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GENETIC TRANSFORMATION OF THE GENUS *Helianthus* BY *Agrobacterium tumefaciens* .

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ABSTRACT

A genetic transformation method has been developed in *H. annuus* and in the interspecific hybrid *H. annuus* x *H. tuberosus* using *Agrobacterium tumefaciens* . Cotyledonary explants of *H. annuus* and leaf explants of *H. annuus* x *H. tuberosus* were inoculated with *A. tumefaciens* carrying a disarmed *Ti* plasmid containing the Cauliflower Mosaic Virus (CaMV) 35S-GUS fusion gene with the nopaline sinthase (NOS) neomycin phosphotransferase II (NPT II) gene. On selection medium containing 25 mg l^{-1} of kanamycin, the inoculated leaf explants of the interspecific hybrid formed meristematic centers with buds and embryo-like structures, that successively developed into putative transformed shoots, on medium without growth regulators. Under optimal conditions the highest transformation frequency was 4.7 percent. Cotyledonary explants of *H. annuus* formed meristematic centers with buds on medium containing kinetin, IAA and 25 mg l^{-1} of kanamycin. Successively, at low frequencies (2.3%) putative transformed shoots were obtained on a medium without growth regulators.
Histochemical staining for the GUS activity provided evidence for transformation in different tissues and organs of regenerated plants. Integration of foreign DNA into genomic *H. annuus* x *H. tuberosus* DNA was demonstred for NPT II gene by polymerase chain reaction (PCR) and DNA slot blot hybridization.

INTRODUCTION

The genus *Helianthus* includes a number of species which have a great potential value as source of oil, protein and energy production. Consequently, there is interest in the development of genetic manipulation systems useful for the transfer of novel traits into the crops.
It has been found that *H. annuus* x *H. tuberosus* leaf explants (Pugliesi et al., 1992) and *H. annuus* cotyledonary explants (Pugliesi et al., 1991) have a high potential for efficient plant regeneration. Notwithstanding that *H. annuus* and *H. tuberosus* are natural host for *Agrobacterium* Spp. (Braun, 1941), there are few reports describing the transformation of the genus *Helianthus* employing the *Agrobacterium* -mediated technique (Everett et al., 1987; Schrammeijer et al., 1990).
In this paper we describe a procedure for a *Agrobacterium* -mediated genetic transformation for stable introduction of two marker genes into plant regenerated from the interspecific hybrid *H. annuus* x *H. tuberosus* , and into shoots regenerated from cotyledonary explants of *H. annuus.*

MATERIALS AND METHODS

Plant material and tissue culture conditions.
Leaves, of the interspecific hybrid *H. annuus* x *H. tuberosus* , obtained from regenerated plants, were cultured on regeneration media based on MS basal composition (Murashige and Skoog, 1962), 3% sucrose, 0,8% Bactoagar (Oxoid, Ltd England), containing 0.2 mg l^{-1} BAP (Benzylaminopuryne) and 0.2 mg l^{-1} NAA

(1-naphthaleneacetic acid) (Pugliesi et al., 1992). Cotyledonary explants of *H. annuus* inbred line R857 were sterilized and succesively cultured on MS basal composition, 3% sucrose, 0.8% Bactoagar (Oxoid, Ltd England) containining 4 mg l^{-1} kinetin and 0.4 mg l^{-1} IAA (3-indolacetic acid) (Pugliesi et al., 1991).
The pH of media was adjusted to 5.7 with 1M NaOH or 1M HCl prior to autoclaving at 1.1 kg cm^2 pressure and 120 °C for 15 min. Antibiotics were filter sterilized and added to media, after autoclaving.

Agrobacterium strain.

