DIRECT GENE TRANSFER TO SUGARBEET LEAVES

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ABSTRACT

Sugarbeet transformation methods in the public domain are not readily reproducible and yield low transformation frequencies. These methods utilize sugarbeet cotyledons, shoot basal tissues, and hypocotyl callus generated in tissue culture for gene transfer experiments. We developed a particle bombardment transformation method that uses leaves from greenhouse grown plants. Leaf discs or squares were excised from surface sterilized FC607 leaves and placed on B1 medium with added mannitol and sorbitol. Leaf tissues were bombarded with the *uidA* (GUS) gene under control of the osmotin (OSM) gene promoter or with the enhanced green fluorescent protein (EGFP) gene fused to the double 35S CaMV promoter. After 2 days, leaf fragments were transferred to B1 medium (2 fragments per petri plate) and cultured at 31 °C in the dark. Two days after bombardment, 1 to 30 GUS$^+$ units per leaf fragment were observed. With the EGFP gene, 20 to 150 fluorescent cells per leaf fragment were visualized with an epifluorescence microscope. Both GUS and EGFP expression decreased significantly during the first 2 weeks of culture. After 6 to 8 weeks, embryogenic callus was removed from the bombarded leaf discs and analyzed for GUS expression. Presence of the GUS and the selectable marker (*npt* II) gene was confirmed by PCR analysis. GUS$^+$ shoots regenerated from several GUS$^+$ calli. The advantages over previously published transformation methods include an abundant source of leaf material from greenhouse grown plants, the ease of handling leaf material in tissue culture, and the overall rapid regeneration of transgenic shoots within three months.

INTRODUCTION

Sugarbeet improvement by conventional breeding has been a challenging task because of many biological complexities such as allogamy and incompatibility. Genetic engeenering methods involve direct introduction of foreign genes into plants and thus circumvent problems associated with sexual methods. Nonproprietary methods of sugarbeet transformation that include *Agrobacterium*-mediated gene transfer using shoot-base tissue (Lindsey and Gallois, 1990), cotyledonary node explants (Krens et al., 1996), or embrygenic callus from seedlings (D'Halluin et al., 1992) are plagued by low transformation frequencies and lack of reproducibility (Snyder et al., 1999). Our attempts to transform commercially important sugarbeet cultivars using particle bombardment of hypocotyl callus that was developed with a highly regenerative tissue culture clone REL-1 (Snyder et al., 1999) were unsuccessful. Therefore,

we explored alternative explants for use with the particle bombardment method for transformation of sugarbeet breeding lines.

MATERIAL AND METHODS

Greenhouse-grown plants of sugarbeet breeding line FC607 (Smith & Ruppel, 1980) were used for transformation experiments. Leaf discs (9 mm) were excised from surface sterilized expanding leaves 4 h before transformation. Fifteen to 20 explants were arranged in the center of a petri plate on B1 medium (Doley & Saunders, 1989) with 44.6 g/l mannitol and 44.6 g/l sorbitol. Leaf fragments were bombarded up to 3 times with gold particles coated with plasmid DNA (Ingersoll et al., 1996; Biolistic Particle Delivery System PDS-1000/He, Bio-Rad, Hercules, CA). Three plasmids were used for particle bombardment, pGT109, pGV130 and pARS108. pGT109 and pGV130 contained the reporter gene *uidA* (GUS) fused to either the tobacco osmotin (Osm) or the potato proteinase inhibitor II (Pin2) gene promoter, respectively, and the selectable marker gene NOS-*npt* II for kanamycin (KM) resistance (Snyder et al., 1999). pARS108 carried as a reporter gene enhanced green fluorescent protein (EGFP) fused to the double CAMV 35S gene promoter (Randall P. Niedz, USDA-ARS, Ft. Pierce, FL). Two days after bombardment, two explants per petri plate were placed on B1 meda with varying concentrations of KM and incubated in the dark at 31°C for 8-9 weeks. Regenerated calli and shoots were maintained on B1 medium at 25°C under a 16 h diurnal photoperiod provided by fluorescent lights (cool-white, 30 $\mu mol/m^2s$).

GUS activity was assayed by incubating the tissues in an XGluc solution at 37° C overnight (Jefferson et al., 1987). EGFP was visualized using an epifluorescence microscope (Nikon SMZ 1500) with FITC/EGFP filter providing excitation wavelengths 460-500 nm.

Putative transformants were screened for the presence of the *uidA* and *npt* II gene by PCR (Smigocki & Hammerschlag, 1991).

RESULTS

1.- TRANSFORMATION WITH THE UIDA GENE

Transient GUS activity in leaf fragments bombarded with pGT109 showed 0-170 expression units (blue spots) per plate 1-2 d after transformation. There was no significant difference in number of GUS^+ units per plate between plates bombarded at 1100 psi and 1350 psi. No blue staining was observed in control, untransformed explants. To obtain stable transformants, explants bombarded once with pGT109 or pGV130 were transferred 2 d after transformation to B1 medium without selection or with 10-100 mg/l KM. Leaf fragments showed expansion during the first week of culture on all media. Callus growth begun to appear on the wounded edges 5 weeks after bombardment in explants placed on medium without selection. By week 7, 48-100% of these explants formed big, friable, yellow calli (Table 1), most of which regenerated adventitious shoot and, to a lesser extent, somatic embryos. Regeneration of calli was much

slower when leaf fragments were cultured on B1 medium with 10-30 mg/l KM. At week 7, small number of these explants formed 1-2 very small friable calli without shoots. Some of the explants developed calli and shoots after an additional 7 weeks of culture, presumably when the KM content in the medium was reduced due to degradation. None of the explants cultured on medium with 100 mg/l KM regenerated callus or shoots even after 14 weeks of culture.

