GENE TRANSFER IN SUNFLOWER: COMPARISON OF DIFFERENT TECHNIQUES

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Abstract

We achieved transient *GUS* expression in elettroporated hypocotyl protoplasts of *Helianthus annuus* L. We also used *Agrobacterium tumefaciens* as a vector for the introduction of engineered T-DNA in protoplasts. We temporarily co-cultivated virulent agrobacteria with protoplasts to introduce *DapA* (isolated from *Bacillus subtilis*) and *NPTII* (antibiotic resistence) genes, both driven by the CaMV 35 S promoter. Up to now we have obtained some transformed calli selected in media containing 50 mg/l kanamycin. Protoplasts offer some advantages for genetic manipulation for the absence of the cell wall, but the major drawback is the difficulty to regenerate plants from isolated protoplasts. So we used a different approach to obtain genetic transformation in sunflower. First of all we established a regeneration procedure for cotyledons of mature seeds, then we used these regenerable explants for microprojectile bombardment with a biolistic equipment. Up to date we have some putative transformed shoots growing on selective medium. Molecular analysis to confirm transformation are in progress.

Introduction

In sunflower the establishment of procedures for genetic transformation is still at the beginning. There are some examples of transient expression of reporter genes (Schrammeijer *et al.* 1990; Moyne *et al.* 1989; Kirches *et al.* 1991) and stable transformation (Everett *et al.*, 1987). Different delivery techniques for foreign DNA introduction were used: direct DNA transfer in protoplasts (Moyne *et al* 1989; Kirches *et al.* 1991), *Agrobacterium tumefaciens*-mediated transfer (Schrammeijer *et al.* 1990), particle gun bombardment of different kind of explants and tissues (Bidney *et al.* 1992; Burrus *et al.* 1992). One of the common problem to all these methods is the difficulty to regenerate plants from transformed explants. Therefore we have developed a method for plant regeneration from protoplasts (Trabace *et al.* 1995) and cotyledons. We have used them as a target for genetic transformation. We have studied different gene transfer methods: co-culture of protoplasts with *A. tumefaciens*, direct gene transfer in protoplasts by electroporation, particle gun bombardment of cotyledons. In our study we used the *GUS* gene as a reporter marker for transient expression and *DapA* gene for stable transformation experiments.

Material and Methods

The genotypes used in this work were HA 89 and the Ro 1941 hybrid obtained from I.C.C.P.T. Fundulea, Romania.

Protoplasts

Isolation and culture. Seeds were surface-sterilezed with 0.1% $HgCl_2$ for 5 min and then rinsed several times in sterile distilled water. Seeds were germinated onto half strength MS salts with 0.5% sucrose and solidified with 0.7% agar , pH 5.6. Seven day-old etiolated hypocotyls were used for protoplast isolation. Hypocotyls were cut into 2-3 mm sections and incubated for 14 h at 25 °C in a mixed enzyme solution containing 0.35% Cellulase R-10, 0.02% Macerozyme R-10 and 0.05 % Driselase. After digestion protoplasts were purified by flotation on Ficoll 12%. (Chanabe *et al.*, 1989). Treated and non treated protoplasts were resuspended in L4M (Lenee and Chupeau, 1986) and cultivated in agarose droplets at a final density of 5×10^4 protoplasts/ml (Burrus *et al.*, 1991).

Electroporation. In a first series of experiments we tried to find an accetable compromise between survival rate of protoplasts and electropermeation of the cellular membrane. For this purpose protoplasts were resuspeneded in the electroporation buffer (glucose 10% , HEPES 10 mM, $CaCl_2$ 4mM, spermidine 0.2 mM, pH 7.2). and subjected to different electrical treatments in presence of Evans blue. Sunflower protoplasts were resuspended in the ice cold electroporation buffer at a concentration of 3×10^5 cells/ml; 300 µl of protoplast suspension were mixed with plasmid pBI 221 (CaMV 35S, GUS, NOS) at a concentration of 20 µg/ml and electoporated (Hoefer Progenetor II) using a ring electrode made of stainless steel and 2.5 mm high anode platinum; the distance between electrodes was 6 mm. Different combination of pulse duration and number of pulses were applied with a constant electrical field strength (see results). After a post pulsation incubation of 20 min at 22 °C protoplasts were washed and resuspended in the culture medium. After 24 hour culture protoplasts were subjected to the *GUS* fluorimetric assay according to Jefferson (1987).

