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Carrot (*Daucus carota*) hypocotyl transformation using *Agrobacterium tumefaciens*

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Summary *Daucus carota* hypocotyl sections were transformed with *Agrobacterium tumefaciens* LBA4404 containing CaMV 35S promoter, ß-glucuronidase coding sequence and the nopaline synthase (Nos) poly adenylation sequences in Bin 19. Sliced sterile seedling hypocotyl segments were preincubated for 2 days, co-cultivated with *Agrobacterium* for an additional 2 days, and then transferred to medium containing 100ug/ml of kanamycin and 400ug/ml carbenicillin. In 6 weeks kanamycin resistant calli were obtained in 5.8% of the explants from one variety. Calli were subcultured on solid medium, and in 4 weeks introduced into suspension culture. NPTII and Southern blot analysis confirmed that three selected lines were transformed with 1-3 copies of the GUSII construction. GUS activity in transformants was 5 to 250 fold over background.

Keywords: *Agrobacterium*- Somatic Embryogenesis- Electroporation- ß-Glucuronidase

Abbreviations: NPT II neomycin phosphotransferase II; Nos nopaline synthase; GUS ß-glucuronidase; CaMV cauliflower mosaic virus; 2,4-D 2,4 dichlorophenoxyacetic acid; PMSF phenylmethylsulfonyl fluoride

Introduction

Somatic embryogenesis in carrot has been used as a model system of plant development for over 20 years (Steward et al. 1964). Upon removal of 2,4-D, formation of globular, heart and torpedo stage somatic embryos results. Morphologically normal root, shoot and cotyledonary structures later arise and can mature into normal plants (Ammirato 1983; 1987). Employing carrot somatic embryogenesis, developmentally regulated mRNAs and proteins have been described (Sung and Okimoto 1981; Choi et al. 1987; Wilde et al. 1988).

While traditional methods of gene transfer have been used to study the developmental regulation of seed storage proteins (Beachy et al. 1985; Sengupta-Gopalan et al. 1985), the dissection of immature petunia or tobacco embryos to study less prevalent gene products is difficult. The isolation of large quantities of staged carrot somatic embryos is simple and provides an advantage over the use of in vivo grown embryos. Therefore, a transformable carrot somatic embryogensis system would facilitate the study of gene expression during development.

Earlier reports of carrot transformation have used mature tap roots or 8 week old suspension cells (Cardarelli et al. 1985; Ryder et al. 1985; Van Sluys et al. 1987; Scott and Draper 1987). The latter method requires a feeder layer to support the growth and proliferation of the transformants. In addition, the cell culture must be 8-10 weeks from *in vitro* initiation when treated with *Agrobacterium*.

Here we report a simple and reliable method for carrot transformation and somatic embryogenesis. Freshly germinated carrot hypocotyls are sliced and precultured for 2 days on medium with 2,4-D, followed by *Agrobacterium* co-cultivation for an additional 2 days. The tissue is then subcultured on kanamycin and carbenicillin. Within 6 weeks kanamycin resistant calli can be recovered and subcultured. The callus is then established in suspension culture. Somatic embryogenesis of the transformed cells can be induced by lowering the cell density and growing in the absence of 2,4-D.

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Materials and methods

Plant Materials. Carrot seeds: Danver's 126, Chantenary Red Cored, Scarlet Nantes (Northrup King Seed Co.) and Imperator 58 (Southland Garden Seed) were purchased in a local Safeway market. Seeds were disinfected with 10% Clorox with 1 drop of Tween 20 /500 ml. After 10 minutes the seeds were washed four times in sterile deionized water and then placed on MSr medium (1/2 strength Murashige and Skoog (1962) macro salts, 1X microsalts, 2g/L sucrose, 8g/L glucose, MS vitamins with an additional 0.4mg/L thiamine and 8g/L Difco Bactoagar). One week old carrot hypocotyls were sliced into sections of 10-15mm and placed on 2B5 medium (B5 medium of Gamborg et al. 1968 with 9uM 2,4-D and 8g/L Difco Bactoagar).