Table 1. *Results of biolistic transformation of sugarbeet leaf fragments with Osm-GUS and Pin2-GUS gene constructs.*

Construct	Experiment	KM concentration (mg/l)	Number of bombarded explants	Explants with callus (%)[1]	GUS⁺ callus	GUS⁺ shoots
Osm-GUS	T21	0	34	100[2]	1	0
	T9	0	27	48[2]	1	3
		100	6	0	0	0
Pin2-GUS	T25	0	115	66[2]	1	1
	T12	0	32	100[2]	1	1
		10	3	67[3]	0	0
		20	6	17[3]	0	0
		25	8	50[3]	2	0
		30	8	38[3]	0	0

[1] *determined 8 weeks after transformation.*

[2] *large, friable, yellow callus with shoots.*

[3] *small, friable callus, no shoots.*

All calli and shoots regenerated on bombarded leaf fragments were subjected to histochemical GUS test for the *uidA* gene expression. Results showed that 6 calli were GUS⁺, while none of the shoots showed the *uidA* gene expression (Table 1). Four GUS⁺ calli were regenerated on medium without KM, while 2 GUS⁺ calli were obtained on medium with 25 mg/l KM. All GUS⁺ calli were chimeric i.e. sectored for the presence of blue color. GUS⁺ calli were isolated and maintained on B1 medium as separate lines. During next 4 weeks they regenerated shoots which were tested for *uidA* gene expression. Results showed that GUS⁺ callus line from experiment T9 regenerated 3 GUS⁺ shoots (T9 a, f and g), while callus lines from experiments T12 and T25 each regenerated 1 GUS⁺ shoot. Efficiency of transformation, calculated as number of GUS⁺ shoots/number of bombarded leaf fragments, ranged from 0.9% to 11.1% (Table 2).

Table 2. *Sugarbeet transformation efficiency following particle bombardment of leaf fragments.*

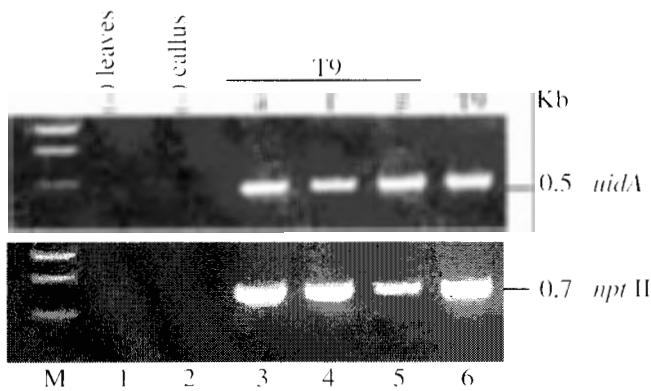
Experiment	Construct	Transformation efficiency %
T9	Osm-GUS	11.1 (3/27) [1]
T12	Pin2-GUS	3.1 (1/32) [1]
T25	Pin2-GUS	0.9 (1/115) [1]

[1] *number of GUS⁺ shoots/number of bombarded leaf fragments*

PCR analyses of three GUS⁺ shoots and one callus line revealed the expected size fragment for uidA (0.5 Kb) and npt II (0.7 Kb) genes (Fig. 1).

Fig 1. *PCR amplification of uidA (0.5 Kb) and npt II (0.7 Kb) gene fragment in Pin2-GUS transformed shoots (lanes 3-5) and callus (lane 6).*

Lane 1, untransformed leaves; lane 2, untransformed callus; M, DNA size markers.



2.- TRANSFORMATION WITH THE EGFP GENE

All leaf fragments bombarded with the pARS108 which carried the CaMV35S-EGFP gene showed fluorescence 2 d after transformation, while control explants that were not bombarded did not fluoresce. Fluorescence was observed in epidermal and stomatal cells. The number of fluorescent cells per fragment ranged from 20-30, 30-100, and 40-150 for tissues bombarded once, twice or three times, respectively. This number declined to only several cells per explant 6-8 d after bombardment. Three weeks after transformation, almost all newly generated callus showed green fluorescence that was also observed in control, untransformed tissues. Eight weeks after transformation bombarded leaf fragments regenerated calli and adventitious shoots, some of which showed green fluorescence. Fluorescence was also obtained in control, nontransformed tissues. Fluorescent calli and shoots that regenerated on all tissues were analyzed by PCR for the EGFP gene. Results showed that none of these tissues contained the expected EGFP gene fragment.

CONCLUSION

Particle bombardment of leaf explants provided a simple and efficient procedure for transformation of *B. vulgaris* breeding line FC607. Tranformed plants were obtained as early as 3 months after transformation. Such rapid production of transformed plants is desirable since studies have shown that long periods in tissue culture lead to somaclonal variations. This also means that a large number of independent transgenic lines can be produced rapidly, a crucial factor for systems with relatively low transformation efficiency. The EGFP gene may prove to be a better reporter gene than the GUS gene for sugarbeet transformation because EGFP expression can be analyzed nondestructively and used for visual selection of transformed tissue. However, further optimization is needed due to the observed autofluorescence of untransformed sugarbeet tissues.

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