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Transformation and regeneration of carrot (*Daucus carota* L.)

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Abstract

A protocol is presented for the efficient transformation of carrot (*Daucus carota* L. cv. Nantaise) by *Agrobacterium tumefaciens*. The binary vector contained the marker gene β-glucuronidase (GUS), driven by the 35S promoter of cauliflower mosaic virus, and the *nptII* gene, which confers kanamycin resistance. Highest T-DNA transfer rates were obtained by co-cultivating bacteria with hypocotyl segments of dark-grown seedlings on solidified B5 medium containing naphthaleneacetic acid and 6-benzylaminopurine. After 2 days, bacterial growth was stopped with antibiotics. Two weeks later, the explants were placed on agar containing the kanamycin derivate geneticin; antibiotic-resistant calli developed during the following 4 weeks. Suspension cultures were obtained from resistant calli and plants regenerated via somatic embryogenesis in liquid culture. The majority of plants were phenotypically normal and, depending on the *Agrobacterium* strain used, harbored single or multiple copies of the T-DNA. About equal levels of GUS activity were found in different organs of young plants up to 6 weeks after embryogenesis. In leaves of older plants, GUS activity was markedly reduced, whereas the activities in phloem and xylem parenchyma cells of developing tap roots were still high and fairly uniform. Thus, the 35S promoter may be a useful tool to drive the expression of transgenes in developing carrot storage roots.

Introduction

Carrot (*Daucus carota* L.), a member of the Umbellifereae, is a biennial plant grown for its edible tap root. It is one of the most important vegetables, with 13.5 million tonnes grown in 1991 for human consumption [5], representing 3% of world-wide vegetable production [12]. Under optimal conditions, 2–6 kg of roots can be harvested from 1 m^2 of soil after 90–120 days of growth [14].

Carrot tap roots are rich in vitamin A and fiber. On average, the mature storage organ is 88% water, 6–8% sugar, 1–2% fiber, 0.7–1.2% protein, 1% ash, and 0–3% fat. Total sugar is the sum of glucose, fructose, and sucrose [22]. The ratio between di- and monosaccharides is genetically determined and varies from cultivar to cultivar [6]. Starch grains were observed under the microscope and the amounts found were dependent on the physiological state of the plants [22,12].

Of commercial interest are plant lines with a high yield and a high sugar level for producing fuel alcohol or sugar, low levels of reducing sugars, and high resistance to diseases, insects and nematodes [22]. Furthermore, of high interest are plants expressing antibodies or synthetic vaccines to be used orally as passive immunization [16]. The introduction of these traits into *Daucus* cultivars by genetic engineering is thus clearly important and is dependent on a fast, easy, and efficient protocol for plant transformation and regeneration. An overview of world carrot seed value and root production area [5] shows that Nantes is by far the most grown root type (53% and 74%, respectively). Thus, carrot plants of the type Nantes, such as the cultivar Nantaise used in our studies, are ideal targets for future genetic manipulations.

Carrot is used in our laboratory to study metabolism and partitioning of sucrose, and we have cloned genes for polypeptides involved in sucrose biosynthesis, transport, utilization and storage [28]. The use of

mRNA antisense technology [18] to unravel the contribution of each of these polypeptides in sucrose metabolism and partitioning also requires an efficient transformation and regeneration system [32].

Various methods for the delivery of DNA into carrot cells have been described, including the use of *Agrobacterium tumefaciens* [26, 31, 1, 21, 8]. However, initial experiments in our laboratory showed that published methods did not generate large numbers of independent transgenic cell lines (Lorenz and Sturm, unpublished results). To develop a more efficient protocol, we tested different growth media, hormone combinations and concentrations for their ability to induce the formation of transgenic calli, and different cells and tissues for their susceptibility to two different *Agrobacterium* strains. The influence of different antibiotics on callus formation and embryogenesis was also examined. Plants were regenerated via somatic embryogenesis in liquid medium and GUS expression patterns analyzed in regenerated plants transformed with binary vectors containing the GUS gene (*uidA*) [13] driven by the 35S promoter of cauliflower mosaic virus (CaMV) [2].

Material and methods

Plant material and tissue culture

Seeds of carrot (*Daucus carota* L. cv. Nantaise) were obtained from Hild (Mambach, Germany). If not stated otherwise, seedlings and carrot cell cultures were grown aseptically in the dark. Semi-solid growth media usually contained 0.8% agar and 3% sucrose. Cell cultures of cv. Nantaise were obtained as described by Guzzo *et al.* [10], modified at several points. Briefly, seeds were sterilized consecutively for 15 min in 70% ethanol and 10% sodium hypochlorite. After extensive washing in sterile water, the seeds were germinated on agar (0.8%) at 24 °C for 7 days. The hypocotyls of about 50 7-day-old seedlings were cut into segments ca. 1 cm long and immediately transferred into 50 ml B5 medium [7] containing 3% sucrose and 0.1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). After 3 weeks of continuous shaking at 24 °C and 120 rpm, the liberated cells were collected on a 100 μm mesh, centrifuged (150 $\times$ g for 10 min) and resuspended in fresh medium. Rapidly growing cell cultures were subcultured weekly.

