Evaluation of the biolistic transformation method for commercially important sugarbeet breeding lines

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Introduction

Conventional breeding of sugarbeet is difficult since it is a biennial and a highly heterozygous plant. Genetic improvement of sugarbeet using biotechnology has progressed slowly since currently available methods of transformation (D'Halluin et al., 1992; Hall et al., 1996; Krens et al., 1996; Lindsey et Galloi, 1990) are not readily reproducible or cultivar independent. A biolistic sugarbeet transformation method (Snyder et al., 1999) was developed in this laboratory using embryogenic hypocotyl callus of a tissue culture clone REL-1. This clone has high regeneration potential *in vitro*, however, it is not suitable as a breeding line for rapid genetic improvement of commercially important sugarbeet lines. Therefore, we tested the feasibility of using the biolistic transformation method with several commercially important sugarbeet breeding lines.

Materials and Methods

Tissue culture media

Experiments were performed using MS medium (Murashige and Skoog salts, B5 vitamins, 1.0 mg/l panthotenic acid, 0.01 mg/l biotin, 0.5 g/l MES, 30 g/l sucrose, and 5.0 g/l agar gel, pH 5.8) supplemented with different plant growth regulators as indicated in Table 1.

Table 1. Composition of tissue culture media

	Plant Growth Regulators (mg/l)				Other
Medium	BAP	NAA	ABA	TIBA	additions (g/l)
GM	1	-	-	0.5	-
B1	1	-	-	-	-
B2	2	-	-	-	-
B1A2	1	-	2	-	-
SIM	0.3	0.1	-	-	-
SIMM	0.3	0.1	-	-	adenine sulfate 0.05 mannitol 44.6 sorbitol 44.6

Plant material

Sugarbeet tissue culture line REL-1 (Saunders at al., 1992) and breeding lines C69, C78, Z731, C76895 (obtained from Dr. R. Lewellen, ARS, Salinas, CA) and FC709-2 (Panella, 1999) were used. To determine the seed germination potential, seeds were either planted in soil and kept at 23°C in a growth chamber or sterilized in a 20% commercial bleach with 0.01% SDS solution for 40 min, washed with sterile water and germinated on GM medium at 27°C in the dark for three weeks. Determination of the regeneration potential was done by using hypocotyl (1 cm) and cotyledon (3 mm) fragments of each line that were excised from germinated seedlings and placed on B1 medium (11 hypocotyl pieces or 25 cotyledon pieces per plate) at 30°C in the dark for 5 weeks.

To determine the influence of medium composition on regeneration potential of C69 explants, seeds were germinated on GM or B2 (D'Halluin et al., 1992) medium and hypocotyl or cotyledon fragments were maintained on B1 or B2 medium with 3% or 8% sucrose, or on SIM, B1A2, or GM medium. Explants were also isolated from 2 to 6.5 week old C69 seedlings with red or green hypocotyls to determine the influence of age, hypocotyl color, and position of explants on seedlings on callusing and regeneration potential.

Particle bombardment

Particle bombardment was performed using a transformation vector carrying the bacterial isopentenyl transferase (*ipt*) gene fused to the promoter from the potato proteinase inhibitor II-K (PI-II) gene and neomycin phosphotransferase (*npt II*) gene for kanamycin selection of transformants (Smigocki et al., 1993). Embryogenic hypocotyl callus of line C69 was used for transformation as described by Snyder et al. (1999). Briefly, callus was harvested from hypocotyl fragments grown on B1 medium for 6 to 8 weeks, spread on a sterile filter disc overlaying solidified SIMM medium (Table 1) and after 4 h of incubation was subjected to biolistic treatment with DNA-coated gold particles. The filter discs with callus were cultured on B1 medium with 200 mg/l kanamycin (Km) for four weeks and then on B1 medium without Km. Subcultures were made every two weeks. After 3 months, green, viable callus was harvested and cultured on B1A2 medium until shoots formed. Shoots were propagated on media containing Km (150 mg/l). To generate cotyledon callus for biolistic transformation, cotyledon pieces (3x3 mm, 25 per plate) were cultivated for 6 to 8 weeks on B1 medium. Callus was harvested and used in the transformation protocol as described above. In 16 independent experiments, this transformation protocol was compared with seven different procedures that included varying the length of selection on kanamycin and plant growth regulator composition in media used for cultivation of bombarded callus (Fig. 3).

