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A FOCUSING DEVICE FOR BIOLISTIC TRANSFORMATION OF SUNFLOWER (*Helianthus annuus* L.) COTYLEDONS

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SUMMARY

In the past few years several attempts have been made to obtain sunflower genetic transformation using different tissues such as meristems, shoot axes, hypocotyls, cotyledons of young seedlings and immature embryos. Transgenic plants were obtained in some instances but the efficiency and reproducibility were low. The common problem was the difficulty to establish an efficient regeneration system strictly correlated with the transformation system. Several reports indicate sunflower cotyledons as one of the most effective explants for plant regeneration but unlike other sunflower tissues this material is not easily transformed by *Agrobacterium*. Particle bombardment is an important tool for monocot transformation and could be an interesting alternative also for sunflower cotyledons. Particle delivery system currently sold commercially has a limited focusing ability whereas cotyledons exhibited a confined regenerable area restricted to the embrionic axes. To overcome this problem a stainless steel focusing device that can be applied to the DuPont/BioRad PDS1000/He apparatus was constructed. This device was tested in a series of β-glucuronidase expression carried out on sunflower cotyledons, cv. HA89 and experimental lines. Endogenous GUS-like activity was abolished by changing the sample buffer and by performing the reaction at 56°C after having pre-equilibrated explants and buffer for 90 min. The device enabled to confine and concentrate more than 90% of the transformation events in a ring-shaped area at 5-6 mm from the target center. Without the device transformation was very low and scattered over a much wider surface. On the basis of these data an appropriate arrangement of the cotyledons could improve dramatically the probability to transform competent cell for regeneration; this could be even more evident if sunflower genotypes with a high frequency of shoot regeneration are used.

Key words: **Sunflower, cotyledon, plant regeneration, genetic transformation, biolistic focusing device**

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INTRODUCTION

In the past few years several attempts were made to obtain sunflower genetic transformation using different tissues such as meristems (Schrammeijer *et al.*, 1990), shoot axes (Malone-Schoneberg *et al.*, 1991), hypocotyl sections (Everett *et al.*, 1987 and Hartman, 1991), cotyledons of young seedlings and immature embryos (Laparra *et al.*, 1995). Transgenic plants were obtained in some instances (Everett *et al.*, 1987; Schrammeijer *et al.*, 1990; Malone-Schoneberg *et al.*, 1991; Espinasse-Gelhner, 1992), but the efficiency and reproducibility of the transformation process were low. The common problem was the difficulty to establish an efficient regeneration system coupled with transformation (Laparra *et al.*, 1995). For instance, protoplasts can be easily transformed *via* PEG or *Agrobacterium tumefaciens*, but the very low regeneration capacity (10^{-4}) dramatically reduces the possibility to obtain stable transformed plants; otherwise a multitude of shoots can be induced on cotyledons of young seedlings (Knittel *et al.*, 1991) but this material is not easily transformed by *Agrobacterium* (Laparra *et al.*, 1995) unlike other sunflower tissues without such regeneration ability (Escandon and Hahne, 1991). Particle bombardment could be an effective alternative to obtain sunflower transformation (Vischi *et al.*, 1996) also in combination with *A. tumefaciens* co-culture (Bidney *et al.*, 1992; Laparra *et al.*, 1995). Sunflower cotyledons appear to be one of the most effective explants for plant regeneration (Knittel *et al.*, 1991; Fiore *et al.*,1997) but as like other regeneration systems in sunflower appear to be direct, i.e., without an intervening callus phase and thus only affects a relatively small proportion of the cells in the explant (Laparra *et al.*, 1995). Particle delivery system currently sold commercially has a limited focusing ability whereas the ability to distribute particles could be essential in explant with a confined regenerable area (Torisky *et al.*, 1996). In the present paper a regeneration system from cotyledon is presented in conjunction with a possible transformation system based on particle gun with an adapter to focus gold particles in the region close to the embryo axes, i.e., the regenerable area of cotyledon explants.