Callus formation from root and hypocotyl segments of 7-day-old seedlings was tested on semi-solid MS medium [19] containing 0.5–2.0 mg/l naphthaleneacetic acid (NAA) together with 0–0.5 mg/l 6-benzylaminopurine (BAP) or 0.1–1.0 mg/l 2,4-D, and on semi-solid B5 medium [7] containing 0.25-2.0 mg/l NAA together with 0.25-0.75 mg/l BAP or 0.1-1.0 mg/l 2,4-D. The influence of different antibiotics on callus formation was tested: 0.05-20 mg/l geneticin (G418), 0.5-100 mg/l paromomycin or kanamycin, 200 or 400 mg/l carbenicillin, and 250 or 500 mg/l vancomycin or claforan (cephotaxime). After several weeks at 24 °C, calli were transferred onto new plates. Selection of fast-growing calli led to homogeneously growing tissues that were subcultured every 4 weeks.

Bacterial strains and binary vectors

Agrobacterium tumefaciens strain LBA4404, containing the helper *vir* plasmid pAL4404 [11] and the binary plasmid p35S GUS INT [30], and strain GV3101, with the *vir* plasmids pPM6000 and the binary plasmid pLRG [24], were used for carrot transformation. LBA4404 was grown in LB medium and GV3101 in YEB medium [25].

Co-cultivation of explants with agrobacteria and selection of transformed cells

Suspension-cultured cells were filtered through a 100 μm mesh, collected by low-speed centrifugation, and resuspended in 50 ml fresh medium. Agrobacteria (500 μl of an overnight culture) and acetosyringone (final concentration 100 μm, stock solution 100 mM in DMSO) were added. After co-cultivation for 2 days, the cells were harvested by low-speed centrifugation, washed once with fresh medium and stained for GUS activity by using a cyanide-containing X-gluc solution at 37 °C for up to 24 h [17].

Callus tissue was cut with a scalpel into small pieces and about 0.2 g of tissue was mixed with 100 μl of an overnight culture of agrobacteria containing 100 μm acetosyringone. The mixture was transferred onto agar plates containing semi-solid growth medium. After 2 days co-cultivation, GUS activity was determined by staining.

Sterile carrot tap root slices (2–3 mm thick) were put into an overnight culture of agrobacteria. After vacuum infiltration at 15 kPa for 5 min, the slices were dried on filter paper and placed for 2 days on agar plates containing growth media. After co-cultivation, the slices were washed twice with sterile water and

exposed to the GUS staining solution under reduced pressure.

Hypocotyls or roots of 7-day-old seedlings were cut into segments of about 1 cm and dipped into an overnight culture of agrobacteria. After a few minutes, the seedling segments were briefly dried on filter paper and placed on semi-solid B5 medium containing 1 mg/l NAA and 0.5 mg/l BAP. After 2–3 days of co-cultivation, the segments were washed with liquid B5 medium and put onto semi-solid B5 medium, containing hormones, 200 mg/l claforan, and 200 mg/l vancomycin. After 2 weeks, the explants were transferred to fresh agar containing geneticin (10 mg/l) in addition to the reagents described above. After 4–6 weeks, fast-growing calli were split into small pieces and put onto fresh agar of the same composition.

Plant regeneration

Carrot cells in liquid culture were obtained by transferring 1–2 g of callus tissue crushed into small pieces into in a 250 ml flask containing 50 ml liquid B5 medium with 0.1 mg/l 2,4-D, 10 mg/l geneticin, and 200 mg/l claforan. The fast-growing cell suspension cultures were subcultered weekly by transferring 10 ml of the suspension to 50 ml of fresh medium; slow-growing cultures were subcultured at intervals of 2 weeks.

Somatic embryogenesis was induced in a suspension of single cells and small cell clusters harvest on a 100 μm mesh sieve and low-speed centrifugation. Briefly, the harvested cells were washed once with 10 ml hormone-free B5 medium and resuspended in 40 ml hormone-free B5 medium containing 200 mg/l claforan. Somatic embryos, visible 2 weeks after induction, were selected manually and transferred onto plates with semi-solid B5 medium containing 1.5% sucrose, 10 mg/l geneticin and 200 mg/l claforan. The plates were not sealed with parafilm. After 2 weeks in a phytotron with a 16/8 h day/night cycle, 24 °C, and 85% relative humidity, plantlets were transferred to soil in 20 cm deep pots. Initially, the pots were covered with a thin light-permeable plastic foil and kept in phytotron boxes with a 16/8 h day/night cycle and 22 °C. The foil was removed after 4 weeks when most plants had formed the first foliage leaves.

