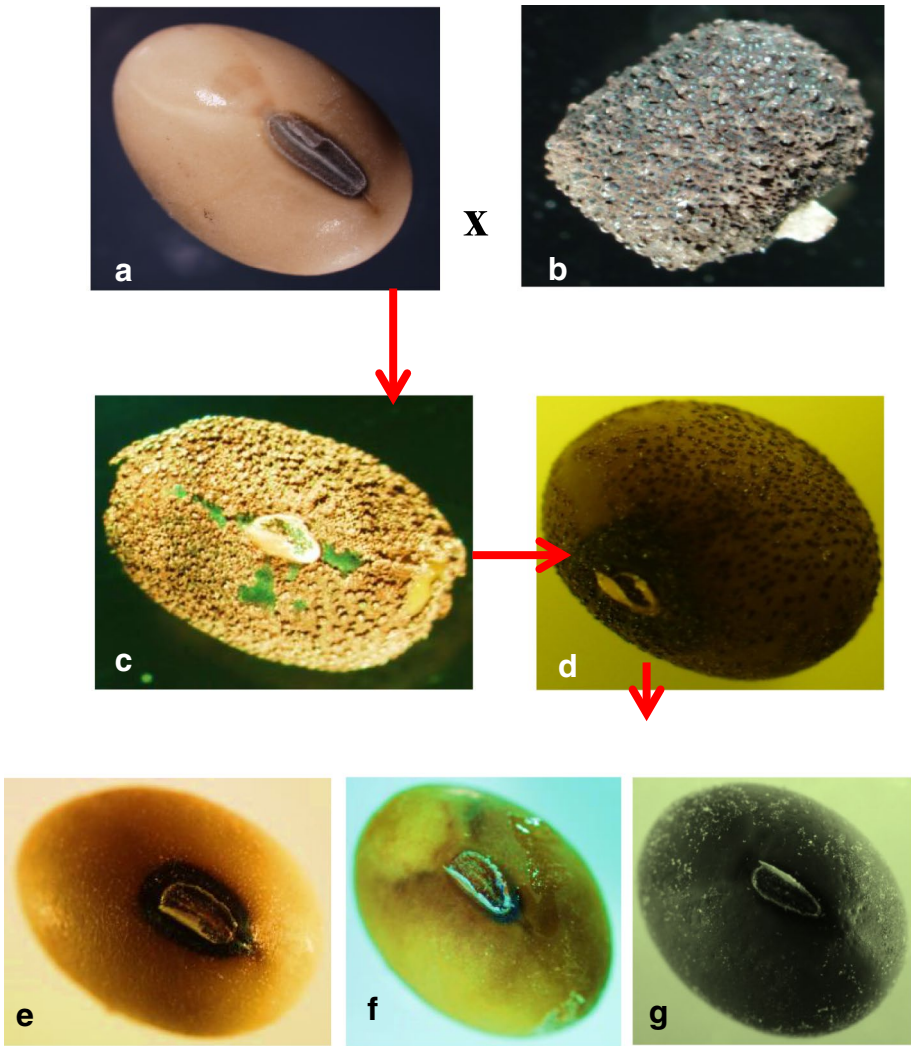

Fig. 9 Seed coat structure of *Glycine max* cv. 'Dwight' and *G. tomentella* PI 441001 and changes in seed coat structure during subsequent generations. **a** Seeds of 'Dwight' with smooth seed coat, *yellow*, *black* hilum and round seed, **b** seed of PI 441001 with rough (reticulate-net) seed coat, back color and flat seed; **c** seed of amphidiploid showing *brown* see coat with grainy structure, **d** BC_2 seed showing *brown* seed coat color with less pronounced grainy structure than those observed in amphidiploid seed, **e** BC_3 seed produced from plant 06H1-3 showing *tan* seed coat with *black* hilum like 'Dwight', **f** BC_3 seed produced from plant 06H1-1 showing *greenish-brown* seed coat, **g** BC_3 seed produced from plant 07H1-26 showing *black* seed coat like PI 441001. All BC_3 seeds were slowly nearing to 'Dwight' seed coat structure (color figure online)

Table 4 Chromosome segregation in BC_3F_2 plants with $2n = 41$ and $2n = 42$ chromosome from 'Dwight' × PI 441001