MATERIALS AND METHODS

Plant material

Experiments were carried out with the commercial inbred CMS HA89 and the restorer lines 3294 and 3277, kindly supplied by Dr. M. Baldini, University of Udine.

Seeds were surface sterilized in 70% ethanol for 2 min, and in 4% sodium hypochlorite for 1 h and then rinsed throughly with sterile distilled water. Sterilization was repeated after 24 hours. The seed coat was removed and the seeds were maintained for 48 h in the dark on an agar-solidified medium (H_0) at 25°C, to detect contaminants. Cotyledons were then excised and cultured onto an agar-solidified

basal MS medium (H_1) containing 1.0 and 4.0 mg l^{-1} of IAA and BAP, respectively. Cultures were kept at 25°C with a photoperiod of 16/8^h day/night. After 2-3 weeks regenerated shoots (2-3 mm) were transferred onto a hormone free MS medium (H_2). Developing shoots (10-20 mm) were rooted onto a third-strength MS medium (H_3), potted in peat and hardened in the greenhouse. Alternatively regenerated shoots were micrografted according to Trabace *et al.* (1995).

Biolistic

Particle gun. For particle bombardment the cotyledons with regeneration primordia (2-3 days) were placed in 60 x 15 mm petri dishes in three concentric circles having 8, 24 and 40 mm diameter, respectively (Figure 1). Shots were done with or without a focusing device (Figure 2) applied to the DuPont/BioRad PDS 1000/He gun. The focusing device was built according to the specifications of Torisky *et al.* (1996) with slight modifications. The distance between rupture disk and macrocarrier (gap distance) was adjusted to 6 mm, the distance between the macroprojectile and the stopping screen (macrocarrier travel distance) to 3 mm, the distance between the stopping screen and the biological target (target distance) was 120 and 150 mm. The pressure used for bombardment was adjusted to 1100 psi in combination with two gold particle size (1.0 and 1.6 µm). The chamber vacuum was 25" Hg. For each shot 25-50 cotyledons were used and each treatment was repeated 3 times.

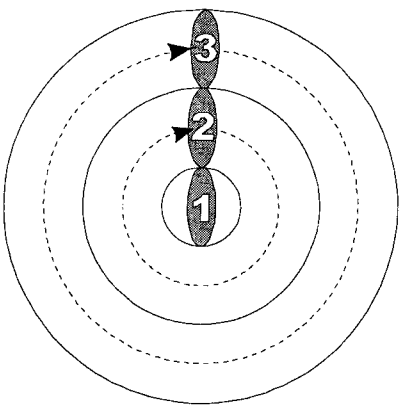

Figure 1: Spatial arrangement of cotyledon for particle bombardment.

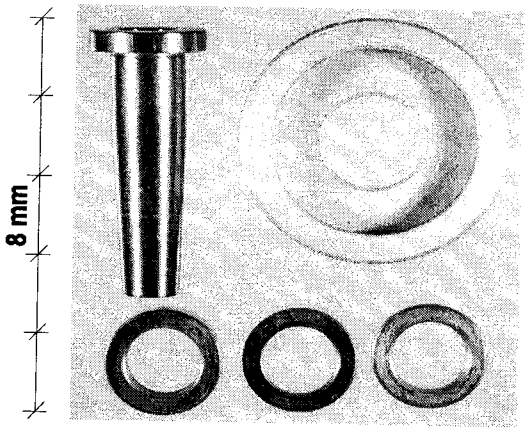

Figure 2: Stainless steel focusing devices with retaining spool (right) and spacer rings (bottom).

Plasmid. The pBI 221 plasmid (Clontech Laboratories), cloned in *E. coli* JM 101 (Promega) was used for transformation; pBI 221 contains the *uidA* gene coding for GUS under control of CaMV 35 S promoter. Plasmid DNA was extracted with Qiagen Maxi-prep and an aqueous solution with a 1 µg μl^{-1} concentration of DNA was prepared.