T-DNA transfer and transformation efficiencies

The frequency of T-DNA transfer was defined as the number of blue cells or blue spots on explants (hypocotyl and root pieces), relative to the total number of cells or explants used. Blue stripes on hypocotyl or root pieces were counted as a single event. The transformation frequency (%) was defined as the number of antibiotic-resistant calli, relative to the total number of co-cultivated explants.

DNA gel blot analysis

Plant DNA was isolated from leaves or calli as described by Murray and Thompson [20]. Leaf tissue and callus cells contained about 200 μg and 20 μg DNA per gram, respectively. DNA was digested with *Hin*dIII, separated on an agarose gel (10 μg/lane) and transferred onto a nylon membrane (Hybond-N, Amersham, UK). The blots were hybridized with a ^{32}P-labeled GUS gene and exposed to X-ray films using standard procedures [25].

Results

Efficiency of Agrobacterium*-mediated T-DNA transfer to carrot cells*

The efficiency for T-DNA transfer to different cell types and tissues of carrot were determined with two strains of *A. tumefaciens* with different genetic backgrounds (Table 1). LBA4404 is derived from Ach5 and produces octopine [11], whereas GV3101 originated from C58 and produces nopaline [24]. Strain LBA4404 harbored the helper *vir* plasmid pAL4404 [11] and the binary plasmid p35S GUS INT with an intron in the coding region of the β-glucuronidase (GUS) marker gene, preventing GUS expression in bacteria [30]. Strain GV3101 contained the *vir* plasmid pPM6000 and the binary vector pLRG with an intron-less GUS gene [24]. In both binary vectors, the GUS gene was driven by the 35S promoter of CaMV. The T-DNA of the binary vectors also contained the *nptII* gene, conferring kanamycin resistance to transformed plant cells. T-DNA transfer to plant cells was measured by a histochemical GUS assay. No blue staining was observed, when only bacteria with the intron-containing GUS gene were incubated in a solution of X-gluc or after carrot tissues were co-cultivated with a transfer-defective form of GV3101 (Figure 1A, D) in which the *virD2* gene of the *vir* plasmid was mutated [24].

Callus cells were transformed at a very low rate, independent of the addition of acetosyringone [27] or whether the calli were broken or cut into small pieces. The transformation rate of suspension-cultured cells

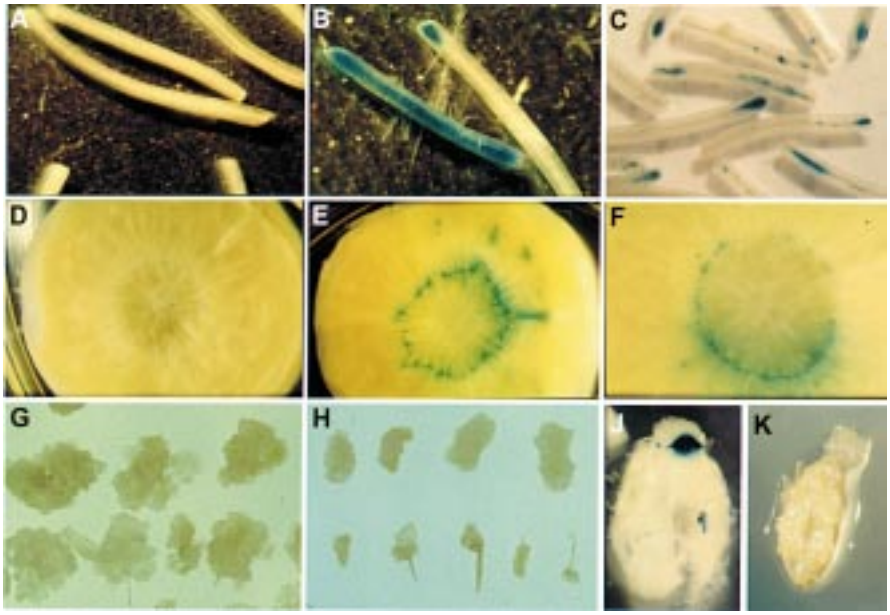

Figure 1. Histochemical detection of GUS activity in explants of the carrot cultivar Nantaise. Segments of hypocotyls (A, B) and roots (C) of 7-day-old seedlings, and tap root slices of 12-week-old plants (D-F) were co-cultivated for 2 days with the transfer-deficient *Agrobacterium* strain GV3101 (pLRG and pPM6000K) (A, D) or the transfer-proficient strain GV3101 (pLRG and pPM6000) (B, C, E, F), and subsequently stained for GUS activity. Root segments of 7-day-old seedlings were cultivated on B5 (G) or MS medium (H) for 4 weeks. Staining of a callus, derived from a hypocotyl segment 2 weeks after co-cultivation, is shown in J. After 4 weeks on selection medium, geneticin resistant calli were visible (K).