BC_2F_1	$2n$	BC_3F_1	$2n$	BC_3F_2	$2n$ number (%)				Total
					40	41	42	43	
06H1-1[a]	58	07H6-3	41	07ST2[b]	148 (71)	57 (27)	4 (2)	0	209
		07H6-21	41	08ST3	68 (69)	25 (26)	5 (5)	0	98
		07H6-20	41	08ST4	72 (72)	26 (26)	2 (2)	0	100
06H1-3	56	07H5-8	41	07ST1	91 (64)	46 (32)	6 (4)	0	143
09H1-40	56	10H269-2	41	11H22	24 (60)	15 (38)	1 (2)	0	40
		11H2-15	41	12H6	10 (77)	3 (23)	0 (0)	0	13
06H1-1	58	06H6-38	42	08ST5	17 (42)	14 (35)	9 (23)	0	40
		07H6-25	42	08H35	23 (52)	14 (32)	5 (11)	2 (5)	44
09H1-40	56	10H269-7	42	11ST2	21 (37)	22 (38)	14 (25)	0	57

[a] Hybrid

[b] ST (SoyTom); H (Hybrid)

The progenies of three BC_3F_1 plants with $2n = 42$ chromosomes were not completely fertile. Number of viable seeds set ranged from 40 to 57 (Table 4). BC_3F_1 plants with $2n = 43$ or higher were male sterile and set mature pods with seeds after backcrossing to 'Dwight' (Table 5).

Fig. 10 Three morphologically distinct plants derived from BC_3F_1 plants with $2n = 41$ chromosomes (MAALs). **a** Morphology of one BC_3F_2 plant (07H6-3) showing morphological traits like 'Dwight', **b** morphology of one BC_3F_2 plant (07H6-21) showing branching growth habit and pods were arranged on the branches, **c** Morphology of one BC_3F_2 plant (07H5-8) showing morphological features like 'Dwight' (color figure online)

Production of BC_3F_2 plants

We examined cytologically progenies of five BC_3F_2 plants (Table 4). Chromosome numbers in the progenies of 07ST2 ($2n = 41$) were $2n = 40$ (71 %), $2n = 41$ (27 %) and $2n = 42$ (2 %). This pattern was observed in other 5 BC_3F_2 plants (Table 4). Plants of 07ST2 segregated for purple and green coleoptyl; purple coleoptyl plants produced purple flowers and green coleoptyl produced white flowers while all plants of 08ST3, 08ST4 and 07ST1 produced purple flowers.

Three 42-chromosome plants were self-fertile (08ST5, 08H35, 11ST2). Plant 08ST5 and plant 11ST2 segregated plants for $2n = 40$, 41 and 42 chromosomes. This segregation indicates that these plants are likely to contain 2 different PI 441001 chromosomes and we expect to produce two different 41-chromosome plants. The frequency of 40-chromosomes, as expected, was lower (37–52 %) than those observed in the selfed progenies of plants with $2n = 41$ chromosomes (Table 4).

Production of BC_4F_1 plants

BC_3F_1 plants with $2n = 43$–49 were male sterile because they did not produce selfed pods. These plants were backcrossed to 'Dwight'. Pod set on BC_3F_1 plants required a large number pollination attempts and crossability rate of only few plants was recorded because we had many sterile plants (Table 4 and data not presented). The seed set ranged from 1 (07H66-33, 08H36-22) to 42 (07H6-17). Plant 07H6-16 ($2n = 44$) produced 23 seeds from 89 flowers pollinated. Chromosome segregation in BC_4F_1 was $2n = 40$, nine plants; $2n = 41$, eight plants; $2n = 42$, five plants; and $2n = 43$, one plant. Of the nine 41-chromosome plants, one plant (08H18-8) with $2n = 41$ chromosomes contained three satellite chromosomes (two from 'Dwight' and one from PI 441001). This plant produced 70 pods and 111 seeds.

The plant 07H6-17 ($2n = 48$) produced 47 seeds from 162 flowers pollinated and 42 seeds germinated and survived. Chromosome segregation in BC_4F_1 ranged from $2n = 41$ to $2n = 45$ (Table 5). The progenies of 07H6-17 produced a plant with $2n = 42$ chromosomes (08H15-29) that showed short petiole and large droopy leaves (Fig. 11a). Flowers were hidden under the leaves; the plant was self–sterile. We have not yet isolated such a unique plant. The plant produced 12 seeds when it was pollinated by 'Dwight'. Chromosome segregation among 12 BC_5F_1 plants was $2n = 40$ (three plants) and $2n = 41$ (nine plants). Compared to 40-chromosome sib (Fig. 11b), two morphologically distinct plants with $2n = 41$ chromosomes were identified (Fig. 11c, d).