A 2 day hypocotyl preculture was allowed in the dark at 20-22°, however transformants have been recovered with a preculture of 5 days. No kanamycin resistant calli were obtained without a preculture period before *Agrobacterium* treatment.

Nicotiana tabacum cv. xanthi seeds were obtained from Dr. T. McKnight. Sterilization and in vitro germination were as described for carrot seeds.

Bacterial Strains and Plasmids. Agrobacterium tumefaciens LBA4404 containing either pRGUSII (Ulian et al. 1988), pBI101.1 or pBI121.1 (Jefferson 1987) were grown overnight in 50ml of M9 salts (Maniatis et al. 1982). The cells were centrifuged at 500xg for 5 minutes and resuspended in 50ml of liquid 2B5 medium.

Plasmids pBI101.1, pBI121.1 and pBI221.1 were kindly provided by Richard Jefferson and pRGUSII by Terry Thomas. All contain the ß-glucuronidase gene either promoter-less (pBI101.1) or downstream from the CaMV 35S promoter. The NosCAT and 35SCAT plasmids were kindly provided by Joe Ecker. Plasmids were prepared using $CsCl_2$ as described (Maniatis et al. 1982).

Co-cultivation of *A. tumefaciens* with carrot tissues. Two day precultured hypocotyls were placed in the bacterial solution for 5 minutes, dried briefly, returned to 2B5 medium and grown at 20-22°C for 2-3 days. After the *Agrobacteria* were evident upon the explants, the hypocotyls were transferred to 2B5 medium containing 100ug/ml of kanamycin and 400ug/ml of carbenicillin. Cultures were incubated in the dark at 20-22°C.

After 6 weeks, kanamycin resistant calli were observed up to 1cm in diameter. To eliminate the possibility of nontransformed callus remaining alive, subsequent subcultures always contained the transformation selective agent kanamycin and carbenicillin to prevent bacterial growth. Under these conditions, all calli growing after the second subculture were resistant to kanamycin in the subsequent transfers.

Following the second transfer, calli were introduced into suspension culture (2B5 less agar) with the above mentioned levels of kanamycin and carbenicillin. Growing conditions were 20-22°C in the dark at 150rpm. Erlenmeyer flasks (125ml) contained 40ml of medium and an initial cell density of not more than 1g fresh weight per flask. Cells were later subcultured on fresh liquid medium containing kanamycin with an initial cell density of 1×10^5 cells/ml.

Protoplasts were isolated from suspension cultures and electroporations done according to Thomas et al. (1989). Electroporation conditions corresponded to 1.5×10^6/ml protoplasts resuspended in 70mM NaCl, 1mM KH_2PO_4 (pH 7) and 4mM $CaCl_2$. A single exponential pulse of 350V at 1050uF was applied, the cells cooled on ice for 5 minutes, and protoplast medium then added. After 16 hours of culture in the dark at 22° the protoplasts were recovered and CAT assays done according to Gorman et al. (1982).

Somatic embryogenesis was initiated as described by Sung and Okimoto (1981). Embryogenesis of the transformed cells was not inhibited in the presence of both kanamycin and carbenicillin at the levels used earlier. After reaching the torpedo stage, the embryos were transferred to MSr solid medium containing 400ug/ml carbenicillin and 100ug/ml of kanamycin, where leaves and roots developed. Later plants were transferred to soil.

Nicotiana tabacum cv. xanthi leaf transformations were performed according to Horsch et al. (1985) without the use of a feeder layer.

Molecular analysis. NPT assays were according to Reiss et al. (1984) with the inclusion of 100ug/ml of PMSF. GUS assays were done according to Jefferson (1987) and quantitated using methyl umbelliferone standards on a Kontron spectrofluorometer. Protein determination was according to Bradford (1976).

