Gene transfer into sunflower (*Helianthus annuus* L.)

M. Burrus*, B. Damm*+, R. Hunold°, H. Laparra, A. Pignard**, G. Hahne

**Institut de Biologie Moléculaire des Plantes et Université Louis-Pasteur*
12, rue du général Zimmer, 67084 Strasbourg, France
*** Sanofi, Elf Bio-Recherches, 31676 Labège, France*
+ Current address: Mogen Leiden, the Netherlands

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SUMMARY:

In an attempt to obtain transgenic sunflower plants, we are investigating different combinations of regeneration systems developped in our laboratory with various transformation procedures: *Agrobacterium tumefaciens* and cotyledons, particle gun and immature embryos, and direct gene transfer in protoplasts. PEG treated-protoplasts are a consistent system to study gene expression, giving a high and reproducible transient expression. Transient expression has been observed in immature embryos bombarded with gold particles. GUS positive zones in regenerated shoots have been detected after *Agrobacterium* coculture of cotyledons.

INTRODUCTION:

Until now, only one paper describes stable transformation of sunflower with subsequent transmission of the introduced gene to the next generation (Everett *et al.* 1987). But the reproducibility of this work is low. Therefore, we are currently investigating different gene transfer methods in sunflower, namely *Agrobacterium tumefaciens*, particle gun and direct gene transfer in protoplasts. Plant regeneration has been obtained in our hands from cotyledons (Knittel *et al.* 1991), immature embryos *via* somatic embryogenesis (Li *et al.* 1988, Jeannin and Hahne 1991), and protoplasts (Fischer and Hahne, submitted). In our study, the uidA gene was used as a reporter marker in transformation experiments. The appearance of blue spots was evaluated at different times following the treatments. For transient expression studies, the CAT and uidA genes were used.

MATERIAL AND METHODS:

The genotype used in this work was Ha 300B.

Cotyledons

Dry seeds were germinated *in vitro* for 1-2 days in sterile water, in the dark. Cotyledons were excised according to Knittel *et al.* (1991). For plant regeneration, the proximal part was cultured on a solid

MS based medium containing 1 mg/l BAP and 0.5 mg/l NAA (Per C). For coculture experiments, the *Agrobacterium* strain GV 2260 containing the plasmid pGUS-INT (obtained from L. Willmitzer, Berlin) was used. Freshly excised cotyledons were incubated in a late log-phase bacteria solution for 20 minutes, blotted dry on filter paper and cultured for 3 days in dim light conditions (16 hours) on different media. Explants were then rinsed in liquid MS medium containing 500 mg/l cefotaxim, blotted dry and cultured on Per C medium supplemented with 500 mg/l cefotaxim.

Immature embryos

Immature embryos of different sizes (1-6 mm) were obtained from greenhouse grown sunflower plants. Embryos were excised without sterilisation and immediately plated on three different media. For zygotic embryo preculture (7, 10, 12, or 14 days) and post-bombardment culture, the following solid media were used (modified after Gamborg *et al.* 1968):

B_4: B5 medium containing 7 mg/l zeatin and 12 % sucrose;

B_9: B5 supplemented with 1 mg/l 2,4-D, 500 mg/l casein hydrolysate, 20 mg/l adenine sulfate, and 18 % sucrose;

B_{10}: similar to B_9, but 2,4-D was exchanged for 7 mg/l zeatin.

For particle bombardment, immature embryos were placed very tightly in a 60x15 mm Petri dish and were held in place by gauze. Experiments were carried out with a gun powder-driven particle gun apparatus and a 8.3 kb plasmid (pCG35S), containing the kan gene, the uidA gene and the CAT gene, all three under control of the 35S promoter (Lepetit *et al.* 1991). Plasmid DNA was adsorbed on freshly prepared gold particles (1.5-3.0 µm, Alfa Products) by adding 10 µl DNA (4 µg per µl in deionized water) to 25 µl particle suspension (400 mg per ml of 50 % glycerol) in a 1.5 ml microfuge tube. $CaCl_2$ (25 µl of a 2.0 M solution) and spermidine free base (10 µl of a 10 M solution) were then added to the suspension and allowed to settle for 30 minutes. After centrifugation, 55 µl of supernatant were removed. The tube was then finger vortexed to suspend the particles and 3 µl of suspension were placed on the front surface of a nylon macroprojectile. Each plate was bombarded two times. The distance between the stopping plate and the Petri dish was 10 cm. For better distribution of DNA coated particles, a 225 µm mesh metallic screen was placed between the stopping plate and the Petri dish.

