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Transformation of cucumber (*Cucumis sativus* L.) plants with *Agrobacterium rhizogenes*

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Summary. Transgenic cucumber plants (*Cucumis sativus* L., cv. 'Straight Eight') were regenerated from roots induced by inoculation of inverted hypocotyl sections with *Agrobacterium rhizogenes* containing the vector pARC8 in addition to the resident Ri-plasmid. The DNA transferred to the plant from the vector (T-DNA) included a gene which encoded the enzyme neomycin phosphotransferase II, and thus conferred on the plant cells resistance to kanamycin. The transgenic plants looked normal and were positive for the neomycin phosphotransferase II. Southern blot analysis of the transgenic plants revealed that all plants contained vector DNA, but only some of them contained DNA from the Ri plasmid.

Key words: Cucumber – Roots – Gene transfer – *Agrobacterium rhizogenes*

Introduction

Sterility barriers between species are perhaps the most limiting factors in plant breeding. They preclude transfer of many desirable traits such as disease, insect and herbicide resistance from sexually incompatible cultivated or weedy plants. This problem is especially acute in cucumber (*Cucumis sativus* L.), where successful crosses can be made only between *C. sativus* and the closely related *C. hardwickii*, but not other species (Deakin et al. 1971). Therefore, the recent advances in genetic engineering would be especially welcome in improvement of this economically important crop. However, the lack of a reliable method for cucumber regeneration in tissue culture (Wehner and Locy 1981; Novak and Dolezelova 1982; Malepszy and Nadolska-Orczyk 1983), has been the major limiting factor.

Due to the ease of regeneration of plants in the Solanaceae, the majority of efforts in genetic engineering (Fraley et al. 1983; Herrera-Estrella et al. 1983; Bevan 1984; Horsch et al. 1985; Jones et al. 1985) has been limited to plants in this family, most of which are not food crops. The most widely used method of gene transfer is via a disarmed form of the Ti plasmid of *Agrobacterium tumefaciens* (Zambryski et al. 1983). An alternative tool for gene transfer is *A. rhizogenes*, which differs from *A. tumefaciens* by inducing roots rather then tumors (Chilton et al. 1982; David et al. 1984).

In this paper we show that *A. rhizogenes* can be used successfully to transfer a foreign gene into cucumber plants. The efficient, reproducible, and facile methodology opens new avenues in genetic improvement of cucumbers by permitting the transfer of agronomically desirable genes. Additionally, selectable marker genes such as kanamycin resistance, introduced by this method, should facilitate somatic hybridization of cucumbers with other species.

Materials and methods

Plant material

Seeds of cucumber cultivar 'Straight Eight' (ARCO Seed, Brooks, Oregon) were sterilized in 10% (v/v) Clorox (commercial bleach containing 5.25% sodium hypochlorite) with a drop of Tween 80 for 10 min, then rinsed 3 times in sterile, distilled water and placed in sterile petri plates (approx. 30 per plate) lined with moist Whatman No. 3 filter paper. To assure uniform and rapid germination of the seeds, plates were kept for 24–30 h at 27 °C, in the dark. Germinated seeds (radicle length approx. 5 mm) were placed aseptically in Magenta boxes (six seeds per box) containing 40 ml of TM-1 medium (Shahin 1985) supplemented with 150 mg/l of carbenicillin (Sigma), and incubated for 5 days in a growth chamber, at 21 °C night, 26 °C day, 14 h photoperiod (4,500 lux).

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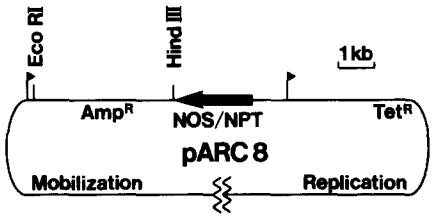

Fig. 1. The disarmed vector plasmid used in the transformation of cucumber plants. The vector contains a wide host range replicon (to permit the replication of the plasmid in both *Escherichia coli* and *Agrobacterium*), a bacterial origin of transfer (which permits the vector to be mobilized by a helper plasmid), markers encoding resistance to tetracycline (Tet®) and ampicillin (Amp®), which allow selection of bacteria containing the vector. The unique restriction enzymes sites Eco RI and Hind III facilitate the insertion in vitro of "foreign" DNA fragments into the vector. The flags mark the termini sequences which *Agrobacterium* uses to delimit the DNA it transfer to plant cells. The NOS/NPT is a selectable marker which confers resistance to kanamycin in transformed plant cells. This chimeric gene consists of the neomycin phosphotransferase (NPT) coding region, flanked by the nopaline synthase (NOS) promoter and terminator