Co-culture with Agrobacterium tumefaciens. We used the *Agrobacterium* strain EHA 105 containing a modified PBI 121 plasmid in which the *GUS* gene was replaced by *DapA* gene (isolated from *Bacillus subtilis* and codifing for an enzyme involved in the regulation of the lysine metabolic pathway), whereas the *NPTII* gene was left unalterated in order to maintain its function as selective agent. The chimaeric constructs was driven by CaMV 35S promoter. Protoplasts were resuspended in the liquid medium L4 at a concentration of 1×10^5 and inoculated with *A. tumefaciens* with a numerical ratio of 100/1 between bacteria and protoplasts. After 18 h co-culture at 25 °C protoplasts were rinsed in the liquid L4 medium containing 500 mg/l Cefotaxim and 250 mg/l vancomycin. Protoplasts were then cultured in L4 agarose droplets covered by liquid L4 medium supplemented with 50 mg/l kanamycin. Liquid medium was refreshed every week until callus development. Putative transformed calli selected on kanamycin were analized to confirm transformation using PRC amplification.

Cotyledons.

Isolation and culture. Seeds were surface sterilized (see above) and soaked for 24 hours in sterile water. Cotyledons were then excised and cultured onto an agar solified basal MS medium containing 4 mg/l BAP and 1 mg/l IAA. Cultures were kept at 25 °C with a photoperiod of 16/8 hours d/n. After 2-3 weeks regenerating shoots (2-3 mm) were transferred on a hormone free MS medium. Developing shoots (10-20 mm) were rooted onto one third strength MS medium and then potted in peat and hardened in the greenhouse.

Biolistic transformation. For particle bombardment the cotyledons with regeneration primordia (7-10 gg) were cut into 2-3 pieces and placed very tigthly in 60 x 15 mm Petri dishes. Experiments were carried out with pDAHPS, a plasmid containing the *DapA* gene and the *HPTII* gene (hygromycin resistance) under the control of the CaMV 35S promoter. Plasmid DNA was adsorbed to 1.0 μm gold particles (3 mg gold particles/50 μl water) by adding in order 5 μl of DNA (1μg /μl), 50 μl of 2.5 M CaCl2 and 20 μl of 0.1 M spermidine. After vortexing for 3 minutes microcarriers were washed and resuspended in 60 μl of 100 % ethanol. Aliquots of 10 μl were used for each bombardment. The target materials were bombarded one or two times, using a Bio-Rad Biolistic PDS-1000/He particle delivery system. Different combination of rupture disk (1100-1300 psi) and distance between macrocarrier and stopping screen (6-8 cm) were used. After bombardment explants were cultured on shoot induction medium supplemented with 20 mg/l hygromyicin.

Results and Discussion

Electroporation of protoplasts. Several electropermeabilization experiments with the dye, Evans Blue, were carried out; significant levels of coloured protoplasts were found with an electrical field strength of 215 volts/cm, while higher levels of voltage caused a drastic reduction in survival rate of protoplasts. So this field strength was fixed while the number and duration of pulses could vary. A good compromise between protoplast viability and membrane permeation was found with 3-4 pulses of 100-300 msec., so we tested GUS expression under similar conditions, while varying number and duration of pulses, but keeping field strength constant at 215 volts/cm. In Tab. 1 some electroporation treatments are summarized, 4 pulses of 300 msec gave rise to a considerable increase in β-glucuronidase activity. These results confirm the possibility to use electroporation of protoplasts as a mean to delivery exogenous DNA in sunflower (Kirches *et al.*. 1991). In our experiments we used hypocotyl isolated protoplasts that previous studies indicated as suitable for plant regeneration (Burrus *et al.*, 1991; Krasnyanski and Menczel, 1993; Trabace *et al.* 1995), whereas the electrical parameters used by Kirches *et al.* (1991) for mesophyll protoplasts were not suitable for our material. In particular high electrical field strength dramatically reduced the viability of hypocotyl protoplasts, and PEG-mediated DNA transfer, by Negrutiu *et al.* (1987) found more effective in transforming protoplasts of *N. tabacum* and *N. plumbaginifolia,* caused low survival rates of sunflower hypocotyl protoplasts (data not shown).