Plant 07H6-26 ($2n = 47$) produced 50 seeds from 385 flowers pollinated. Twenty-one seeds germinated and 17 plants survived. Chromosome numbers in BC_4F_1 plants (08H38) ranged $2n = 41$–44 (Table 5). Plant 07H6-43 ($2n = 43$) contained 3 satellite chromosomes, was self-sterile and produced four viable seeds. We obtained only four seeds from plant 07H6-32 ($2n = 46$) after pollinating 231 flowers. We failed to produce BC_4F_1 seeds from 07H6-35 ($2n = 46$) after pollinating 136 flowers.

Five BC_3F_1 plants ($2n = 42$–47) were obtained from plant 06H1-3. Plant 07H5-6 ($2n = 46$) and 07H5-10 ($2n = 48$) failed to produce pods after backcrossing to 'Dwight'; 338 flowers of plant 07H5-6 were pollinated and 283 flowers of plant 07H5-10 were pollinated. By contrast, pod set was observed when 07H5-33 ($2n = 47$) was pollinated by 'Dwight' and 200 hybrid seeds were produced. Of the 57 seeds germinated from 07H5-33, 34

Table 5 Chromosome segregation in BC_4F_1 from three BC_2F_1 plant from 'Dwight' x PI 441001

BC_2F_1	BC_3F_1	2*n*	BC_4F_1	2*n* Number							Total
				40	41	42	43	44	45	46	
06H1-1 $2n = 58$	07H6-16	44	08H18	9	8	5	1	0	0	0	23
	07H6-17	48	08H15	0	8	12	14	7	1	0	42
	07H6-22	46	08H17	5	7	10	5	2	1	0	30
	07H6-23	46	08H23	4	6	6	6	0	0	0	22
	07H6-26	47	08H38	0	6	5	4	2	0	0	17
	07H6-27	47	08H37	4	5	2	0	0	2	0	13
	07H6-28	44	08H45	2	5	3	4	0	0	0	14
	07H6-30	49	08H16	0	0	0	1	2	0	0	3
	07H6-32	46	09H54	3	1	0	0	0	0	0	4
	07H6-33	46	09H55	0	1	0	0	0	0	0	1
	07H6-42	44	09H53	7	10	2	0	0	0	0	19
	07H6-43	43	09H57	1	0	3	0	0	0	0	4
06H1-3 $2n = 56$	07H5-5	44	07H7	17	21	4	3	0	0	0	45
	07H5-26	43	08H11	3	5	2	1	0	0	0	11
	07H5-28	45	08H10	3	1	7	5	2	0	0	18
	07H5-33	47	08H8	5	4	6	6	4	1	0	26
	07H5-34	42	08H9	2	5	0	0	0	0	0	7
07H1-26 $2n = 56$	08H36-1	43	09H87	4	4	4	2	0	0	0	14
	08H36-2	?	09H88	0	0	3	2	0	0	0	5
	08H36-3	45	09H89	0	0	0	2	0	0	0	2
	08H36-14	45	09H91	0	1	4	0	0	0	0	5
	08H36-15	47	09H92	1	0	1	0	1	0	0	3
	08H36-17	42	09H93	3	2	0	0	0	0	0	5
	08H36-19	44	09H94	7	5	3	2	0	0	0	17
	08H36-22	45	09H96	1	0	0	0	0	0	0	1
	08H36-29	45	09H100	0	0	2	0	0	0	0	2
09H1-40 $2n = 56$	10H269-1	43	11H3	10	7	1	0	0	0	0	18
	10H269-3	45	11H4	2	3	7	3	1	0	0	16
	10H269-5	46	11H5	1	2	0	0	1	0	2	6
	10H269-9	43	11H6	4	10	13	3	0	0	0	30
	11H2-1	46	12H19	0	0	1	0	1	0	0	2
	11H2-2	44	12H20	0	1	0	1	1	0	0	3
	11H2-8	45	12H21	0	2	0	0	0	0	0	2
	11H2-4	43	12H2	0	10	1	0	0	0	0	11
	11H2-5	44	12H3	5	6	1	0	0	0	0	12
	11H2-10	42	12H5	4	1	0	0	0	0	0	5