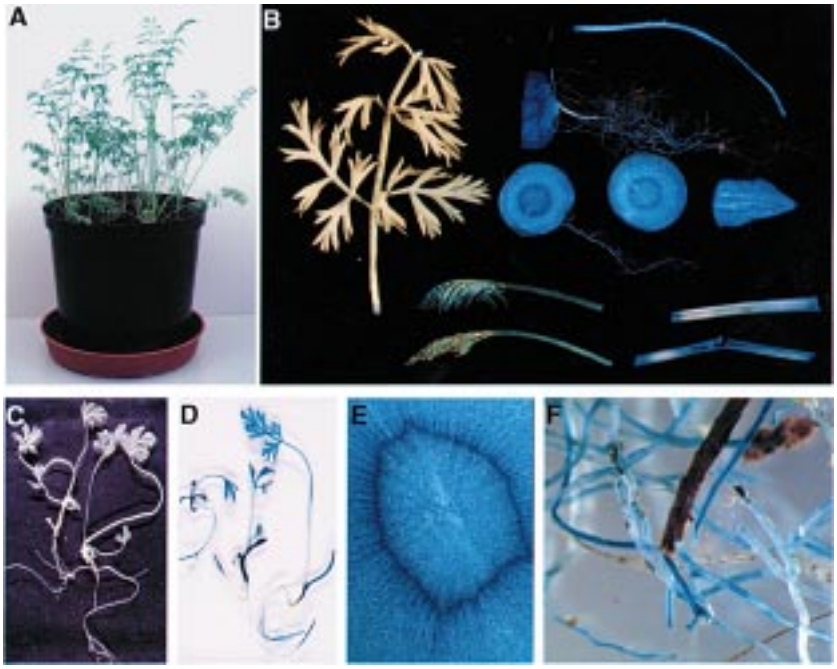

Figure 4. Histochemical detection of GUS activity in transgenic plants expressing the β-glucuronidase gene driven by the CaMV 35S promoter. Ten-week-old plants transformed with the *Agrobacterium* strain GV3101 (pPM6000) shown in A were dissected into different organs and stained (B). In clockwise orientation starting at the top: a tap root tip, tap root segments with fibrous roots, stems, newly developing leaves, and an old foliage leaf. Magnifications of the core of a tap root (E) and of fibrous roots (F) are also shown together with GUS activities in 3-week-old untransformed (C) and transgenic plantlets (D).

was 0.1% or lower. Vacuum infiltration of tap root slices from 12-week-old plants with the *Agrobacterium* strain GV3101 resulted in a large number of GUS-expressing cells. More than 40% of the slices showed blue spots, predominantly in the ring of intrafascicular cambium (Figure 1E, F). LBA4404 was 20 times less effective than GV3101. Hypocotyl pieces of 7-day-old seedlings were transformed by both *Agrobacterium* strains with high efficiencies. Usually, GV3101 led to GUS activities at both ends of the explants and LBA4404 to blue staining all along the vascular bundles. The efficiency of T-DNA transfer from GV3101 to root segments was also very high, but that of LBA4404 was much lower. A 2-day preincubation of the root and hypocotyl pieces on agar plates with growth media before co-cultivation with bacteria, as described by Thomas et al. [29], resulted in a fourfold decrease in the transformation rate. Seed germination on half-strength MS medium, as described by the same author, or addition of acetosyringone, had no effect on T-DNA transfer efficiencies. Hypocotyl segments from seedlings grown in the light were slightly less susceptible than segments from dark-grown plants (data not shown).

Thus, hypocotyl segments of 7-day-old dark-grown seedlings were highly susceptible to both *Agrobacterium* strains. As they also can be obtained easily in aseptic culture, they were used for all further studies. Because of frequent difficulties with the sterilization and handling of root slices, their usage was not continued.

Selection of transgenic carrot cells

Preliminary results indicated that the published concentrations of aminoglycoside antibiotics were not optimal for inhibition of cv. Nantaise cell growth. Therefore, hypocotyl and root pieces of 7-day-old seedlings were placed on callus-inducing medium (B5 medium with 1 mg/ml NAA and 0.5 mg/ml BAP) containing antibiotics at different concentrations and the number of calli formed within 4 weeks was counted (Figure 2). Callus formation stopped at geneticin (G418) and paromomycin concentrations of 10 mg/l, which is about tenfold lower than the growth-inhibitory concentrations of kanamycin (100 mg/l). The antibiotics carbenicillin, claforan and vancomycin used to kill agrobacteria had no effect on callus formation at concentrations up to 200 mg/l.