Vector plasmid and Agrobacterium strain

We used the A4 strain of *A. rhizogenes* containing, in addition to the resident Ri-plasmid, a vector (pARC8) derived from the Ti plasmid of *A. tumefaciens* (Simpson et al. 1986) (Fig. 1). The construction of this system was based on the binary vector concept described by Hoekema et al. (1983). The selectable marker in pARC8, which conferred resistance to kanamycin, was NOS/NPT, a chimeric gene constructed from Tn5 neomycin phosphotransferase (NPT) coding region flanked by the nopaline synthase (NOS) promoter and terminator.

Inoculations

The inoculum consisted of four-day-old culture grown at room temperature in the dark, on AB medium (Chilton et al. 1974) supplemented with 5 ml/l of tetracycline (Sigma) (to select for the bacterial Tet® marker on the vector). The inoculum was collected on a sterile bacteriological loop and smeared gently on the cut surface of inverted hypocotyl sections (approx. 2 cm long) placed in hormone-free Murashige and Skoog (MS) medium (Murashige and Skoog 1962). The plates were then sealed and incubated at 27 °C, under continuous light (2,500 lux). One week after inoculation, the hypocotyl sections were cut above the agar surface and transferred into hormone-free MS medium supplemented with 100 mg/l of the antibiotic cefotaxime (ampicillin analogue which is not modified by the β-lactamase encoded by the Amp® gene on the vector pARC8) (Calbiochem), followed by incubation in the same conditions.

Plant regeneration

Roots (5 to 10 mm in length) produced on the inoculated surfaces were excised and placed on a medium consisting of MS salts, 5 µM 2,4-Dichlorophenoxyacetic acid (2,4-D) (Sigma), 5 µM α-Naphthaleneacetic acid (NAA) (Sigma), 2 µM 6-Benzylaminopurine (BAP) (Sigma) and 0.7% agar, followed by incubation for 2–3 weeks under continuous light (3,500 lux), at 27 °C. In the second approach this medium was supplemented with 25 mg/l of kanamycin (Sigma) to select transformed plants. Embryoids that appeared on the root surface were detached and transferred to MS medium with 5 µM NAA and 2 µM BAP, 0.7% agar, and incubated in the same conditions for 10–14 days. Mature embryoids were transferred onto hormone-free MS medium (1% agar) on which shoots were produced. In all media pH was adjusted to 5.8 prior to autoclaving at 121 °C for 15 min. To eliminate bacterial carry-over, media contained 100 mg/l of cefotaxime. Regenerated plantlets were transplanted to a mixture (1:1, v/v) of peatlite (Jiffy Products Co., West Chicago, IL.) and soil.

Neomycin phosphotransferase assay

Frequency of transformation by vector DNA was assessed in the regenerated plants using an assay for NPT (Reiss et al. 1984). Small pieces (approx. 25 mm²) of leaf tissue were used in the assay. The assay was performed twice, initially on plantlets on the hormone-free medium, and later on plants, 2–4 weeks after they had been potted in the soil mixture.

Isolation of DNA and Southern blot analysis

Southern blot analysis (Southern 1975) was used to confirm the integration of the NOS/NPT gene in the DNA of the NPT-positive plants. The DNA was isolated from 2 g (fresh weight) of young leaf tissue according to the procedure of Saghai-Maroof et al. (1984), then digested with Hind III and electrophoresed on an agarose gel, blotted and probed essentially as described by Thomashow et al. (1980). Plasmid pNEO 105 (Simpson et al. 1986), containing the chimeric gene NOS/NPT, was used as the probe of the transferred portion of the vector DNA. Southern blot analysis was also used to investigate the extent of the Ri-plasmid DNA transfer into the DNA of the NPT-positive plants. Plasmids pFW94 and pFW41 (Huffman et al. 1984), which are clones of the Ri plasmid T-DNA and were kindly supplied by Frank White, were used as probes to determine the presence of the T_L-DNA and T_R-DNA, respectively.