plants reached maturity. Twenty-six plants were identified cytologically and plants segregated for $2n = 40$, 41, 42, 43, 44, 45 (Table 5). Plant 07H5-5 with $2n = 44$ chromosomes was male sterile but produced 45 seeds when pollinated by 'Dwight'. The progenies of plant 07H7 segregated as: $2n = 40$ (17 plants), 41 (21 plants), 42 (4 plants), and 43 (3 plants). Compared to 'Dwight', some of the plants with $2n = 41$ were distinct such as plant 07H7-9 was tall and late, plant 07H7-11 ($2n = 41$) was early and produced small seeds, plant 07H7-37 ($2n = 41$) was bushy with many branches; leaves were yellow, long and narrow; and the flowers had a long calyx. The plant was male sterile but produced 19 seeds when crossed with 'Dwight'. The resulting BC_5F_1 plants segregated for tan and black pod color.

Plant 07H1-26 ($2n = 56$) produced 43 BC_3F_1 seeds and 32 seeds germinated. Chromosome numbers in BC_3F_1 plants ranged from $2n = 42$–49 (Table 5). Eleven plants survived but nine plants were backcrossed to 'Dwight' because they were self-sterile. Some plants produced a few pods after many pollination attempts while other plants failed to produce any hybrid pods. Chromosome number in BC_4F_1 ranged $2n = 40$–44 (Table 5).

Fig. 11 Morphological features of plants derived from a plant with $2n = 42$ chromosomes. **a** Plant 08H15-29 ($2n = 42$) showing large droopy leaves that look like "lettuce", **b** Plant 09H70-2 with $2n = 40$ showing normal growth and pod set; **c** plant 09H70-4 with $2n = 41$ with slower growth and poorer pod set than diploid sib, **d** plant 09H70-12 with $2n = 41$ showing larger leaves compared to disomic and MAAL (09H70-2) sibs (color figure online)

Plant 09H1-40 ($2n = 56$) produced 36 BC_3F_1 seeds. Twenty seeds germinated and were identified cytologically (Table 3). Chromosome number ranged from $2n = 41$ to 48. Thirteen BC_3F_1 plants survived and produced seeds. Although a small number of population from two BC_3F_1 plants with $2n = 41$ (10H269-2, 40 seeds; 11H2-15, 13 seeds) was cytologically examined, but plants segregated for $2n = 40$, 41 and 42 (Table 4). We pollinated a large number of flowers of BC_3F_1 plants with 'Dwight' but failed to produce many seeds.

Isolation of morphological variants

Morphological variants among 40-chromosome plants were identified in BC_3F_2 and BC_4F_2 generations, described below

1. White flowers: Purple and green coleoptyl were first observed in BC_3F_2 populations of plant 07ST2 which was derived from BC_2F_1 plants 06H1-1 (Fig. 12a). Plants with purple coleoptyl produced purple flowers (Fig. 12b) and plants with green coleoptyl produced white flowers (Fig. 12c). White flowers also appeared in BC_3F_2 and BC_4F_2 populations derived from BC_2F_1 plants 07H1-7, 07H1-14, 07H1-38, and 09H1-40 and also in the populations derived from *G. tomentella* PI 441001 and *G. max* cv. 'Dwight' hybrids. Both parents have purple flowers (Fig. 2b, e). Allele test of newly isolated white flowers from this study with known white flower genes (*w1* and *w4*) is in progress.
2. Delayed flowering: Since all the plants were growing in the hydroponic benches with same light regime (13 h photoperiod), it was easy to identify plants that were not flowering. One plant 07ST2-132 ($2n = 40$) derived in BC_3F_2 from a BC_2F_1 plant 06H1-1 was a climber and did not flower while other plants produced flowers and pods (Fig. 13a, arrow). Stem cuttings were rooted and transplanted in soil in pots. Plants flowered when they were moved to a greenhouse under 8 h day-length and produced flat seeds with black seed coat (Fig. 13a, insert bottom right). Seeds shattered after pods matured. This trait is in PI 441001.
Plant 10H154-2 ($2n = 41$) was derived from BC_4F_2 populations of a BC_2F_1 plant (07H1-27). This plant

Fig. 12 BC_3F_2 population of plant 07ST2 segregating purple and green coleoptyl (**a**), **b** *purple flowers* produced on plant with purple coleoptyl, **c** green coleoptyl produced *white* flower (color figure online)

was a climber with thick stem and vigorous vegetative growth. Leaves were long, narrow, and dark-green and produced few flowers in 13 h photoperiod (Fig. 13b). This plant was multiplied easily by stem cuttings. Cuttings rooted even in water. Rooted cuttings with robust vegetative growth were transferred to a greenhouse under 8 h daylength and flowered profusely. Pod color was tan, seeds were large and seed coat was black (Fig. 13b, insert top right).