The inoculation of the leaves explants was carried out using the disarmed *Agrobacterium tumefaciens* strain GV2260 (Debleare et al., 1985), containing the *Ti* plasmid with the Cauliflower Mosaic Virus (CaMV) 35S GUS fusion gene and the nopaline synthase (NOS) neomycin phosphotransferase II (NPTII) gene conferring the kanamycin resistance. To prevent the expression of the GUS gene in *Agrobacterium* a portable intron derived from the potato ST-LS1 gene was introduced into the GUS gene (Vancanneyt et al., 1990).
Agrobacterium was cultured in YEB medium, pH 7.2 (Zaenen et al., 1974) containing 150 mg l^{-1} rifampicin (Sigma, St Louis) and 150 mg l^{-1} kanamycin sulphate and grown overnight at 25 ±1 °C on rotary shaker (100 rpm).

Co-coltivation of *A. tumefaciens* and leaf disk of *H. annuus* x *H. tuberosus.*

Following the application of the leaf disk transformation methods (Horsh et al., 1985), leaf explants were co-cultured for 1, 2, 3, 5 and 7 days on regeneration medium. After co-cultivation the leaf explants were transferred into the same medium containing 25 mg l^{-1} kanamycin and 200 mg l^{-1} cefotaxime (claforam, Roussel Maestretti) to inhibit bacterial growth.
The frequency of adventitious buds and embryo like structures regeneration was determined 30-35 days after infection. The transfer to fresh selection medium was repeated every three weeks to prevent multiplication of *Agrobacterium.* Successively, for normal development, clusters of buds were transferred to MS medium without growth regulators containing the previously decribed concentration of kanamycin and cefotaxime. Elongated shoots (10-15 mm) were transferred to root inducing medium (MS salts and vitamins with reduced sucrose concentration (1.5%), 200 mg l^{-1} cefotaxime and 25 mg l^{-1} kanamycin). The effective transformation frequency were calculated as number of explants producing transformed plants (based on GUS activity) on total of the cultured explants. After rooting, the putative transgenic plantlets were tranferred to the greenhouse into pots containing a mixture of vermiculite, peat and soil covered with plastic bags to mantain high umdity (70-80%) and grown until maturity.

Co-coltivation of *A tumefaciens* and cotyledonary explants of *H.annuus.*

After the dissection, from 2 day old germinated seeds (Pugliesi et al., 1991), cotyledonary explants were soaked in a bacterial suspension and transferred in co-coltivation medium containing Kinetin (4 mg l^{-1}), IAA (0.4 mg l^{-1}). The time of co-cultivation was 3 days in Experiment 1, Experiment 2, Experiments 4 and Experiments 5; 5 days in Experiments 3.
Cotyledons were precultured for two days in regeneration medium withouth kanamicin and then transferred in co-cultivation medium in Experiments 4 and Experiments 5. The feeder layer (suspension culture of *Medicago sativa*) was utilized in the Experiment 3 and Experiment 5.
After co-coltivation the cotyledonary explants were transferred in the same medium containing 200 mg l^{-1} cefotaxime and 25 mgl^{-1} of kanamycin. Four -six explants were placed on the medium.
The frequency of adventitious buds formation was determined three weeks after infection. Successively cluster of buds were transferred in medium without growth regulators with the same concentration of kanamycin and cefotaxime. The transfer into the fresh selection medium was repeated every three weeks.

Enzime expression assays.

Kanamycin resistance was tested by the ability of leaf tissue of individual putative transformed plants of *H. annuus* x *H. tuberosus* , to produce callus and to regenerate shoots on regeneration medium containing 25 mg l^{-1} kanamycin. Leaf sections from non transformed plants were used as control. Shoots formation was scored after four-five week. Elongated shoots from this material were rooted on medium with kanamycin and analyzed for GUS activity.

The GUS activity was assayed, by histochemical reaction (Jefferson, 1987), in callus and in different tissue of transformed plants of *H. annuus* x *H. tuberosus* and transformed shoots of *H. annuus* .

DNA analisys

Total genomic DNA was extracted from young expanded leaf tissue (250-1000 mg) of putative transformed plants of *H. annuus* x *H. tuberosus* and untransformed control using the procedure described by Dellaporta et al. (1983). For detection of specific DNA sequences, the polymerase chain reaction (PCR), useful as a rapid check for transformation (Lassner et al, 1989), was performed according to manufacturers instructions using Perking Elmer Amplitaq Recombinant Taq DNA polymerase.