The antibiotics were also tested on the growth of carrot cells transformed with the *nptII* gene. Hypocotyl

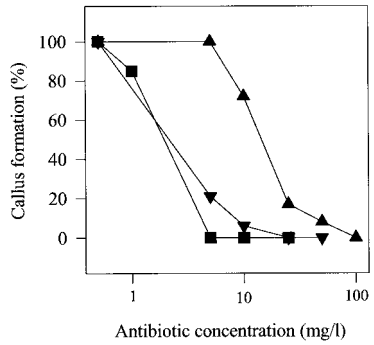

Figure 2. Effects of antibiotics at different concentrations on callus formation from hypocotyl segments of 7-day-old seedlings. Geneticin (■), paromomycin (▼), and kanamycin (▲).

segments of 7-day-old seedlings were co-cultivated for 2 days with LBA4404 and transferred after washing onto plates with B5 medium. To inhibit the growth of bacteria, the medium contained claforan and vancomycin. Two weeks later, the pieces were transferred onto agar plates of the same composition plus 10 mg/l geneticin. After 4–6 weeks, numerous yellowish calli were clearly visible, whereas large parts of the tissues had turned brown (Figure 1K). In contrast, in the presence of kanamycin (100 mg/l) neither color nor growth differences were observed between transformed and untransformed cells. Therefore, in all further studies, geneticin was used for the selection of transformed cells.

Optimization of cell culture conditions

About 800 hypocotyl pieces were co-cultivated for 2 days with the *Agrobacterium* strain GV3101, washed and then transferred to selection medium described in the carrot transformation protocol published by Gogarten et al. [8] containing MS medium with 2 mg/l NAA, 0.3 mg/l BAP, vancomycin (200 mg/l), and claforan (200 mg/l). Kanamycin used in the original protocol was replaced by geneticin (10 mg/l). Only three large calli with GUS activity developed within 9 weeks, whereas numerous small explant sections not forming visible calli stained blue in the presence of X-gluc. To optimize the formation of transgenic calli, different growth media, and different hormone combinations and concentrations were tested. No improvements were made on MS medium [19]. In contrast, on B5 medium [7] more than 90% of the explants developed calli (Table 2). Callus formation on B5 medium occurred all along the explant pieces, whereas on MS medium

Table 1. Efficiencies of T-DNA transfer expressed as numbers of blue cells or blue spots on explants relative to the total number of cells or explants used. Suspension cells, callus tissue or explants were co-cultivated for 2 days with agrobacteria. After histochemical staining for GUS activity, blue cells or spots were counted

Cell and tissue types	*Agrobacterium* strains	treatments	Amount or number of explants	Blue spots	T-DNA transfer frequencies (%)
Callus	GV3101 [a]	chopped	2 g	6	very low
Callus	LBA4404 [b]	chopped	2 g	5	very low
Cell suspension culture	GV3101	acetosyringone	50 ml	nc	$<0.1^{*}$
Cell suspension culture	LBA4404	acetosyringone	50 ml	nc	0.1^{*}
12-week-old plants:					
Tap root slices	GV3101	vacuum infiltration	93	38[c]	41
Tap root slices	LBA4404	vacuum infiltration	121	2[c]	2
7-day-old seedlings:					
Root segments	GV3101	–	175	170	95
Root segments	LBA4404	–	80	4	5
Hypocotyl segments	GV3101	–	170	218	128[d]
Hypocotyl segments	LBA4404	–	130	145	112[d]

nc, not counted; *, estimated.
[a]Strain GV3101 with the helper *vir* plasmid pPM6000 and the binary plasmid pLRG.
[b]Strain LBA4404 with the helper *vir* plasmid pAL4404 and the binary plasmid p35S GUS INT.
[c]Number of tap root slices with blue spots. [d]Values higher than 100% were obtained because some explants segments had more than one blue spot.

this mainly occurred at the segment ends (Figure 1G and H). Efficient formation of calli on hypocotyl or root pieces required 1–2 mg/l NAA and 0.5–0.75 mg/l BAP. The average transformation efficiency was about 20%. The plant growth regulator 2,4-D, frequently used in carrot tissue culture, gave best results at low concentrations (0.1 mg/l) but the calli that formed within 4 weeks were about fourfold smaller than those formed in the presence of optimal concentrations of NAA and BAP (data not shown).

Plant regeneration

Somatic embryogenesis was induced in rapidly growing, transgenic suspensions by omitting hormones. Only a few globular and heart stages embryos formed when the medium contained 10 mg/l geneticin and, therefore, it was omitted during embryo formation. Claforan and vancomycin at concentrations up to 200 mg/l had no effect.

Embryogenesis was already possible 3 weeks after initiation of suspension cultures and reached an optimum 3–12 weeks later. Some cell lines needed a longer adaptation of up to 2–3 months until they proliferated rapidly, and other lines did not grow in liquid culture. Embryos at the torpedo stage were transferred to solidified medium containing geneticin and claforan and exposed to light. The embryos of most lines developed further into small plantlets with cotyledons and primary roots, whereas in control experiments nontransformed embryos of the same stage became brown and died. After 2 weeks, plantlets with green cotyledons were transferred to soil because further development, especially of the roots, was inhibited in agar-containing synthetic media. Usually, 20–50% of the explants transferred to soil formed foliage leaves within 4 weeks. In contrast, on synthetic medium only 1% of the explants continued to develop. After this stage of development, the growth rate of transgenic carrots in soil was indistinguishable from that of nontransformed plants.