Particle preparation. Forty mg of gold were first resuspended in 1 ml of absolute ethanol, centrifugated at 10000 rpm for one minute and the pellet resuspended in 1 ml distilled water; 5 µl of pBI221, 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine were added in order, under continuous vortexing, to 50 µl of gold suspension. The solution was then centrifuged and the supernatant removed. A volume of 250 µl of absolute ethanol was first added, and after centrifugation and elimination of the supernatant, the pellet was resuspended in 60 µl of absolute ethanol. For each shot 3.5 µl of the last solution was spread onto the macrocarrier within a circle having a 20 mm diameter.

Histochemical GUS assay

Transient expression of the β-glucuronidase coding gene was examined 2 days after transformation. Staining was carried out according to Hodal *et al.* (1992) to eliminate GUS intrinsic activity (Hu *et al.*, 1990). Explants were submerged in water at 55°C for periods of 1.5, 2 and 3 hours and then incubated in the prewarmed staining solution for 15 hours.

Data analysis

All experiments were performed at least three times in order to measure the effect of uncontrolled source of variations. Transient expression was determined for each concentric circle counting the number of blue spots. The analysis of variance was performed to a factorial design with 4 variables (genotype, target distance, gold particle size, concentric circle) in order to detect statistical differences between treatments and their interactions.

RESULTS AND DISCUSSION

The data concerning the regeneration capacity (n° of shoots / n° of cultured cotyledons) of the three genotypes on trial are reported in Table 1. Restorer line 3294 and CMS HA89 showed consistently a regeneration ability of 15% and 10% respectively, whereas in our conditions no regeneration was ever obtained with the restorer line 3277.

Table 1: Genotypic effect on shoot regeneration and *in vitro* rooting

Genotype	Cultured cotyledons	Number of shoots	%	Rooted plants	%
HA 89	120	13	10.1	10	80
3294	120	18	15.0	5	30
3277	120	0	0.0	0	0

Cotyledons exhibited a confined regenerable area, i.e., embryonic axes, but with a difference between the two genotypes: HA89 regenerated only one shoot per cotyledon while 3294 up to five. *In vitro* rooting was more difficult for 3294 than HA89

but in both cases shoot micrografting solve any rooting problem with the achievement of complete fertile plants.

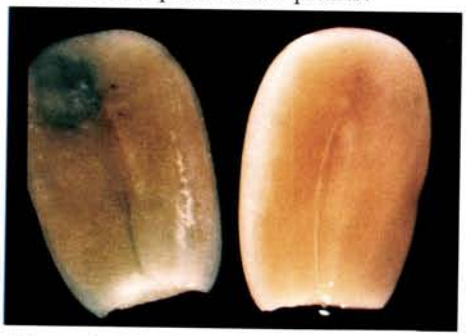

Figure 3: GUS-like activity on sunflower cotyledons before (left) and after (right) thermal inactivation at 55°C for 2 hours.

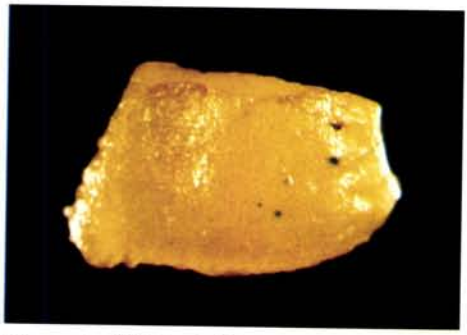

Figure 4: GUS foci following bombardment without the focusing device.

The bacterial GUS gene *uidA* was used as reporter gene to study the effect of the focusing device for DNA delivery into the cotyledons of sunflower. GUS histochemical assay performed according to standard protocol (Jefferson *et al.*, 1987) resulted in a uniform blue staining of the whole cotyledon in controls. In Figure 3 the endogenous GUS activity in cotyledon of sunflower is shown. GUS intrinsic activity was completely eliminated after thermal inactivation at 55°C for 2 hours, whereas shorter periods were not sufficient for removing the GUS background.