Agrobacterium-mediated transformation. Plating efficiency of protoplasts was registered every week (Tab. 2). After 2 weeks non transformed protoplasts in presence of kanamycin stopped to divide at the microcolony stage. On the contrary, protoplasts co-cultivate with *A. tumefaciens* actively divided on the selective medium to form visible calli. Primer-directed enzymatic amplification was chosen to confirm the presence of the *DapA* gene in the calli because little genomic DNA available. DNA amplification of the putatively tranformed calli grown on selective medium shown the expected 810 bp band corresponding to *DapA* gene (Fig.1). Some of these calli exhibit regeneration primordia and are now cultivate to regenerate complete plants.

Biolistic of cotyledons. Cotyledons have a considerable potential for regenerating shoots (Knittel *et al.* 1991), so they can represent suitable explants for biolistic transformation.

Our experiments confirm their regeneration potential and that genotype exerts a pronounced effect on the regeneration frequency (Tab. 3).
Up to now we have some putatively transformed shoots actively grown on selective medium; are in progress molecular analysis to confirm transformation.

Conclusions

Protoplasts offer some advantages for genetic manipulation for the absence of the cell wall and moreover transformation of single cells may avoid the chimaeric problems associated with transformation of multicellular tissues (Schremmeijer *et al.*, 1991). Our first results demonstrate the possibility to use electroporation as a mean to introduce exogenous DNA in sunflower protoplasts, although further studies are still necessary to optimize the electroporation technique and the regeneration system for electroporated protoplasts. In our knowledge co-culture of protoplasts with *A. tumefaciens* is a first attempt in sunflower, vancomycine and Cefotaxim were effective to eliminate *Agrobacterium* but determined a reduction in protoplast division frequency. Despite this reduction we obtained some transformed calli , so a refiniment of this tranformation technique seems very promising for sunflower, also considering the present possibilities of plant regeneration from sunflower protoplasts (Burrus *et al.*, 1991; Fischer *et al.*, 1992; Kransnyanski and Menczel, 1993; Trabace *et al.*, 1995). However cotyledon culture seems to be a more reliable regeneration system for sunflower (Knittel *et al.*, 1991; Chraibi *et al.*, 1992; Ceriani *et al.*, 1992) and these explants represent an ideal target for microprojectile bombardment. The good results obtained in several species and the preliminary results reported in this paper point out biolistic technology as the incoming technique to obtain transgenic plants in *Helianthus annuus* L.

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ILLUSTRATIONS

Tab. 1. Electroporated protoplasts. Fluorimetric assay of β-glucuronidase activity. (Electrical field strength : 215 volts/cm).

Treatment	N° of pulses	Pulse duration (msec)	Enzymatic activity (pmol/h)	Viability (%)
control	0	0	0.0	58.9
1	3	50	6.0	51.6
2	4	50	7.7	39.8
3	3	100	5.1	40.9
4	4	100	5.9	18.7
5	4	300	19.7	17.5

Tab.2. *A. tumefaciens*-mediated transformation of protoplasts. Plating efficiency (%) of protoplasts (genotype Ro 1941) cultured on selective medium (kanamycin 50 mg/l).

days after plating	*A. tumefaciens* co-cultured protoplasts	Non treated protoplasts
7	16.0	23.6
14	28.5	17.9
21	29.3	5.2

Tab. 3. Regeneration of plants from cotyledon explants. Genotype dependence of shoot induction.

Genotypes	Type	Regenerated shoots/ cultured cotyledon (%)
Ro 1941	Hybrid	17.5
Ha 89	Imbred;cms	7.7

Fig. 1. DNA analysis from sunflower callus transformed with *A. tumefaciens*. Lanes 1-4 calli selected for kanamycin resistence: lanes 5-6 untransformed controls.