DNA was extracted from carrot cultures according to DeRose (personal communication). Tissue was frozen, ground in liquid N_2 and placed in a 50ml Falcon tube. For 10g (fwt) of tissue, 30ml of CTAB buffer (1% hexadecyltrimethylammonium bromide (CTAB), 0.7M NaCl, 50mM Tris HCl (pH 8), 10mM EDTA, 100mM Na metabisulfate, 1% soluble polyvinyl pyrrolidone, 40mM dithiothreitol, 10ug/ml of proteinase K) at 55°C was added. Just after the tissue had thawed, 20ml of chloroform:isoamyl alcohol (24:1) was added and the solution mixed. The slurry was incubated at 55-60°C for 60 min, centrifuged (500xg 5 min), and the aquaeous phase reextracted with chloroform:isoamyl alcohol. The aqueous fraction was removed and solid ammonium acetate added to 2.5M. Once the salt was dissolved, the DNA was precipitated with 1 volume of isopropanol at 20-22°C, recovered by centrifugation and washed in 70% ethanol and 60mM Na acetate. The DNA was dissolved in sterile water and precipitated twice as an ammonium salt.

DNA concentrations were determined with Hoechst dye 33258 (Labarca and Paigen 1980). Aliquots of DNA (4-10ug) were restricted for 4 hours with BamH1 and EcoR1 (BRL) according to the manufacturer's directions with 0.5ug/reaction of heat denatured RNase A (Sigma). Samples were separated in a 1% agarose gel and then blotted overnight in 20X SSC onto Gene Screen Plus. After a 30 second UV treatment (Khandjian 1987), membranes were prehybridized overnight at 45°C in 50% formamide, 5X Denhardts, 5X SSC, 0.2% SDS, 50mM phosphate buffer pH 6.8, 100ug/ml yeast tRNA, 50ug/ml polyA (Pharmacia) and 10ug/ml polyC (Pharmacia).

A 2.1kb BamH1, EcoRI fragment from pBI221.1 containing the GUS-Nos poly adenylation sequence was isolated, gel purified and nick translated to 2 X 10^9 cpm/ug (Maniatis et al. 1982). The concentration of probe in the overnight hybridization was 10ng/ml. Following hybridization the membranes were washed at 60°C in 4X,2X,1X, and 0.1X SET (SET: 0.15M NaCl, 0.02M TrisHCl (pH 8), 0.002M EDTA and 0.5% SDS). Autoradiographs were developed after 4 days exposure at -70°C with a DuPont Cronex Quanta III intensifying screen.

Results

Regeneration and transformation.

The toxicity of kanamycin was determined using nontransformed carrot cell suspensions. As shown in Figure 1, kanamycin above 10ug/ml restricts, but does not prevent packed cell volume increase. It is during the subsequent subculture to fresh kanamycin containing medium (levels from 10-200ug/ml) that growth as measured by packed cell volume becomes completely inhibited (data not shown).

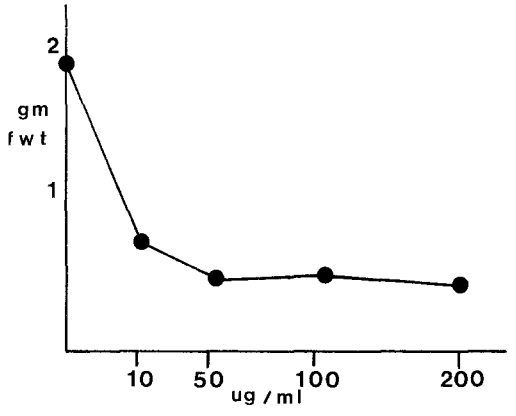

Fig 1. Kanamycin effect on packed cell volume of nontransformed carrot suspension cultures. Cells were subcultured after 14 days to fresh 2B5 medium at a cell density of 1 X 10^5 cells/ml and grown in kanamycin is indicated on the X axis. Measurements were taken 3 weeks after culture.

Following *Agrobacterium* co-cultivation, kanamycin selected and resistant calli were produced after 6 weeks of culture. Of the original calli obtained on kanamycin selection, 94% continued to grow on kanamycin containing medium after subculture. Transformants were then grown in suspension culture and later induced to undergo somatic embryogenesis. The embryos were easily regenerated into whole carrot plants as described earlier. Use of kanamycin in each subculture eliminated nontransformed cell growth. Tobacco transformants were also obtained with the pRGUSII construct using routine methods (Horsch et al. 1985).