DNA gel blot analysis

To test for the presence and integrity of transgenes, DNA isolated from transgenic plants or callus tissue was subjected to Southern blot analysis. DNA was digested with *Hin*dIII, which cleaves once in the T-DNA between the GUS gene and the *nptII* gene (Figure 3). The GUS gene was used as a probe. A different

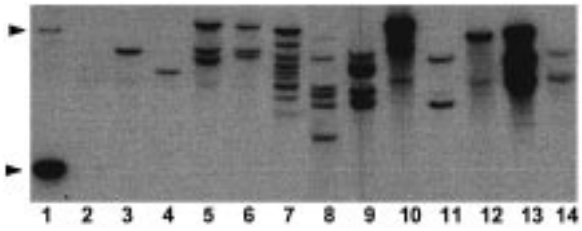

Figure 3. Southern blot analysis of DNA isolated from transgenic plants (5 μg/lane) regenerated from independent geneticin-resistant cell lines. The DNA was restricted with *Hin*dIII and the blot probed with the open reading frame of the GUS gene labeled with ^{32}P. A partially restricted pBI121 plasmid [13] (lane 1), DNA from an untransformed carrot plant (lane 2), and DNA from 12 independent transgenic plants transformed with the *Agrobacterium* strain GV3101 (lanes 3 and 4) or LBA4404 (lanes 5–14) was analyzed. The arrowheads mark DNA fragments of 13.0 kb (upper band) and 2.1 kb (lower band).

Table 2. Efficiency of callus formation on segments of carrot seedlings on different growth media. Segments of roots or hypocotyls from 7-day-old seedlings were placed on different callus-inducing media and calli were counted four weeks later.

Tissue types	Growth media	Callus formation in %
Hypocotyls	MS [a]	48
Roots	MS	35
Hypocotyls	B5 [b]	99
Roots	B5	98

[a]Murashige and Skoog [19], containing 2 mg/l NAA and 0.3 mg/l BAP. [b]Gamborg *et al.* [7] containing 1 mg/l NAA and 0.5 mg/l BAP

T-DNA integration pattern was found for each independent transformant (Figure 3). When the *Agrobacterium* strain LBA4404 was used, the majority of transformants had multiple inserts, predominantly 3–5. Only one single-copy insertion was found among 15 independent transgenic lines. In contrast, all three cell lines obtained by transformation with the *Agrobacterium* strain GV3101 had single copies of the T-DNA.

Expression of the GUS gene driven by the 35S promoter in transgenic carrot plants

Geneticin-resistant calli transformed with the *Agrobacterium* strain GV3101 contained the GUS gene driven by the CaMV 35S promoter. GUS activity in the different transgenic cell lines varied markedly, ramping from 4 to 22500 nmol 4-methylumbelliferone (4-MU) per minute per mg protein, with an average of 6000 nmol min^{-1} mg^{-1}. The GUS expression patterns in two independent transgenic plants with single-copy inserts and high GUS activities were identical. Uniform GUS activities were detected in roots, cotyledons and foliage leaves of young plants up to 6 weeks (Figure 4D), whereas no blue color was observed when nontransformed carrots plants were stained (Figure 4C). GUS expression was low in older foliage leaves and was in some cases restricted to the vascular tissues (Figure 4B). The staining activity was not increased by addition of 20% methanol to the staining solution [15], by preincubation of mature leaves for 15 min in 70% acetone [23], or by brief (1 min) soaking in diethyl ether followed by three rinses in 95% ethanol [3], all in order to enhance penetration of the X-gluc substrate. High and uniform levels of GUS activity were found in the large phloem and xylem parenchyma cells of the tap roots (Figure B, E) and in fibrous roots (Figure 4F). The smaller cells of, for example, the intrafascicular cambium of tap root slices were most intensely stained, probably due to higher specific GUS activities.

Discussion

A comparison of published protocols for the generation of transgenic plants of carrot [26, 31, 1, 21, 8] showed that *Agrobacterium*-mediated DNA transfer, selection of transformed cells with the antibiotic kanamycin, and plant regeneration via somatic embryogenesis were the preferred methods. In each of the protocols, various types of carrot cells and explants, bacterial strains and transformation vectors were combined. The results obtained (see for example [29]) indicate that the identification of 'best-matching partners' and optimal cell culture conditions are essential for the development of an efficient carrot transformation system. The importance of these factors was nicely demonstrated by the studies of Pawlicki *et al.* [21] and Balestrazzi *et al.* [1], who showed that hypocotyls of a French carrot cultivar were not susceptible to the *Agrobacterium* strain LBA4404 harboring pGV2269, whereas the transformation of the same tissue from a Dutch cultivar with the strain A281 containing pGA471 worked very well. We tried several of the published protocols to transform the carrot cultivar Nantaise, widely used

in Europe and the USA, and found that they did either not work at all or only at very low efficiencies.