Results and discussion

The A4 strain of *A. rhizogenes* containing the vector pARC8 was infectious on cucumbers as indicated by dense, cream-colored callus that appeared on the inoculated surfaces 7–10 days after the inoculation. One to two weeks later this callus produced roots. There was no root formation in control, uninoculated hypocotyl fragments which produced small amounts of white, loose callus.

A total of 691 roots harvested from the inoculated hypocotyl sections were plated on the embryo-inducing medium. Plantlets were regenerated from 64 roots. Twenty two plantlets (each regenerated from a separate root) were positive in the test for the neomycin phosphotransferase II (NPT-positive). These plants remained NPT-positive when assayed a second time after potting in the soil mix.

The low frequency of plant regeneration from roots was the main limitation in the recovery of transformed plants. This was not a result of transformation, however, since about 10% of untransformed roots regenerate plants in the cv. 'Straight Eight' (Trulson and Shahin, in

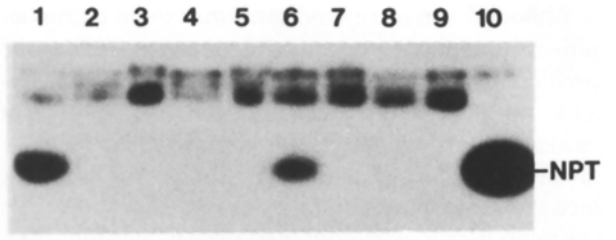

Fig. 2. Neomycin phosphotransferase II test on cucumber plants regenerated from roots induced by inoculation with *A. rhizogenes.* We used the native polyacrylamide gel assay of Reiss et al. (1984). Numbers 1–9 represent samples from randomly chosen cucumber plants regenerated without selection on kanamycin, sample No. 10 is a positive control (bacteria producing NPT). "NPT" indicates mobility of the enzyme

preparation). Without selection for resistance to kanamycin two out of twenty regenerated plants were NPT-positive (for example see Fig. 2), whereas when kanamycin (25 mg/l) was added to the embryo-inducing medium more than half of the regenerated plants were NPT-positive. The addition of kanamycin did not affect the regeneration process of the transformed tissue, but did not prevent regeneration of some NPT-negative plants. This concentration of kanamycin also allowed some growth of control, nontransformed roots, which however, did not regenerate plants in the presence of kanamycin. In preliminary experiments we increased kanamycin concentration to 50 mg/l, but this concentration of kanamycin slowed plant regeneration and increased the number of abnormal plantlets.

Southern blot analysis of the DNA from the NPT-positive plants confirmed the integration of the vector T-DNA in cucumber DNA (Fig. 3). Each transformed plant appeared to contain a single copy of foreign DNA as indicated by presence of two bands corresponding to two border fragments resulting from the Hind III digest (see Fig. 1). Among the plants tested for the integration of the Ri-plasmid DNA, which can be integrated into plant genome in two fragments, T left (T_L) and T right (T_R) (White et al. 1985), two plants did not contain any Ri-plasmid T-DNA, one plant had a 5.7 kb fragment of the T_R-DNA and two plants had different amounts of the T_L-DNA (Table 1). This would suggest that our procedure does not select strongly for or against T_L-DNA or T_R-DNA. However, further experiments are required to study the possible influence of vector structure on the extent of the Ri T-DNA transfer.

Although some plants had thicker leaves and shorter than normal internodes, the transgenic cucumber plants did not show abnormalities in leaf morphology (Fig. 4), such as leaf wrinkling, reported in tobacco, carrot and morning glory transformed with *A. rhizogenes* (Tepfer 1984). Upon maturation, however, the transgenic cucumbers displayed varying degrees of