DNA preparation and Polymerase Chain Reaction

Qiagen plasmid purification kit was used for preparation of plasmid DNA for biolistic transformation (Qiagen, Santa Clara, U.S.A). Presence of the *ipt* gene in putative transformants was tested by polymerase chain reaction (PCR) using primers that specifically amplify a 514-basepair fragment of the *ipt* coding sequence (Smigocki and Hammerschlag, 1991) or 700-basepair fragment from the *npt II* sequence. High-molecular weight DNA for PCR was isolated by the method of Dellaporta et al. (1984). PCR was performed in 50 µl reactions with 0.4 µM of

each primer, 0.2 mM of each dNTPs, 1 X PCR buffer supplied with 1.5 mM $MgCl_2$, and 2.5 units of *Taq* DNA polymerase (Perkin Elmer Corp, Norwalk, CT). After initial denaturation at 94^0C for 4 min, 30 cycles of 94^0C, 55^0C, 72^0C for 1 min each were carried out followed by final extension step at 72^0C for 7 min. PCR reaction products (20µl) were electrophoresed on 1% agarose gel.

Results and Discussion

Seed germination

Germination of seeds *in vitro* is often hampered by fungal and bacterial contamination. Our seed contamination rates varied from 5 to 30% depending on the line and batch of seeds used. Contamination rate was not reduced by adding the antifungal agent Benomyl (10 mg/l) to the germination medium. The overall seed germination rate *in vitro* was reduced by 30 to 70% as compared to germination frequency in soil.

Hypocotyl and cotyledon organogenesis

All sugarbeet lines generated callus on hypocotyl explants, but shoot regeneration occurred only on hypocotyls from C69, C78, and Z731 breeding lines and the control line REL-1 (Fig. 1A). Hypocotyls of line C69 had the highest callusing (expressed as percent of explants with callus) and organogenic potential (expressed as percent of organogenic calli), although both were lower than the REL-1 control. Cotyledons of both REL-1 and C69 lines proved to be a good source of organogenic callus (Fig. 1B). The other sugarbeet lines developed only hard, nonregenerative callus. Lines REL-1 and C69 regenerated the highest average number of shoots per explants for both hypocotyls and cotyledons (Fig. 2).

Influence of medium composition on regeneration potential of C69 explants

To potentially increase the rate of shoot regeneration from C69 hypocotyl and cotyledon explants, standard media used for seed germination (GM) and explant cultivation (B1) were compared with several others. Results in Table 2 show that when seeds were germinated on GM medium and the hypocotyl explants plated on SIM medium, a 70% rate of callus induction was observed as compared to 56% on B1 medium. B1 or SIM medium was also best for generating callus from cotyledons of seeds germinated on GM or B2 media. The age of the seedlings, hypocotyl color or position of explants on seedlings had no influence on callus production and shoot regeneration from the C69 line.

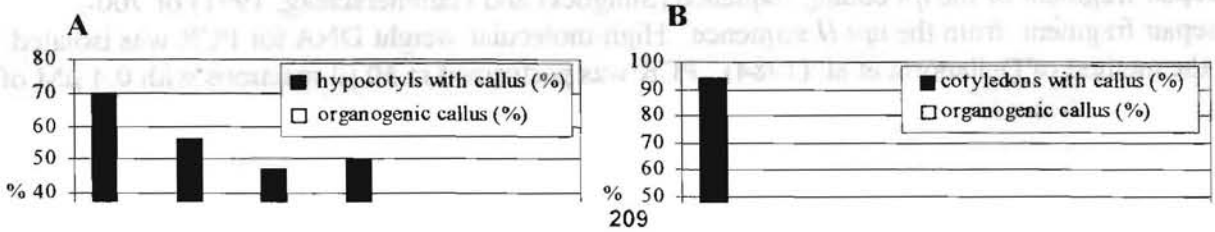

Fig. 1. Callusing and organogenesis of hypocotyl (A) and cotyledon (B) exlants of six sugarbeet lines 5 weeks after isolation.

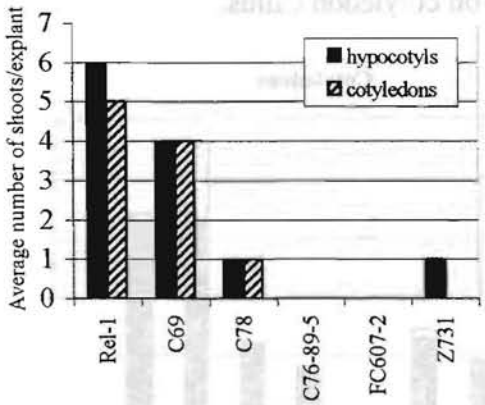

Fig. 2. Average number of shoots per hypocotyl (solid bars) or cotyledon (hatched bars) explant of six sugarbeet lines (200 explants of hypocotyls or cotyledons were used). Shoots were scored 6 weeks after isolation.