Table 2: Analysis of variance on the spot data obtained with the focusing device

Source of variation	Df	MS
Genotype	2	106.72**
Distance	1	22.01
Gold	1	63.92*
Ring	2	101.83**
Genotype x Distance	2	5.20
Genotype x Gold	2	71.35*
Genotype x Ring	4	46.34*
Distance x Gold	1	4.42
Distance x Ring	2	6.33
Gold x Ring	2	14.83
Genotype x Distance x Gold	2	5.94
Genotype x Distance x Ring	4	2.83
Genotype x Gold x Ring	4	16.37
Distance x Gold x Ring	2	2.09
Genotype x Distance x Gold x Ring (Error)	4	7.77
Total	35	

None transformation events were observed after particle bombardment without the focusing device of more than one thousand cotyledons. Only in one experiment, restorer line 3277 when bombarded with 1.6 µm gold particle and with the target distance of 120 mm, exhibited a few number of blue spots (Figure 4). These data confirm the difficulty to penetrate the cotyledon further than epidermis (Laparra *et al.*, 1995) as only the denser gold particles (1.6 µm) were able to penetrate into deeper cell layers to give rise to transformation events.

On the other hand bombardment with the focusing device intensified the impact of the blast on target tissue and gave rise to different results according to the different variables considered in this study. The factorial analysis is reported in Table 2; significant differences were detected among genotypes (G), gold particle diameter (P), distance from the target center (D) and the interactions G x P and G x D. Genotype 3277 showed the best transient expression with a mean number of 6 spots per cotyledon.

Table 3: Mean number of the spots in relation to the variety and gold particle size

	Genotype			
Gold particle size	L3277	L3294	HA89	Mean
1.0 µm	2.69	2.19	1.30	2.06
1.6 µm	10.96	1.57	1.65	4.72
Mean*	6.83 a	1.87 b	1.47 b	3.39

* Means with the same letter do not differ at P<0.01

In Table 3 data reported the comparison between the two sizes of gold used in this study; 1.6 µm gold particle gave the best results in each combination of parameters used, only in genotype 3294 there was a slight difference in favor of 1.0 µm gold particle.

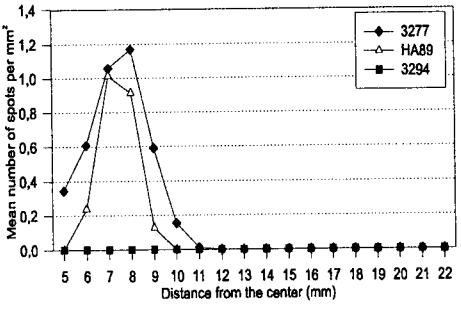

Figure 5: Distribution of blue spots after bombardment with a target distance of 120 mm.

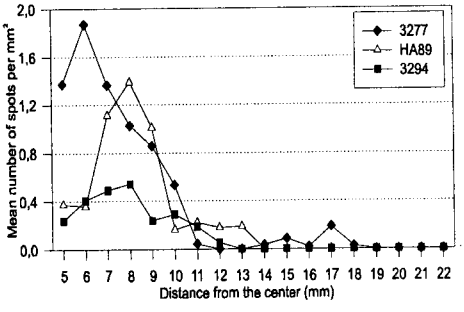

Figure 6: Distribution of blue spots after bombardment with a target distance of 150 mm.

Significant genotype x gold interaction is due to the high value of 3277 line when associated to gold particles of 1.6 µm. The distribution of transient expression in relation to distance from the center is represented graphically in Figures 5, 6 for target distance of 120 and 150 mm, respectively. For each target distance gen-

otype 3277 significantly differed from the other two genotypes, approximately 90% of the transformation events were concentrated in the cotyledons distributed in the second concentric circle in a consistent pattern of GUS foci defined in a ring of approx. 4 mm (Figure 7). Increasing the target distance from 120 mm to 150 mm there was a slight shifting of the ring of maximum transient expression between 5 and 6 mm diameter from the center (Figures 5, 6). The center of the target area, i. e., the first concentric circle, shows only weak transformation probably due to cell damage as focusing device intensified the impact of gold particles on the target tissue.