Two different *Agrobacterium* strains with different Ti plasmids, both containing the marker gene β-glucuronidase (GUS) driven by the 35S promoter of CaMV, and the *nptII* gene leading to kanamycin resistance, were tested. Both strains efficiently transformed hypocotyl and root segments of 7-day-old seedlings but apparently had different cell type specificities. Transformation with GV3101 predominately gave rise to GUS staining at the wound sites, whereas transformation by LBA4404 was all along the vascular tissues. Use of a transfer-deficient *Agrobacterium* strain and an intron-containing GUS gene confirmed that GUS activity was due to gene expression in plant cells and not in cells of bacteria. Cells of the intrafascicular cambium of tap root slices were also efficiently transformed but because of problems with tap root sterilization and handling, they were not used further. In contrast, callus and suspension cells were only poorly susceptible to the two bacterial strains.

Formation of transgenic calli on hypocotyl and root segments of seedlings was increased 20- to 100-fold by changing the hormones in the growth medium from the plant growth regulator 2,4-D to a mixture of NAA and BAP, and the growth medium composition from MS medium to B5 medium, containing less nitrogen. Only meager results were obtained when kanamycin was employed for the selection of transformed cells. Marked improvements were made when the more potent kanamycin-derivative geneticin was used. Best results were obtained when selection on geneticin was applied at about 2 weeks after callus induction. The addition of acetosyringone, an inducer of the bacterial virulence genes and, thereby, a stimulator of genetic plant transformation [27], had no influence on transformation efficiencies of cv. Nantaise. Similar findings were made by Wurtela and Bulka [31] and may be due to high levels of phenolic compounds present in the explants, rendering any additions ineffective [21]. Under optimal conditions, our protocol led to transformation efficiencies of about 20%, which was in our hands at least 100-fold more effective than the published protocols. Transgenic plants with tap roots of 1 cm in diameter were obtained in about 8 months.

Southern blot analysis showed that all geneticin-resistant plants contained between one and several copies of the T-DNA. Single insertions were preferentially found in cells transformed with the *Agrobacterium* strain GV3101, whereas in most transgenic cell lines originating from transformation with LBA4404 multiple copies were inserted. Whether this can be generalized must await further testing of larger populations of independent transformants. Our finding is in contrast to a report by Grevelding *et al.* [9] showing a lack of correlation between the T-DNA copy number in transgenic *Arabidopsis* plants and the *Agrobacterium* strains and transformation vectors used. Instead, a relationship between copy number and explant type used for transformation was reported. High copy numbers were found in plants derived from the transformation of leaf disks, and single insertions in plants originating from transformed roots explants. The authors argued that the 35S promoter was less active in older leaves than in roots and, therefore, more copies of the T-DNA were required to allow for efficient drug resistance. Analysis of DNA from transgenic carrot plants generated with the published carrot transformation protocols stated above showed multiple copies of T-DNA in the majority of independent transformants.

The CaMV 35S promoter is thought to be constitutively expressed in different plant tissues and cell types at different developmental stages [2]. In carrot, histochemical analysis of GUS activity in young plants up to 6 weeks after embryogenesis showed uniform staining patterns, and is in agreement with the literature. In contrast, only low GUS activity was found in older foliage leaves. Whether this was due to lower expression of the GUS gene or reduced stability of GUS transcripts or polypeptides in this organ is not clear. In developing tap roots, GUS activity remained high. With the exception of the intrafascicular cambium and cells of the phloem, GUS activities were fairly uniform in root tissues. Histological inspection of the tap root [4] showed that it consists mainly of phloem and xylem parenchyma cells, which are large and rather uniform in size. In contrast, the cells of the cambium and the phloem are much smaller, leading to higher specific GUS activities and, thereby, to a more intense blue staining. Our results indicate that the 35S promoter may be a useful tool to drive transgene expression in developing carrot storage roots.