3. Plant 10H204-4 ($2n = 41$) was also vigorous, produced large leaves and petioles. Stem, leaves, and pods contained grey and dense pubescence (Fig. 13c). Seeds were flat with a reddish-brown seed coat (Fig. 13c, insert top right). This plant was derived from BC_3F_2 populations of BC_2F_1 plant 07H1-27.
4. Resistant to soybean rust: Plant 10H154-2 ($2n = 41$) and its progenies 12ST4 with $2n = 40$ chromosomes were resistant to soybean rust when tested on by detached leaves (G. L. Hartman, personal communication). Plant 12ST4-5 was crossed with a soybean cultivar 'Komata' (PI 200492) that carries *Rpp1* gene. The F_2 population segregated for 15 resistant: 1 susceptible ratio. This suggested us that 12ST4-5 has different rust resistant gene than *Rpp1*. Allele test with known rust resistant genes (*Rpp2*, *Rpp3*, *Rpp4*, *Rpp5* and *Rpp6*) is in progress.

Discussion

Poor intersubgeneric crossability between wild perennial *Glycine* species and *G. max* is a post-fertilization problem because pollen tubes reached to the eggs 24 h post-pollination (Singh and Hymowitz 1987). Pod set in crosses between 40- and 78-chromosome *G. tomentella* and *G. argyrea* ($2n = 40$) accessions and soybean cultivars was recorded in all cross combinations except in 'Clark 63' × PI 441001, 'IA 3010' × PI 446998 and 'IA 3010' × PI 505151crosses. It is likely that we did not hybridize a sufficiently large number of flowers of these soybean cultivars to determine if pod set was possible for these combinations (Table 2). Although pod set is common for these wide crosses, all pods will abort before maturity and immature seeds must be removed aseptically and