Protoplasts

Seven day-old axenic hypocotyls were used for protoplast isolation. Hypocotyls were shredded and incubated in enzyme mixture (0.2% Macerozyme R10, 1% Cellulase R10, 0.25% Driselase in 334 mM KCl, 14 mM $CaCl_2$, 0.7 g/l MES, pH 5.7) for 14 hours, with low agitation. After digestion, protoplasts were filtered and rinsed twice. Treated and non-treated protoplasts were cultured in liquid L4 medium (Lénée and Chupeau 1986) at a final density of 5. 10^4 /ml

Electroporation was performed in a solution containing 0.6M mannitol and 0.1mM $CaCl_2$, with 10^6 protoplasts and 40 micrograms of pCG35S. Different voltage conditions and pulse durations were tested, ranging from 0 to 1400 V/cm and from 3 to 10 msec.

For PEG transformation, protoplasts were incubated in 334 mM KCl, 125 mM $CaCl_2$, 0.7 g/l MES, pH 5.7 for 30 minutes at 4°C. They were suspended in 0.25 M mannitol, 15 mM $MgCl_2$, 0.1 g/l MES pH 5.6 at a density of 2.10^6/ml. 20 micrograms plasmid DNA/ 0.5 ml protoplast suspension were added, followed by PEG (40% PEG, 0.1 mM $(NO_3)_2Ca$, 0.4 M mannitol, pH 7.0). After an additionnal 20 minute incubation, the suspension was step-wise diluted with KCl 334 mM, $CaCl_2$ 125 mM, MES 0.7 g/l, pH 5.7, and followed by centrifugation. The pellet was then suspended in L4 medium.

GUS and CAT assays

Extracts of protoplasts cultivated for 24 hours were subjected to the GUS fluorometric assay described by Jefferson (1987). Results are expressed in pMoles MU/ min/ µg protein extract. The histological assay was performed according to Jefferson (1987).
The kit furnished by Dupont was used for the quantitative CAT assay. Results are expressed in cpm/ hour/ µg proteins.

RESULTS AND DISCUSSION:

We are trying to combine our efficient regeneration systems with transformation procedures in order to obtain stably transformed sunflower tissue and finally fertile transgenic sunflower plants.

Cotyledons and *Agrobacterium*

Sunflower cotyledons are suitable explants for coculture experiments with *Agrobacterium tumefaciens*. Detection of transformed tissue by histological GUS assays showed that, in more than 95% of the cases, the transformed cells were localized on a particular region of the cotyledon, i. e. the border between cotyledon and petiole. This region also shows high regeneration ability (Knittel *et al.* 1991). In a series of experiments, we have investigated various coculture conditions, i.e. coculture media, coculture duration, bacteria density, acetosyringone, for their effects on transformation frequency. The most important factor was the composition of the coculture media (Table 1). When coculture was done on a hormone-free medium, the frequency of transformation (number of explants transformed as well as number of transformed cells per explant) was maximal. However, these transformed cells belong to a non regenerating callus. Whereas, on a hormone containing medium, the frequency of transformed explant was lower, yet the transformed cells were part of organized tissue. Newly formed primordia were allowed to develop into shoots, without selection. Some of the emerging structures showed blue zones on the stems or the leaves. Further studies were carried out on the feeder effect (Table 2). Coculture on a feeder-layer made from a 7 day-old sunflower cell suspension led to an increase of the number of blue spots per explant, but had no clear effect on the average of transformed explants.

Protoplasts and direct gene transfer

Initial experiments focused on gene transfer optimization. We compared two methods commonly used for transient expression studies, electroporation and PEG transfection.

Table 1: Effect of the coculture medium on cotyledon transformation frequency

Coculture medium	% Transformation
MS med.+KNO3 5g/l+BA 1mg/l+NAA 0.5mg/l	36
MS med.+ KNO3 5g/l	80
MS med.	87
1/10 Schenk and Hildebrandt med.	99

Table 2: Effect of sunflower feeder layer (FL) on cotyledon transformation frequency

Coculture medium	Transformed explants (%)	Blue spots/explant
MS med.	75	5
id. + FL	76	40
id. + BA 1mg/l+NAA 0.5mg/l	69	5
id.+BA 1mg/l+NAA 0.5mg/l+FL	75	40

Figure 1: Effect of voltage on transient expression in electroporated sunflower (125 µF, 1 pulse)