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References

1. Balestrazzi A, Carbonera D, Cella R: Transformation of *Daucus carota* hypocotyls mediated by *Agrobacterium tumefaciens*. J Genet Breed 45: 135–140 (1991).
2. Benfey PN, Ren L, Chua N-H: The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J 8: 2195–2202 (1989).
3. Dong J, Teng W, Buchholz WG, Hall TC: *Agrobacterium*-mediated transformation of Javanica rice. Mol Breed 2: 267–276 (1996).
4. Esau K: Developmental anatomy of the fleshy storage organ of *Daucus carota*. Hilgardia 13: 175–226 (1940).
5. FAO: FAO Yearbook: Production. Food and Agriculture Organization of the United Nations, Rome (1991).
6. Freemann RE, Simon PW: Evidence for simple genetic control of sugar type in carrot (*Daucus carota* L.). J Am Soc Hort Sci 108: 50–54 (1983).
7. Gamborg OL, Miller RA, Ojima K: Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151–158 (1968).
8. Gogarten JP, Fichmann J, Braun Y, Morgan L, Styles P, DeLapp K, Taiz L: The use of antisense mRNA to inhibit the tonoplast H^+ ATPase in carrot. Plant Cell 4: 851–864 (1992).
9. Grevelding C, Fantes V, Kemper E, Schell J, Masterson R: Single-copy insertions in *Arabidopsis* are the predominant form of integration in root-derived transgenics, whereas multiple insertions are found in leaf discs. Plant Mol Biol 23: 847–860 (1993).
10. Guzzo F, Baldan B, Mariani P, Lo Schiavo F, Terzi M: Studies on the origin of totipotent cell in explants of *Daucus carota* L. J Exp Bot 45: 1427–1432 (1994).
11. Hoekema A, Hirsch PR, Hooykas PJJ, Schilperoort RA: A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303: 179–180 (1983).
12. Hole CC: Carrots. In: Zamski E, Schaffer AA (eds) Photoassimilate distribution in Plants and Crops, pp. 671–690. Marcel Dekker, New York (1996).
13. Jefferson RA, Kavanagh TA, Bevan MW: GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901–3907 (1987).
14. Krug H: Apiaceae. In: Krug H (ed) Gemüseproduktion, pp. 327–325. Paul Parey, Berlin/Hamburg (1991).
15. Li LC, Qu RD, Kochko DA, Fauquet C, Beachy RN: An improved rice transformation system using the biolistic method. Plant Cell Rep 12: 250–255 (1993).
16. Ma JK-C, Hiatt A, Hein M, Vine ND, Wang F, Stabila P, van Dolleweerd C, Mostov K, Lehner T: Generation and assembly of secretory antibodies in plants. Science 268: 716–719 (1995).
17. Mendel RR, Müller B, Schulze J, Kolesnikov V, Zelenin A: Delivery of foreign genes to intact barley cells by high-velocity microprojectiles. Theor Appl Genet 78: 31–34 (1989).
18. Mol JNM, van der Krol AR, van Tunen AJ, van Blokland R, de Lange P, Stuitje AR: Regulation of plant gene expression by antisense RNA. FEBS Lett 268: 427–430 (1990).
19. Murashige T, Skoog F: A revised medium for rapid growth and bioassays with *Camellia japonica* pollen. Phytochemistry 19: 205–209 (1962).
20. Murray MG, Thompson WF: Rapid isolation of high molecular weight plant DNA. Nucl Acids Res 8: 4321–4325 (1980).
21. Pawlicki N, Sangwan R, Sangwan-Norreel B: Factors influencing the *Agrobacterium tumefaciens*-mediated transformation of carrot (*Daucus carota* L.). Plant Cell Tissue Organ Cult 31: 129–139 (1992).
22. Peterson CE, Simon PW: Carrot Breeding. In: Bassett MJ (ed) Breeding Vegetable Crops, pp. 321–356. AVI, West Port, CT (1986).
23. Rodrigues-Pousada RA, De Rycke R, Dedonder A, Van Caeneghem W, Engler G, Van Montagu M, Van Der Straeten D: The *Arabidopsis* 1-aminocyclopropane-1-carboxylate synthase gene 1 is expressed during early development. Plant Cell 5: 897–911 (1993).
24. Rossi L, Escudero J, Hohn B, Tinland B: Efficient and sensitive assay for T-DNA dependent transient gene expression. Plant Mol Biol Rep 11: 220–229 (1993).
25. Sambrook J, Frisch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
26. Scott R, Draper J: Transformation of carrot tissues derived from proembryogenic suspension cells: a useful model system for gene expression studies in plants. Plant Mol Biol 8: 265–274 (1987).
27. Sheikholeslam SN, Weeks DP: Acetosyringone promotes high efficiency transformation of *Agrobacterium thaliana* explants by *Agrobacterium tumefaciens*. Plant Mol Biol 8: 291–298 (1987).
28. Sturm A, Molecular characterization and functional analysis of sucrose-cleaving enzymes in carrot (*Daucus carota* L.) J Exp Bot 47: 1187–1192 (1996).
29. Thomas J, Guiltinan M, Bustos S, Thomas T, Nessler C, Carrot (*Daucus carota)* hypocotyl transformation using *Agrobacterium tumefaciens*. Plant Cell Rep 8: 354–357 (1989).
30. Vancanneyt G, Schmidt R, O'Conner-Sanchez A, Willmitzer L, Rocha-Sosa M: Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Mol Gen Genet 220: 245–250 (1990).
31. Wurtele E, Bulka K: A simple, efficient method for the *Agrobacterium*-mediated transformation of carrot callus cells. Plant Sci 61: 253–262 (1989).
32. Zrenner R, Salanoubat M, Willmitzer L, Sonnewald U: Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L.) Plant J 7: 97–107 (1995).