The NPTII primers TTC TTT TTG TCA AGA CCG ACC T and TTC GTC CAG ATC ATC CT border a 344 bp DNA fragments from the NPTII gene. Reaction were performed using approximately 100 µg plant DNA in 50 µl volumes. Samples were heated to 96°C 10 min, followed by 30 cycles of 96 °C 1 min, 55 °C 1min, 74 °C 2 min with a final extension step of 74 °C 10 min. Amplified samples were electrophoresed on a 10 g l^{-1} agarose gel. DNA slot blot was performed according to Geri et al., (1992). The filter were hybridized with NPTII fragment ^{32}P labelled and exposed to MP films (Amersham).

RESULTS

Co-cultivation of *Agrobacterium* and leaf explants of *H. annuus* x *H. tuberosus* :production of transformed plants.

Preliminary experiments had established that *H. annuus* x *H. tuberosus* leaf tissue is higly sensitive to kanamycin. Therefore, leaf disk explants were cultured in medium A supplemented with varying levels of kanamycin and scored for callus and bud induction after 28 days of culture. High production of callus and shoots was observed in absence or at low levels of kanamycin (2-8 mg l^{-1}). 10-15 mg l^{-1} kanamycin induced very poor callus formation and few regenerated shoots which, after 40-45 days of culture, resulted in no further growth, with yellowing and final death of the tissue. Callus formation and shoot induction was completely inhibited by the increased kanamycin concentration tested. Bleaching was complete at 25 mg l^{-1} within four weeks and selection procedure was performed by this kanamycin concentration.

The optimal length of cocultivation time for leaf explants of *H. annuus* x *H. tuberosus* was analysed (Table 1). The un-inoculated control leaves did not produce shoots in presence of kanamycin (25 mg l^{-1}), while regenerating shoots were obtained with high frequency (73.7 %) in absence of the antibiotic.

One day of co-cultivation was not sufficient for the achievement of transgenic plants; after 4-5 weeks, an efficient production of buds (24.8%) was observed but after subsequent subcultures in fresh selective medium they resulted in no further development. Using 2 and 3 days of cocultivation the same frequency of bud differentiation (20-30 %) was obtained, however with 3 days, the effective transformation frequency was highest (4.7%). Five or seven days induced the highest bud differentation (50-52 %) but the successive subculture on fresh selection medium caused the development of many white non trasformed shoots and the effective tranformation frequency was reduced.

Transformed shoots, with normal dark green colour and vigorous growth, were transplanted into rooting

Table 1.Transformation frequency in leaf explants of the interspecific hybrids *H. annuus x H. tuberosus* obtained after different days of co-cultivation with *A. tumefaciens* strain GV2260.

days of cocultivation	Total No. of inoculated explants	No of explants producing buds	Frequency of buds differentation (%)	No. of explants producing TP [a]	Transformation frequency (%)
1	125	31	24.8	0	0
2	375	36	27.5	11	2.9
3	129	83	22.2	6	4.7
5	123	64	52.0	5	4.1
7	125	63	50.4	4	3.2
CONTROL (- Km)	80	59	73.7	-	-
CONTROL (+ km)	60	0	0	0	0

[a] TP = transgenic plants with positive GUS activity

medium. Chimeric shoots with white sectors on the leaves were descarded. Rooted plantlets were then transferred into the greenhouse and grown to maturity. These plants were analyzed for kanamycin resistance, GUS activity and used for DNA isolation.

Co-coltivation of *Agrobacterium* and cotyledon explants of *H. annuus* : production of transformed shoots

Time of co-cultivation, utilization of precultured cotyledons and feeder layer were tested on the inbred line R857 of *H. annuus* co-cultivated with *A. tumefaciens* (Table 2). Buds differentiation from the cut edge of cotyledon explants was observed after three weeks of culturing in regeneration medium. The succesive transplanting of cluster buds in the same medium withouth growth regulators induced, after eigth -twelve weeks, the development of transformed shoots (positive to GUS activity). The transformation frequency was higher in experiments 1 and 3 (2.3%) (Table 2).