Different carrot varieties were surveyed in order to determine whether transformation was variety specific. For these experiments either pRGUSII or pBI121.1 were used, both in LBA4404. As shown in Table 1, kanamycin resistant calli were obtained in 5.8% of the explants of Danvers 126 to 0.9% for Scarlet Nantes. For further work, Danvers 126 was used.

Table 1. Transformation rates among some carrot varieties using pRGUSII and pBI121.1*.

Variety	N°explants	N° km^r calli (%)
Danvers 126		
	140	8 (5.8%)
	* 124	7 (5.6%)
Imperator 58		
	104	4 (3.8%)
Chantenary Red Cored		
	94	2 (2.1%)
Scarlet Nantes		
	92	1 (0.9%)

Analysis of transformants.

After subculturing kanamycin resistant calli, NPTII assays were performed using several fast growing carrot lines and tobacco isolates transformed with pRGUSII. All carrot and tobacco transformants tested had demonstratable NPT II activity (Figure 2).

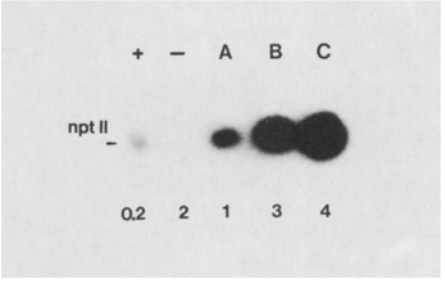

Fig. 2. Neomycin phosphotransferase (NPT II) gel assay of pRGUSII A tobacco (+), nontransformed carrot (-), and carrot pRGUSII A,B and C respectively. The relative protein loads are given below the figure.

A DNA probe from pBI221.1 (see Materials and Methods) was used to characterize the transformants. It was comprised of a portion of the GUS gene and included the Nos poly-A site.

When total genomic DNA from transformed cells was digested with EcoR1 and BamH1, blotted and hybridized with the above described probe, a characteristic 2.1kb band from the GUS Nos-poly A sequence was shown to hybridize. A second Nos-poly A sequence was expected to hybridize and appear at a secondary location, due to the Nos-poly-A site from the NPTII gene in Bin 19 (Jefferson, 1987). Assuming random insertion of the pRGUSII (derived from Bin 19), the resulting hybridization pattern should indicate other bands which correspond to the number of NPT genes (i.e. single or multiple copies) in the carrot genome.

DNA isolated from nontransformed and three independent pRGUSII transformants was then digested with EcoR1 and BamH1, blotted and probed with the above mentioned. In Figure 3, all three isolates contain the GUS Nos-poly A portion. Judging from the intensity of hybridization, pRGUSA and B appear as single insertions while pRGUSC may contain up to 3 copies.

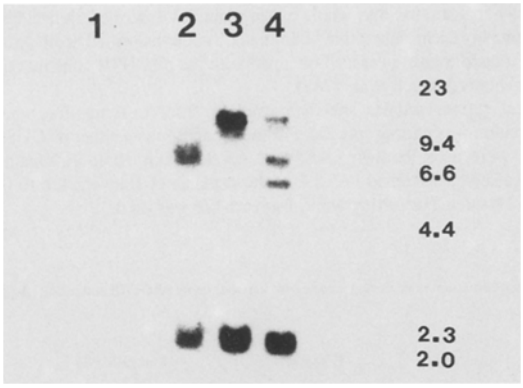

Fig. 3. Southern hybridization of the 2.1kb GUS NOS-poly A probe to DNA isolated from transformed and nontransformed carrot cells. DNA concentration in ug/lane was: lane 1, carrot nontransformed DNA 10ug; lane 2 carrot pRGUSII A 4ug; lane 3 carrot pRGUSII B 10ug, and lane 4 carrot pRGUSII C 10ug. Numbers on the side indicate migration of known standards in kb.

A lower level of GUS activity was observed in the pRGUSII as compared to the pBI121.1 transformed carrots. Tobacco pRGUSII transformants produced levels of GUS activity 10 times higher than their carrot counterparts. Nontransformed carrot cells and cells transformed with pBI101.1 (a promoterless GUS construct) had very low background activity (Table 2).

Table 2. GUS assay results on carrot and tobacco transformants. Assay conditions were according to Jefferson (1987).