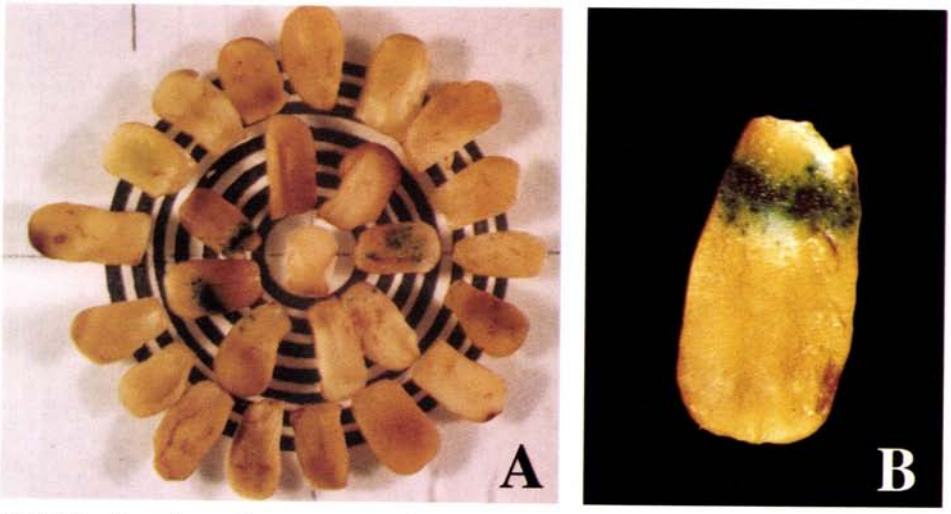

Figure 7: Transformation events after bombardment with the focusing device: a) dispersal of blue spots in the three concentric circles of cotyledons; b) particular of dispersal blue spots in one cotyledon of the second circle.

CONCLUSIONS

The regeneration system used in this work confirms that sunflower regeneration is genotype-dependent and that some problems as rooting ability or premature flowering are problems that need still a solution. In this paper we focused our attention on commercial sunflower genotypes viewing for a possible genetic transformation and despite our regeneration rate was considerately lower than reported in other papers with regeneration of about 70% or more (Fiore *et al.*, 1997) the cotyledon system confirms many favorable features for sunflower regeneration: wide application to many genotypes, consistency, availability of plant material all year long, no need for extra generation of sunflower as with immature embryos. In any case this kind of explant exhibited a very confined area with regeneration ability close to the embryonic axes. In such situation, the standard configuration of PDS1000/He is not well suited for transformation of sunflower cotyledons because

the unpredictable and scattered distribution of gold particles and the difficulty to penetrate the epidermis. The focusing device enabled to confine and concentrate the transformation events in a 4 mm ring-shaped area at 5-6 mm from the target center. On the basis of these data an appropriate arrangement of the cotyledons could improve dramatically the probability to transform competent cells for plant regeneration.

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MEDIO ENFOCANTE PARA LA TRANSFORMACION BIOLISTICA DE COTILEDONES DEL GIRASOL (*Helianthus annuus* L.)