Assay for kanamycin resistence

Leaf explants from several indipendent *H.annuus* x *H.tuberosus* transformed plants were tested for their ability to grown and differentiate shoots on regeneration medium with kanamycin (25 mg l^{-1}). Control leaf

Table 2. Transformation frequency of the *H. annuus* inbred line R-857 obtained by culturing cotyledonary explants with *A. tumefaciens* , strain GV2260.

EXPERIMENTS	Total No. of inoculated explants	No of explants producing buds	Frequency of buds differentation (%)	No. of explants producing TS [a]	Transformation frequency (%)
1	425	144	33.9	10	2.3
2	111	26	23.4	2	1.8
3	385	129	33.5	9	2.3
4	106	20	18.9	1	0.9
5	100	15	15	0	-
CONTROL (-km)	60	43	71.6	-	-
CONTROL (+km)	60	-	-	-	-

[a] Ts = transgenic shoots with positive GUS activity.

pieces on kanamycin completely bleached after four-five weeks. Leaf explants from putative transformed plants remained green and showed at high frequency meristematic centers with differentiating buds (Table 3). After 4-5 weeks from clusters of buds transferring on medium without growth regulators and containing the same concentration of kanamycin, elongate shoots were observed. The shoots were rooted and the plantlets successively assayed for GUS activity. All the analysed plants showed dark blue colour in leaves and stems (Table 3).

GUS expression assays

Histochemical localization of β-glucoronidase were performed in callus, buds, embryo like structures and in different tissues and organs of putative transformed plants of *H. annuus* x *H. tuberosus* and in putative transformed shoots of *H. annuus* . Wide variation in β-glucoronidase activity was observed. Callus and differentiated buds, generally expressed high GUS activity. In a few cases the activity was localized to limitaded sectors indicating possible chimeric conditions. In young leaves of *H. annuus* and in sections of fully expanded mature leaves of *H. annuus* x *H. tuberosus* , GUS activity was observed in the vascular bundles and in the parenchymatic cells. All the cells of young stem sections of *H. annuus* x *H. tuberosus* showed strong GUS activity but roots, and flowers showed only weak blue color. The reproductive organ

Table 3. Regeneration from the control and from different transgenic plants of *H. annuus* x *H. tuberosus* on a medium with 25 mg l^{-1} of kanamycin and β-glucoronidase (GUS) activity of regenerated plants.

Plant	% regeneration on kamamycin	β-Glucoronidase (GUS) activity
CONTROL	0	-
A-2	100.0	+
A-6	33.3	+
A-14	84.6	+
A-15	80.0	+
A-16	60.0	+
B-1	66.7	+
C-1	54.5	+

sections of the interspecific hybrid (tuberous roots) exhibited dark blue color in the epidermal and parenchymatic cells. Small capitula of transformed shoots of *H. annuus* showed the GUS activity on the bractes.

DNA analysis

PCR amplification of the NPTII fragments was performed in putative transformed plants of *H. annuus* x *H. tuberosus*. Non trasformed control plant displayed neither amplified fragments (Fig. 1 Lane 5). DNA from putative transformed plants (Fig. 1 Lane 2, 3, 4) and transformed plant of *Nicotiana tabacum* (Fig. 1 Lane 6) show the presence of NPTII amplified fragments (344 bp).

DNA slot-blots from transformed plant hibridized with the probe NPTII fragment ^{32}P labelled show a positive signal, (Fig. 2, T1-T9) comparable with slot from transformed tissue of *Nicotiatiana tabacum* (positive control) (Fig 2., *Nt* +). No signal has been observed in non transformed plants (negative control) (Fig. 2, C-).