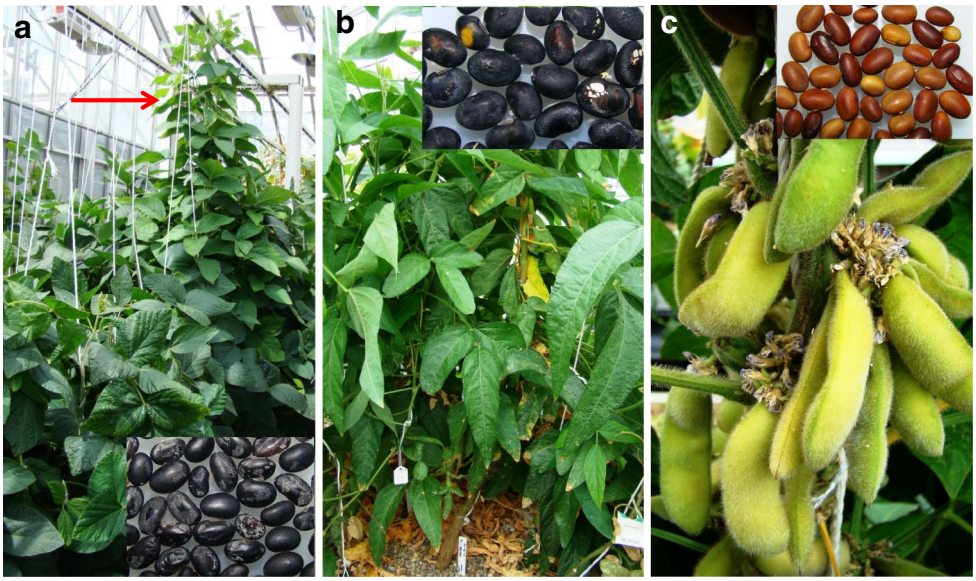

Fig. 13 Morphological features of 3 morphological variants' in lines derived from *Glycine max* cv. 'Dwight' ($2n = 40$) × *G. tomentella* PI 441001 ($2n = 78$) growing in hydroponic benches in greenhouse. **a** plant 07ST2-126 ($2n = 40$) derived from plant 06H1-1 (BC_2) showing climbing growth habit identified by an *arrow*, plant required short day (8 h light) to produce pods and seeds, seeds were flat and black (*insert*). **b** plant 10H154-2 with $2n = 41$ derived from 07H1-27 (BC_2) showing thick stem, long and narrow leaves, droopy branches, produced large *black* seeds (*insert*); **c** plant 10H204-4 with ($2n = 41$) derived from plant 07H1-27 (BC_2) showing pods clustered on each node, pods were flat with dense soft white pubescence, seeds were *reddish- brown* (*insert*) (color figure online)

cultured. Dissected immature seeds turned white and died in the commercial MS basal medium + B5 vitamins and commercial B5 basal medium + B5 vitamins.

Finer and Nagasawa (1988) produced plants through embryogenesis from 7 to 14 days old immature seeds of soybean cv. 'Fayette' using MS salts + B5 vitamins + 60 g sugar\L +40 mg\L 2, 4-D + 6 g\L agar. Using 2, 4-D auxin in medium in our study produced friable calluses and we failed to produce embryogenic calluses. We observed that immature seeds produced embryogenic cultures when 'Dwight', 'Ina', and 'Macon' cultivars were used in the hybridization. By contrast, seeds either died or did not produce embryogenic calluses when 'IA 2052', 'IA 3010', 'Clark 63' and 'Williams 82' were hybridized with *G. tomentella*, PI 446998, ($2n = 40$) from Papua New Guinea. Similarly, we were unable to regenerate plant in crosses with *G. argyrea* (Table 2). We hybridized 'Dwight' with PI 378708 in the late 2013 and F_1 plants were transferred to greenhouse 3 months after immature seeds were cultured. By contrast, immature seeds, cultured in January 2013, of 'Dwight' × PI 559298 (*G. latifolia*) are still producing multiple embryos (January 2015; unpublished results). Bodanese-Zanettini et al. (1996) hybridized nine soybean cultivars with two accessions of *G. canescens* F. J. Hermann, one accession of *G. microphylla* (Benth.) Tindale, six accessions of *G. tabacina* (Labill.) Benth., and three accessions of *G. tomentella*. They produced F_1 plants through embryo rescue in one accession of *G. tomentella* ($2n = 78$) used as a paternal parent. This suggests that we can produce intersubgeneric F_1 hybrid in the genus *Glycine* with only certain accessions of a species. The published results elucidate that certain accessions of 78-chromosome *G. tomentella* are easy to hybridize with the soybean than other accessions. It has been established that plant regenerability trait in soybeans and wild perennial species is genetically controlled. Komatsuda and Ohyma (1988) observed germination capacity of embryos is controlled by genotypes of the cultivars. Hammatt et al. (1986) examined plant regenerability from mature seeds of five accessions of *G. clandestina* Wendl. They observed plant regeneration in two accessions.

Our most successful perennial parents have been $2n = 78$ *G. tomentella* accessions but we were only able to generate F_1 plants from four of six accessions used as parents. F_1 plants were obtained from crosses of PI 378708, 441001, 441008, 583970 and each contained the expected $2n = 59$ chromosomes. Amphidiploid plants induced through colchicine treatment were produced from the 'Dwight' × PI 441001, 'Ina' × PI 441001, and 'Macon' × PI 441001 crosses. Our amphidiploid plants were stunted and produced a few pods with single self-pollinated seeds. Similar results were reported by Newell et al. (1987) for amphidiploids of *G. max* × *G. tomentella*. We failed to generate plants from crossing 'Dwight' by PI 441008, 505258, and 563892. However, we produced viable F_1 plants by a bridge cross ['Dwight' × (F_1