RESUMEN

En pocos ultimos años teniamos muchos ensayos para obtener las transformaciones geneticas de girasol por la utilizacion de diversos tejidos de meristemas: punta de vastago, hipocotil, cotiledon de plantones jovenes y de embriones no madurados. Las plantas transgeneticas fueron obtenidas en pocos casos, pero la eficacia y la repetibilidad eran bajas. El problema comun era la dificultad de establecer un sistema eficaz de regeneracion, que sea liado estrictamente con el sistema de transformacion. Algunos reportes indicaron los cotiledones de girasol como explantados eficaces para la regeneracion de plantas. Entretanto, siendo diferente de otros tejidos del girasol, ese material no se transforma tan facilmente por *Agrobacterium*. El bombardeo de particulas, como modo importante de transformacion en plantas con monocotiledones, puede ser una alternativa interesante para cotiledones de girasol. El sistema de entrega de particulas, que esta vendendose actualmente, tiene la capacidad enfocante reducida, mientras los cotiledones mostraron el campo reducido de regeneracion, que era limitado a los ejes embrionales. Para superar este problema, fue creado el medio enfocante de acero inoxidable, que puede ser aplicado en combinacion con el aparato Du Pont/BioRad PDS1000/He. El dispositivo ha sido testado, a base de la serie de expresion de β-glucuronidase, a los cotiledones de girasol de la linea HA89 y de otras lineas experimentales. Para evitar la actividad semejante a la GUS-actividad endogena, fue cambiado el tampon y la reaccion previa ha sido efectuada a 56°C con los explantados y el tampon equilibrados previamente durante 90 minutos. El dispositivo ha facilitado que mas de 90% eventos de transformacion ocurran dentro de la superficie anular remota de 5-6 mm del centro apropiado. Cuando el dispositivo no era utilizado, la transformacion era baja y ocurria sobre la superficie mucho mas grande. A base de estos datos hemos concluido que la disposicion correspondiente de cotiledones pueda mejorar considerablemente la probabilidad de transformacion de particulas capaces para la regeneracion; eso fue aun mas visible en la utilizacion de los genotipos de girasol que manifestaron alta frecuencia de regeneracion de vastagos.

UN DISPOSITIF DE MISE AU POINT POUR LA TRANSFORMATION BIOLISTIQUE DU COTYLÉDON DE TOURNESOL (*Helianthus annuus* L.)

RÉSUMÉ

Ces dernières années, de nombreuses tentatives ont été faites our obtenir la transformation génétique du tournesol en utilisant différents méristèmes: pousse de germe, hypocotyles, cotylédons de jeunes plants et embryons immatures. Des plantes transgéniques ont été obtenues dans quelques cas mais le taux d'efficacité et de reproductibilité était bas. Le problème commun était la difficulté d'établir un système de regénération efficace lié strictement au système de transformation. Plusieurs rapports indiquent le cotylédon du tournesol comme explant efficace pour la regénération de la plante. Cependant, à la diférence d'autres tissus de tournesol, ce matériel ne se transforme pas si fac-

ilement au moyen de *l'Agrobacterium*. Le bombardement des particules est un outil important pour la transformation des plantes monocotylédones et pourrait être une alternative intéressante dans le cas du cotylédon du tournesol. Le système de livraison des particules qui se trouve actuellement dans le commerce a une possibilité réduite de mise au point alors que les cotylédons ont montré un champ de régénération réduit limité aux axes embryonnaires. Pour surmonter ce problème, un dispositif de mise au point en acier inoxydable a été créé qui peut être utilisé avec l'appareil DuPont/BioRad PDS1000/He. Le dispositif a été testé sur une série d'expression ß-glucuronidase portant sur des cotylédons de tournesol, cv. HA89 et des lignes expérimentales. Pour éviter une activité semblable à celle de l'endogène GUS, le tampon a été changé et la réaction s'est faite a 56°C après que les explants et le tampon ont été équilibrés pendant 90 minutes. Le dispositif a permis que plus de 90% desévénements de transformation soient confinés et concentrés sur une surface de forme circulaire à 5-6 mm du centre visé. Quand le dispositif n'était pas utilisé, le phénomène de transformation était très bas et dispersé sur une surface beaucoup plus grande. Ces données nous permettent de conclure qu'une distribution appropriée des cotylédons peut améliorer considérablement la probabilité de la transformation des cellules aptes è la regénération; ceci pourrait être encore plus évident si on utilisait les génotypes de tournesol ayant montré une haute fréquence de regénération des pousses.