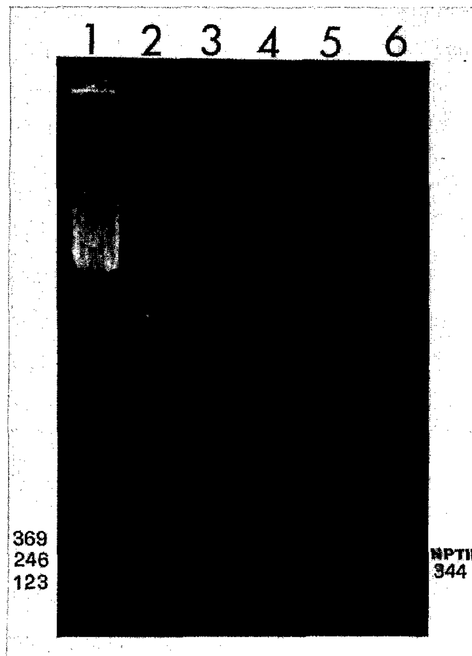

Fig. 1

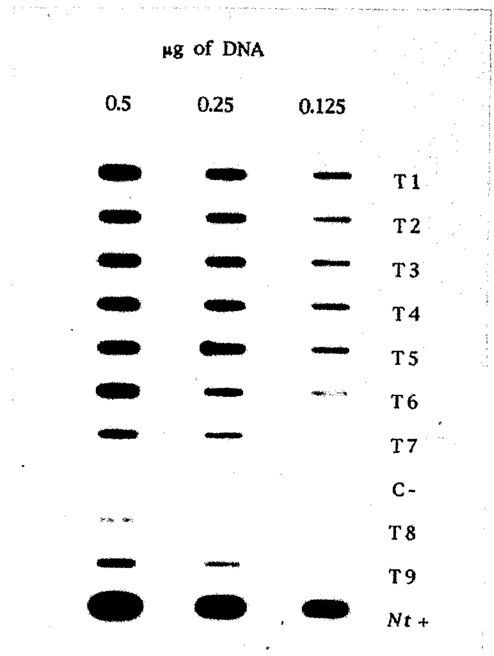

Fig. 2

Fig. 1. 1,0% agarose gel of polymerase chain reaction (PCR) amplified fragments, showing 344 bp neomycin phosphotransferase II (NPTII) fragment. Molecular weight marker123 bp DNA Ladder (BRL) (Lane 1); DNA from transformed plants (Lane 2,3,4); DNA from non transformed plant (negative control) (Lane 5); DNA from trasformed *N. tabacum* (positive control) (Lane 6).

Fig 2. Autoradiography of DNA slot blot hybridized with NPTII fragment ^{32}P labelled. DNA from transformed plants of *H. annuus* x *H. tuberosus* (T1-T9), from non transformed plant of *H. annuus* x *H. tuberosus* (C-, negative control) and from transformed *N. tabacum* (*Nt* +, positive control).

DISCUSSION

Using an *Agrobacterium tumefaciens* binary vector we have shown the simultaneous transfer of two marker genes to leaf cells of the interspecific hybrid *H. annuus* x *H. tuberosus* and in a inbred line of *H. annuus* . In the interspecific hybrids the transformed cells were regenerated to give rooted shoots on medium containing kanamycin. The rooted plants expressed β-glucoronidase activity showing the transfer and integration of an unselected marker gene.

In *H. annuus* transformed shoots were obtained but the premature flowering observed had negative influnce on rooting.

The transformation frequency is a function of many factors including genotype, the strain of the *Agrobacterium* , the selectionable marker, regeneration capacity of the material, co-cultivation time. In the studied material a major problem encoutered is the occurrence of many "escapes". As shown in table 1 and

table 2, on selective medium, high production of differentiated buds (16-52%) was obtained, however only 1-5% of the initial explants were capable to produce transgenic plants (*H. annuus x H. tuberosus*) or transformed shoots (*H. annuus*).

Staining for GUS activity evidentiated differences in colour intensity depending on the organ and tissue tested. Similar differences in gene expression were observed in transgenic plants of other species (Stiekema et al., 1988; Dong et al., 1991). Integration in differential chromosomal positions, physiological or developmental stages of the individual transgenic plants migth influence the level of the expression of the introduced gene(s) (Stiekema et al., 1988).

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