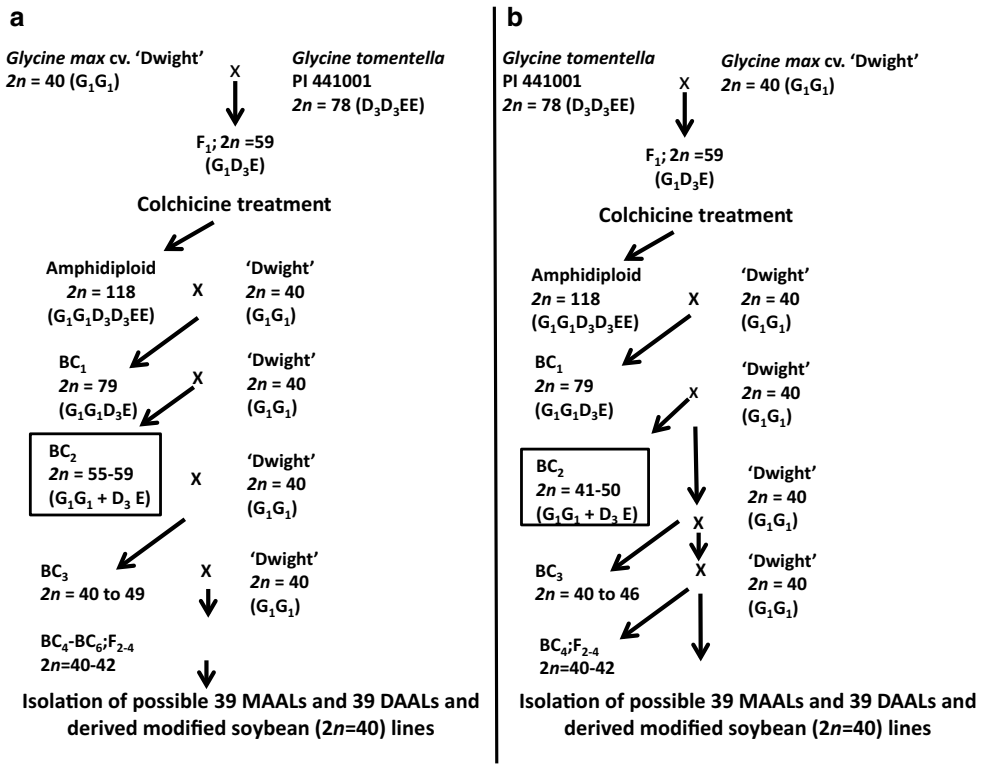

Fig. 14 Diagrammatic sketch for isolating MAALs and DAALs from *Glycine max* cv. 'Dwight' × *G. tomentella* PI 441001 (**a**) and *G. tomentella* PI 441001 × *G. max* cv. 'Dwight' (**b**). Note, chromosome number of plants in BC_2 generation; shown in a *box*

PI 441001 × PI 441008)], a technique that has been used for many wide crosses (Singh 2003), but the flowers on the resulting amphidiploid have no anthers.

The F_1 plants from soybeans × 78-chromosome *G. tomentella* accessions contained expected $2n = 59$ chromosomes. We hybridized Dwight with *G. tomentella* PI 378708 ($2n = 78$) with the objective to produce haploid soybean. Shoemaker et al. (1990) observed elimination of PI 378708 chromosomes in amphidiploid cuttings from the plant produced by Newell et al. (1987). Derived lines carried $2n = 40$ chromosomes with gene introgressed from PI 378708. However, we observed true hybrid with $2n = 59$ chromosomes. Amphidiploid plants induced through colchicine treatment were produced in 'Dwight' × PI 441001, 'Ina' × PI 441001, and 'Macon' × PI 441001. Amphidiploid plants were stunted, produced chasmogamous and cleistogamous flowers (Fig. 5a). Rarely, plants produced mature selfed pods (Fig. 5b) and seeds (Fig. 5c) in all the amphidiploids. However, amphidiploidy was confirmed by counting mitotic metaphase chromosomes (Fig. 5d) and meiotic chromosome pairing (Fig. 5e). Newell et al. (1987) observed mostly one-seeded, cleistogamous pods at a low rate in amphidiploids ($2n = 118$) of *G. max* × *G. tomentella*. Patzoldt et al. (2007) observed that "amphidiploid plants derived from *G. max* and *G. tomentella* cross are normally female sterile and male fertile". However, they did not report meiosis and pollen fertility of amphidiploid clones. Amphidiploid plants reported by Newell et al. (1987) and in this study observed selfed-pods (Fig. 5a–c). Furthermore, this study reports the production of BC_1 plants (Fig. 6a). This demonstrates that eggs in amphidiploids are not sterile.