Table 2. Influence of seed germination and explant cultivation media on hypocotyl and cotyledon callusing and on the regeneration of line C69. Scored 6 weeks after explant isolation.

	Medium for seed germination							
	GM					B2		
	Medium for explant cultivation					Medium for explant cultivation		
	B1 3% sucrose	B1 8% sucrose	SIM	B1A2	GM	B2 3% sucrose	B2 8% sucrose	SIM
Hypocotyls								
% with callus	56.4	44	70	31	32	45	23	36
Callus amount	++++	+++	++	+	+	+	+	+
Embryogenic callus	+	+	+	+	-	+	+	+
Cotyledons								
% with callus	28	25	28	20	15	15	12	25
Callus amount	++++	+	+	++++	+	++++	++	++
Embryogenic callus	+	+	+	+	+	+	+	+

Particle bombardment of organogenic callus

Embryogenic callus generated from hypocotyls and cotyledons of line C69 was subjected to particle bombardment transformation with the PI-II-*ipt* gene construct. In comparison to the standard protocol (B1 + Km 200 mg/l, 4 weeks⟶ B1), the frequency of organogenesis on hypocotyl callus was higher if the tissue was cultured on SIM + Km 200 mg/l medium for four weeks or if the tissue was transferred to GM medium after two weeks of Km selection on B1 medium (Fig. 3A, solid bars). Lower Km concentration in the selection medium (100 mg/l) did not improve the frequency of organogenesis. The highest number of putative transformants on hypocotyl callus was regenerated using the standard protocol (Fig. 3A, open bars). With cotyledon callus, the frequency of organogenesis following particle bombardment was more than 3-fold higher when SIM + Km 200 mg/l was used instead of B1 + Km 200 mg/l as a selection medium (Fig. 3B, solid bars). The average number of putative transformants per plate of cotyledon callus on SIM medium was 12.7 as compared with 0.3 for control callus on B1 medium (Fig. 3B, open bars). Lower Km concentration in the selection medium did not significantly improve the frequency of organogenesis on cotyledon callus.

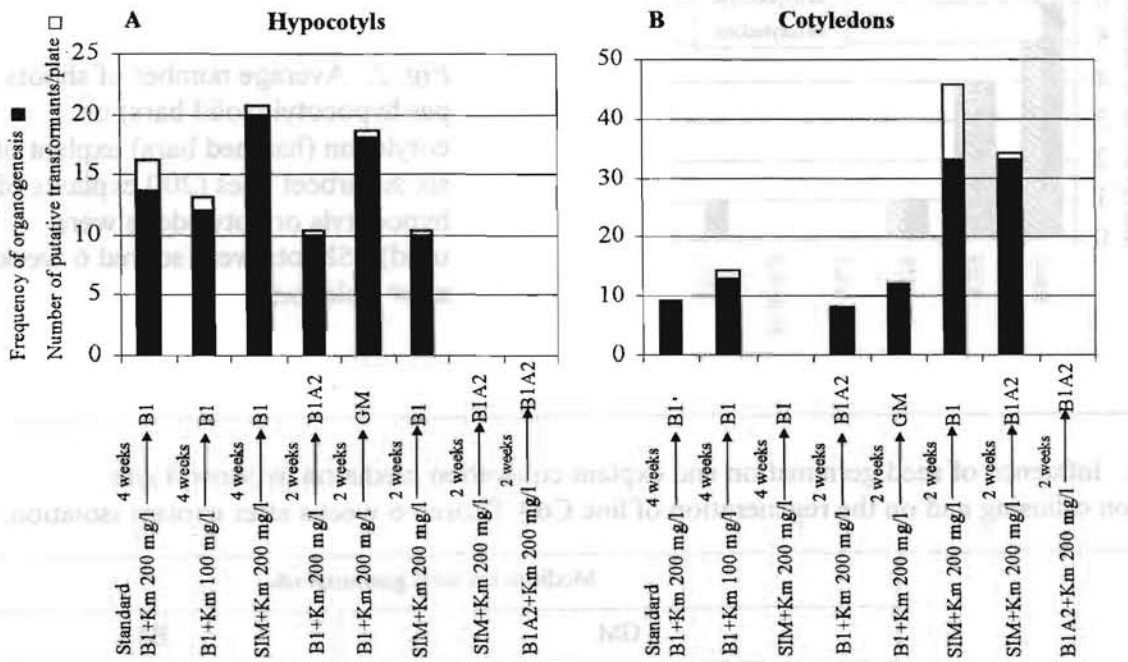

Fig. 3. Influence of the cultivation protocol on the frequency of organogenesis (expressed as a number of plates that regenerated shoots per total number of treated plates) and the average number of putative transformants per plate of the C69 bombarded callus. (A) Hypocotyl callus. (B) Cotyledon callus.