SAMPLE	pmole MUG $min^{-1}mg^{-1}$protein
Carrot nontransformed	1.3
pBI101.1 carrot	1.8
pBI121.1 carrot	252.0
pRGUSII A carrot	11.1
pRGUSII B carrot	10.6
pRGUSII C carrot	12.3
Tobacco nontransformed	0.8
pRGUSII A tobacco	544.9
pRGUSII B tobacco	181.2
pRGUSII C tobacco	388.3

To test the ability of the CaMV 35S promoter to function in pRGUSII carrot transformants, transient expression assays were performed using the Nos and CaMV 35S promoters fused to the CAT reporter gene. These results indicate that nontransformed and transformed carrot cells recognize the CaMV 35S promoter to a similar extent.(Figure 4).

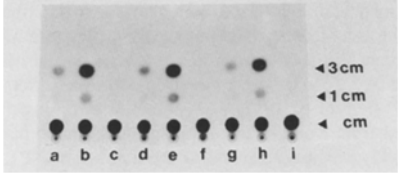

Fig. 4. CAT analysis of electroporated carrot protoplasts from pRGUSII C (a-c), pRGUSII A (d-f) and nontransformed (g-i). Electroporations were according to Thomas (et al. 1989). Cells were electroporated either without DNA (c,f,i), 25ug p35SCAT (b,e,h) or pNosCAT (a,d,g). CAT assays were done the following day. Migration of chloramphenicol (CM) the minor 1-acetyl chloramphenicol (1-CM) and the major reaction product 3-acetyl chloramphenicol (3-CM) are shown.

When nontransformed carrot cells were homogenized with pRGUSII transformed tobacco tissues, the GUS activity was similar to pRGUSII tobacco extracted alone (data not shown). The low expression of GUS activity in pRGUSII transformed carrots does not appear to result from endogenous inhibitors.

Discussion

Nearly 5.8% of carrot hypocotyls treated with *Agrobacterium tumefaciens* Bin 19 can be transformed. A preincubation period of at least 2 days was found to be essential. Carrot hypocotyl preculture may permit the accumulation of phenolic compounds that activate the *vir* genes of the Ti plasmid and stimulate transformation (Bolton et al. 1986).

Carrot hypocotyl transformation was variety dependent, Danvers 126 being the most susceptible. This is surprising as most domestic carrots originated from one of three closely related varieties selected in 1863 (Ammirato 1987).

Many plant transformation and regeneration methods use feeder layers of nontransformed cells (Charest et al. 1988; Horsch et al. 1985; Scott and Draper 1987). The use of feeder layers may provide trace amounts of nutrients and allow for better survival of the transformed cells. Danvers 126 suspension cells are at first growth inhibited by kanamycin (Fig. 1), later growth arrested after subculture. In this same variety, kanamycin selection without a feeder layer provides gradual and effective selection for transformants from hypocotyls. Kanamycin resistant carrot transformants were efficiently recovered and produced measurable levels of enzymatically active NPT II and GUS.

The explaination for pRGUSII expression being 10 fold lower in transformed carrot cells as compared to the pBI121.1 carrots or pRGUSII transformed tobacco cells remains unclear. Levels of GUS expression in pRGUSII transformed tobacco are similar to those reported for pBI121.1 in tobacco (Jefferson 1987).

Variation in gene expression from transformant to transformant has been observed when using the CaMV 35S promoter (Odell et al. 1987; Pierce et al. 1987). Differential transcriptional and/or translational efficiency from one species to the next could also account for this result (Dean et al. 1987; Kozak 1986). We have observed in transient protoplast electroporation experiments that the pRGUSII carrot transformants and nontransformed carrots recognize the CaMV 35S promoter. For this reason, the low level of GUS expression in pRGUSII carrot transformants could not only be due to variation from transformant to transformant, but also to other aspects of gene expression and regulation.

This article provides a simple method for the transformation of carrot hypocotyls with disarmed vectors using *Agrobacterium tumefaciens*. Callus, suspension culture and somatic embryogenesis can all be achieved in the presence of kanamycin selection. This transformation method coupled with the carrot somatic embryogenesis system provides yet another tool for investigating plant development.

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