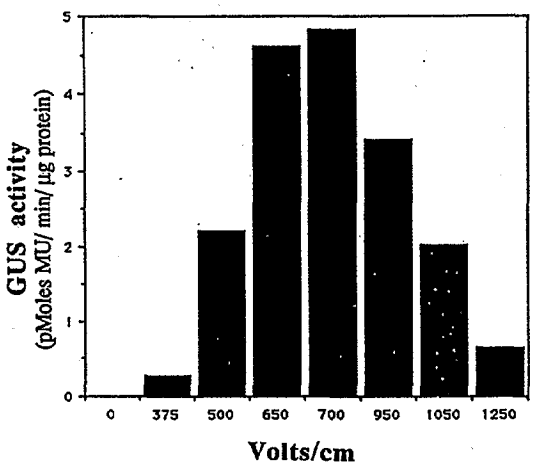
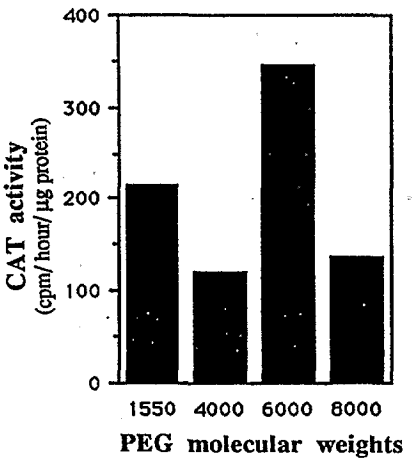


Figure 2: Effect of PEGmolecular weights on transient protopolasts expression in sunflower protoplasts



For electroporation, different voltages were tested (Figure 1). At a capacitance of 125 µF, membrane permeabilization occurred at 375 V/cm. Best expression levels were obtained at 700 V/cm, then decreased in correlation with protoplast lysis.

The PEG transformation procedure described by Negrutiu *et al.* (1987) was adapted to sunflower protoplasts. Among the different molecular weights of PEG (1500, 4000, 6000, 8000), PEG 6000 was the most efficient (Figure 2). We investigated the importance of different parameters: among those tested, carrier DNA, and plasmid concentration increased CAT gene expression, while plasmid linearisation, and heat-shocks (45°C for 5 and 10 minutes) did not have any significant effect. Comparison of PEG treatments and electroporation show that DNA uptake is more efficient using PEG than electroporation. These data are similar to those of Kirches *et al.* (1991) who furthermore reported less damage in electroporated protoplasts than PEG-treated populations. This method was therefore selected for further transformation experiments. Attempts to isolate stably transformed colonies are under way.

Immature embryos and particle gun

Immature embryos are suitable explants for transformation of sunflower using a biolistic gun. Histological GUS assays performed 2.5 days after bombardment resulted in particular high frequencies of transient GUS expression. Twenty eight days after bombardment, stably transformed cells were still detectable in 38 % of embryos examined. The efficiency of transformation, i. e. percentage of immature embryos with blue spots, was mainly influenced by embryo size and media used (Table 3). As a result of transient expression assays, 82.6 % of 1-2 mm small embryos cultured on B_{10} medium were covered by blue spots ranging from 1 to 50 per explant. While zeatin-containing medium (B_4) was inducing calli and shoots on hypocotyls, the B9 medium caused somatic embryogenesis on the cotyledon surface due to 2.4-D and high sucrose level. In some cases, blue spots could be detected on the top of those embryogenic structures. To obtain more uniformly transformed tissues, the zygotic embryos have to be bombarded a few days earlier than our minimal preculture time. A prolongation of preculture on B_{10} medium (up to 14d) did not decrease the

Table 3: Transient GUS expression in 10-14 d precultured immature embryos following particle bombardment: Influence of embryo size and medium.

Embryo size: (mm)	Medium	Immature embryos: tested	+blue spots (%)	Blue spots/ embryo: average	range
6	B4	37	35.1	6.6	1-21
4	B4	58	34.5	2.7	1-10
2	B4	19	47.4	1.8	1-4
1-2	B9	45	68.9	5.7	1-25
1-2	B10	86	82.6	7.8	1-50

Table 4: Transient GUS expression in immature embryos following particle bombardment: Influence of preculture time and medium.

Preculture time/ medium	Embryo size (mm)	Immature embryos: tested	+blue spots (%)	Blue spots/ embryo average	range
7d/B4	6	75	69.3	9.3	1-56
	4	103	54.4	5.8	1-25
	2	10	80.0	14.2	1-40
7d/B10	1-2	25	84.0	6.1	1-14
7d/B4	6	31	32.2	1.9	1-4
	4	45	35.5	2.3	1-7
	2	19	47.4	1.8	1-4
14d/B10	1-2	49	77.5	8.9	1-50

transformation events for the smallest explants (Table 4) but somatic embryos were already preformed. The range of blue spots per embryo increased from 14 to 50. On the other hand, after 7 days on B4 medium precultured explants showed higher transformation frequencies at both the number of blue spots per embryo and the percentage of embryos with transformed cells compared to 14d precultured tissues.

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