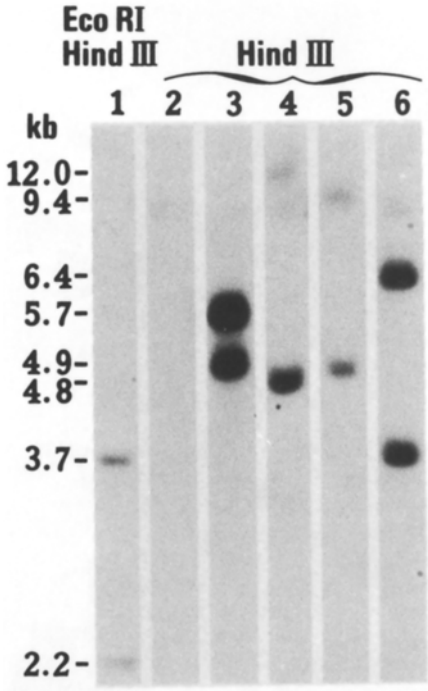

Fig. 3. Southern blot hybridization analysis of cucumber plant DNA demonstrating integration of the vector DNA into the cucumber genome. *Lane 1:* half-copy reconstruction of the chimeric NOS/NPT gene containing Eco RI- and Hind III-digested pNEO 105 which contains the NOS/NPT gene cloned in pBR322 (Simpson et al. 1986). *Lane 2:* Hind III-digested DNA from non-transformed, control cucumber plant. *Lanes 3–6:* Hind III-digested DNA from NPT-positive plants regenerated from transformed roots. The probe is pNEO105

Table 1. Summary of integration of the vector DNA and the Ri-plasmid DNA (T_L and T_R) into DNA of five cucumber plants as a results of transformation with *A. rhizogenes*[a]

Vector	Hind III fragments (kb)		
	Vector T-DNA	T_L-DNA	T_R-DNA
pARC8	5.7; 4.9	0	5.7
pARC8	12.0; 4.8	0	0
pARC16[b]	9.4; 4.9	0	0
pARC16	4.8; 3.9	3.4[c]; 5.9	0
pARC16	6.4; 3.7	3.4	0

[a] DNA isolated from five independent, NPT-positive cucumber plants and digested by Hind III was analysed using Southern blots. The probes were pNEO105 (vector DNA; Simpson et al. 1986), pFW94 or pFW41 (T_L-DNA and T_R-DNA, respectively; Huffman et al. 1984)

[b] pARC16 is a derivative of the pARC8 (Simpson et al. 1986) in which a 9.0 kb Hind III restriction fragments has been inserted at the Hind III site of the T-DNA region (Fig. 1)

[c] Mobility expected for the internal Hind III fragment (H-21) of the T_L-DNA described by White et al. (1985)

Fig. 4. Cucumber plants regenerated from roots. *Left:* control plant. *Right:* transformed cucumber plant

diminution and abscision of male flowers. This may be due to the T-DNA from *A. rhizogenes,* since reduced fertility resulting from transformation was reported in other species (Tepfer 1984). However, we also observed reduced fertility in control plants regenerated from untransformed roots. Thus it is possible that the abnormalities resulted from the selection and regeneration procedures rather than from the T-DNA. Further studies are needed to determine the cause of this variation. If the changes are heritable, they might be beneficial in cucumber breeding, since compact stature and male sterility are desirable in this species (Kauffman and Lower 1976).

Using this method of transformation we recovered transgenic cucumber plants within 10 weeks. This methodology is simpler than the co-cultivation method (Marton et al. 1979) in which plant protoplasts are transformed. The co-cultivation method is labor-intensive and prone to contamination, whereas the methodology presented here consists of a few, simple steps that can be effectively performed in standard laboratories. Furthermore, somaclonal variation frequently results from protoplast culture, whereas organized tissue is known to remain more stable (Shepard et al. 1980; Krens et al. 1982; Horsch et al. 1985).

Although our survey indicates that some cucumber cultivars do not regenerate plants from roots under the specified conditions (Trulson and Shahin, in preparation), we believe that the cultivars capable of root regeneration could serve as intermediates in gene transfer to other genetic backgrounds. Additionally, since the genotypes capable of regeneration from roots represent highly advanced germplasm, genetic engineering performed in this germplasm will help to advance already superior genotypes.

The method of transformation presented here should facilitate somatic hybridization in cucumber. Although the potential of somatic hybridization is well recognized, the lack of selectable markers constitutes a major obstacle in manipulating protoplasts of higher plants (Cocking et al. 1981). The methodology described here should permit the introduction of drug-resistance markers to aid in identification and selection of desirable protoplast fusion products. A recently developed strategy (Van den Broeck et al. 1985) for the transfer to the chloroplast of DNA encoding chloramphenicol-resistance opens additional possibilities for isolating cybrids. Kanamycin and chloramphenicol can be used to select for fusion products with the desired nucleus and chloroplasts. Moreover, the markers would be of great value in transfer of agronomically important genes, since the presence of an easily identifiable marker linked to an agronomically desirable gene would permit efficient selection in tissue culture (Fraley et al. 1983; Herrera-Estrella et al. 1983).

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