Previously derived fertile lines were reported by Singh et al. (1993, 1998) from soybean cv. 'Altona' × *G. tomentella* PI 483218; the F_1 plant was produced by Newell and Hymowitz (1982). Lines derived from this cross came from a BC_1 with $2n = 76$ (Singh et al. 1990) and only 3 BC_2 plants that produced 5 BC_3 plants. This study reports BC_1 with $2n = 79$ and 16 BC_2 plants ($2n = 56$–59) so there is a greater possibility of having more genetic introgression in soybean from *G. tomentella* PI 441001.

Meiotic chromosome pairing at metaphase I observed in this study was bivalents and univalents. Newell and Hymowitz (1982) and Newell et al. (1987) recorded a low frequency of quadrivalent (0–1) and trivalent (0–1). Bodanese-Zanettini et al. (1996) also observed a low frequency of trivalents (0–1) and quadrivalents (0–1) while the majority of microsporocytes showed univalents and bivalents and the range of bivalents was 1–17. The number of bivalents depends upon the stage of metaphase I examined; the

number will change if late metaphase I stage was examined and strength of pressure applied to cells during chromosome slide preparation. The majority of bivalents observed in this study were loosely associated rod-shaped and occasionally bivalents were ring-shaped (Fig. 4c).

The different cytoplasms have differential effects on the performance of the hybrids. The introduction of the nucleus of the cultivated species into the alien cytoplasm could have several potential uses such as production of haploidy, cytoplasmic male sterility, and creating cytoplasmic diversity. The classical examples of producing haploidy are from crosses between *Hordeum bulbosum* L. (wild species; $2n = 14$) and *H. vulgare* L. ($2n = 14$) (Subrahmanyam and Kasha 1973), and *Solanum phureja* Juz. & Bukasov (wild species; $2n = 2x = 24$) × *S. tuberosum* L. ($2n = 4x = 48$) (Hougas and Peloquin 1957). Stable cytoplasmic male sterility in rice (*Oryza sativa* L.) has been identified from crosses of *O. perennis* Moench × *O. sativa* (Dalmacio et al. 1995). This study demonstrates that cytoplasm of *G. max* cv. 'Dwight' is different than the cytoplasm of *G. tomentella* ($2n = 78$), PI 441001. Morphologically, amphidiploid ($2n = 118$), BC_1 of 'Dwight' × PI 441001 were distinct than those obtained from PI 441001 × 'Dwight' (Singh and Nelson 2014). Mature pod set was observed in amphidiploid of 'Dwight' × PI 441001 but it was observed in the reciprocal cross. The BC_1 plant with 'Dwight' cytoplasm was vigorous climber, flowered profusely and was maintained through cuttings while BC_1 plant with PI 441001 cytoplasm was not a climber, leaves were thick and curved and produced mostly cleistogamous flowers. By examining methodology for creating fertile lines of 'Dwight' × PI 441001 and PI 441001 × 'Dwight', it is evident that PI 441001 chromosomes were preferentially eliminated in PI 441001 cytoplasm during BC_2 and BC_3 generations than those observed in 'Dwight' cytoplasm. Figure 14a, b shows diagrammatic comparison of the production of fertile soybean lines, MAALs and DAALs from 'Dwight' cytoplasm (Fig. 14a) and PI 441001 cytoplasm (Fig. 14b). A contrasting difference between both pathways is at BC_2 generation; the pattern of chromosome segregation in BC_2 plants with PI 441001 cytoplasm was observed in BC_3 plants with 'Dwight' cytoplasm. We need to verify these results by producing additional intersubgeneric hybrids using wild perennial *Glycine* species as maternal and paternal parents.

Seed coat structure and color of 'Dwight' (Fig. 9a) and PI 441001 (Fig. 9b) were markedly different. Figure 9 shows the progression of changes in seed coat structure and color of amphidiploid, BC_2 and BC_3 seeds with 'Dwight' cytoplasm. This pattern was not observed in seeds of lines derived from PI 441001 cytoplasm (Singh and Nelson 2014). These results and our previous research (Singh and Nelson 2014) demonstrate that we can access the tertiary gene pool (Harlan and de Wet 1971) for soybean breeding to broaden both genetic and cytoplasmic diversity of future soybean cultivars.

Author contribution statement R. J. Singh: Conceived the idea, conducted the research, and wrote the manuscript. R. L. Nelson: Managed the research program and multiplied derived lines in the field.

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Conflict of interest We have no conflict of interest.

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