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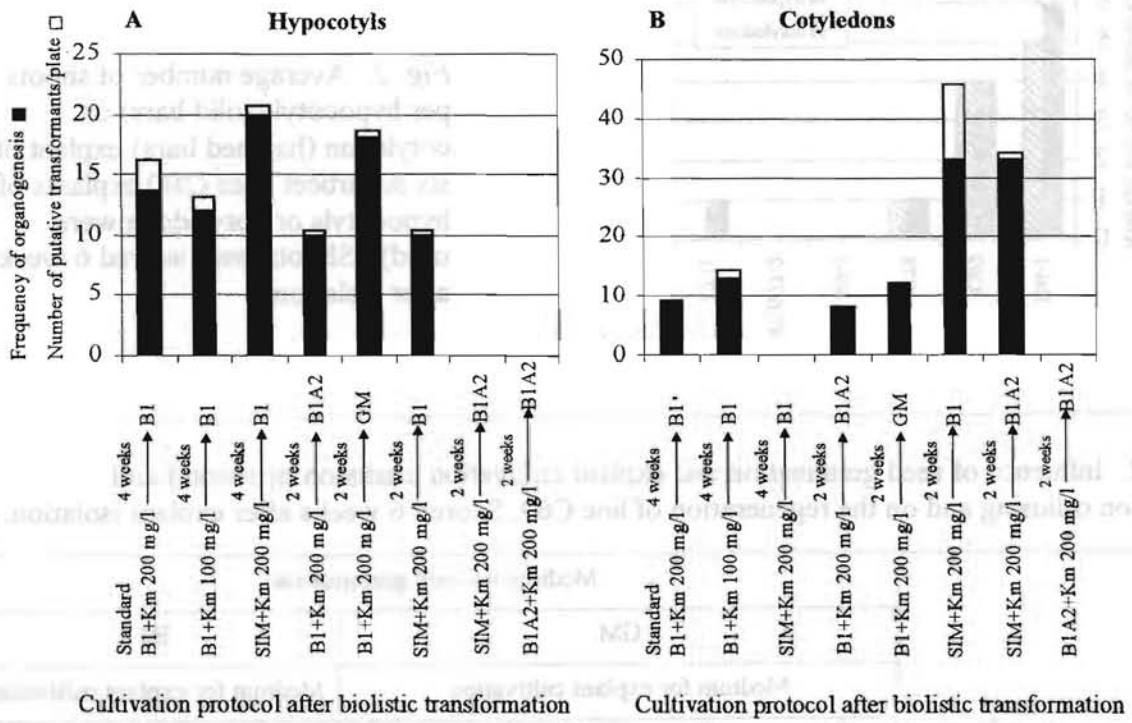

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Molecular analysis of putative transformants

Putative C69 transformants regenerated from hypocotyl or cotyledon callus were screened for the presence of the *ipt* and *npt II* gene by PCR. PCR bands corresponding to the *ipt* (513 bp) and *npt II* (700 bp) gene fragment were detected in 9 out of 28 shoots (32%) (Fig. 4; data not shown). These shoots, however, were vitrified and did not develop into normal plants.

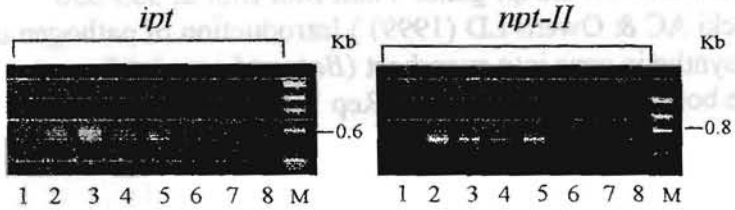

Fig. 4. PCR analysis of putative PI-II-*ipt* transformants. Total DNA was isolated from untransformed C69 plant (lane 1) and putative transformants (lanes 2 to 8) and amplified using primers specific for the *ipt* or *npt II* gene (M; DNA molecular wt marker in Kb).

Conclusions

Of the tested sugarbeet breeding lines, line C69 proved to be the best source of embryogenic hypocotyl callus. Cotyledons of both the REL-1 tissue culture clone and C69 breeding line developed callus that regenerated shoots at a similar rate. The shoot regeneration rate of hypocotyl and cotyledon callus after particle bombardment was improved by initial culturing on SIM medium instead of B1. SIM medium also improved the average number of putative transformants regenerated per plate of cotyledon callus. Putative transformants were shown by PCR to contain the *ipt* and *npt II* gene fragment, but the shoots did not develop into normal plants due to vitrification.

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