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Cultivar dependence of transformation rates in moth bean after co-cultivation of protoplasts with *Agrobacterium tumefaciens*

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Summary. A simplified protoplast regeneration system for *Vigna aconitifolia* was developed. A plating efficiency of 60% was obtained using mesophyll protoplasts from 10-day-old seedlings. By co-cultivation of protoplasts with *Agrobacterium tumefaciens* containing the Ti plasmid derivative pGV 3850:1103 neo kanamycin-resistant colonies were obtained; 23% of the transformed lines showed expression of the nonselected co-transferred nopaline synthase gene. Transformation was confirmed by Southern blot analysis using a non-radioactive detection system. The plant cultivar used was an important factor in determining transformation frequencies since one of the cultivars had an 85 fold higher transformation rate than the other.

Key words: *Vigna aconitifolia* – Co-cultivation – *Agrobacterium tumefaciens* – Kanamycin resistance – Nopaline synthesis

Introduction

Two efficient techniques have been developed for transforming protoplasts deriving from higher plants: the direct gene transfer and the *A. tumefaciens*-mediated gene transfer. By incubation of protoplasts with naked DNA and polyethylene glycol (PEG), the direct gene transfer yielded high numbers of transformants in tobacco (Paszkowski et al. 1984; Hain et al. 1985), particularly in combination with the electroporation (Shillito et al. 1985), and in moth bean (Köhler et al. 1987a). Monocots were also successfully transformed using this technique (Potrykus et al. 1985; Lörz et al. 1985). For the *Agrobacterium*-mediated gene transfer, several procedures were developed ranging from in vivo tumour induction to the in vitro co-cultivation of plant protoplasts (Marton et al. 1979; Wullems et al. 1981; Herrera-Estrella et al. 1983). Gene transfer techniques will have a great impact on legumes, which play an important role in human and animal nutrition. The only report on chimaeric gene transfer to a grain legume using the leaf disc method with *A. tumefaciens,* is that of cowpea *Vigna unguiculata* (Garcia et al. 1986). However, as the species could not differentiate, only transformed calli could be obtained. Using the forage legume *Medicago sativa, Agrobacterium*-mediated gene transfer resulted in transformed plants through stem segment transformation (Shahin et al. 1986).

Here, we report regeneration and transformation of moth bean protoplasts (*Vigna aconitifolia*) by co-cultivation with *Agrobacterium tumefaciens* containing a Ti plasmid with a selectable antibiotic resistance marker (neomycin phosphotransferase) and a non-selectable marker (nopaline synthase gene).

Materials and methods

Protoplast culture

Surface-sterilized seeds of *Vigna aconitifolia* L. (Jacq) marechal cvs IPCMO-560 and IPCMO-909, obtained from S. E. Pawar (Bhabha Atomic Research Centre, Bombay, India), were germinated on MS basal medium (Murashige and Skoog 1962). Primary leaves from 9- to 10-day-old seedlings were used for protoplast isolation. In the leaf material was incubated overnight in a solution of 1% cellulase "Onozuka" R10, 0.2% macerozyme and 0.6 M mannitol. Protoplast purification was carried out as described by Schieder (1984) using sea water as

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the wash solution. The protoplasts were cultured in V47 medium (Binding 1974), supplemented with 1.5 mg/l NAA (naphthylacetic acid) and 0.4 mg/l BAP (6-benzylaminopurine) at a final density of 10^5 protoplasts/ml. Cultures were kept under continuous illumination of about 1,000 lux at 25 °C.

Agrobacterium strain

The *Agrobacterium* strain C58Cl containing pGV 3850:1103 neo was used. The plasmid pLGV 1103 neo, carrying the nopaline synthase promotor fused to the coding region of the neomycin phosphotransferase (NPT) gene of Tn5, is integrated into the Ti-plasmid derived vector pGV 3850 possessing the nopaline synthase gene (Hain et al. 1985). The strain was a gift from Max-Planck-Institute (J. Schell; Köln, FRG).

Co-cultivation

Transformation of regenerating protoplasts was carried out by a modification of the technique of Marton et al. (1979). Protoplasts on day 3 of culture were mixed with 200–300 agrobacteria per protoplast and co-cultured for 48 h. The suspension was then washed with sea water (650 mosmol) and the plant cells were cultured in V47 medium supplemented with 500 mg/l cefotaxim (Hoechst Frankfurt, FRG). The osmotic pressure was reduced by 50 mosmol per week by adding fresh medium.

Selection and regeneration of transformed lines

At 3 weeks after co-cultivation, the cell suspension was mixed with agarose medium and cultured in the bead type culture system (Shillito et al. 1983). A selection pressure was established by adding V47 medium containing 75 mg/l kanamycin. Resistant calli could be distinguished 3–4 weeks after selection started. Growing colonies were transferred to solid V47 medium plus 100 mg/l kanamycin after a further 3–4 weeks. Differentiation was obtained by transferring colonies on MS basal medium without hormones. Shoots which did not develop a root system were transferred to MS agar medium containing 0.5 mg/l NAA, where they developed into complete plants.

Nopaline synthase assay

For this, 100 mg tissue of transformed lines was crushed in extraction buffer, centrifuged, and the supernatant used for enzyme assay as described by Otten and Schilperoort (1978). Standards nopaline and arginine were used as controls and electrophoresis was carried out at 400 V for 45 min. After staining and drying, the spots were visualised under UV light.

Southern blot analysis

Southern blot analysis was carried out with the "Blue gene" kit, a sensitive, non-radioactive detection system. Plant DNA was isolated according to Paszkowski et al. (1984). After restriction with Eco R1 and Hind III, DNA was electrophoresed in 1% agarose gel, transferred to a nitrocellulose filter (Southern 1975) and hybridized with biotin-labelled probe DNA. The procedure for nick translation, hybridization and visualization was carried out according to the manufacturer's instructions.

Results

Protoplast culture and regeneration

The regeneration potential of *Vigna aconitifolia* mesophyll protoplasts was first demonstrated by Shekhawat and Galston (1983). Here, a simplified method was used for isolation of protoplasts and its regeneration. Based on the technique successfully applied to tobacco and Datura (Schieder 1984), a plating efficiency of 60%–70% was obtained. About 10% of the calli showed developing shoot buds after 2 weeks on regeneration medium. The two cultivars used did not show any significant differences in plating efficiency or differentiation potential.

Co-cultivation of protoplasts with Agrobacterium tumefaciens

When protoplasts were co-cultured on the third day with *Agrobacterium tumefaciens* C58Cl pGV 3850:1103 neo, some of the protoplasts had already divided. The protoplasts continued to divide in the presence of *Agrobacteria* at a frequency of 40%–50%, while 60%–70% of the control protoplasts showed divisions. After 4 weeks of culture, selection was started by adding kanamycin to the medium at a concentration of 75 mg/l. After a further 3–4 weeks, green, rapidly growing colonies were detected against a background of dying, brown calli (Fig. 1).

In three independent experiments, the rate of kanamycin resistant colonies was an average of 3.4% for cultivar IPCMO-560 and 0.04% for cultivar IPCMO-909 based on the number of colonies regenerated without selection pressure (Table 1). In each experiment the influence of the plant cultivar on transformation rate was evident.

The transformed colonies were further cultured in the presence of kanamycin (100 mg/l) for 2–4 weeks

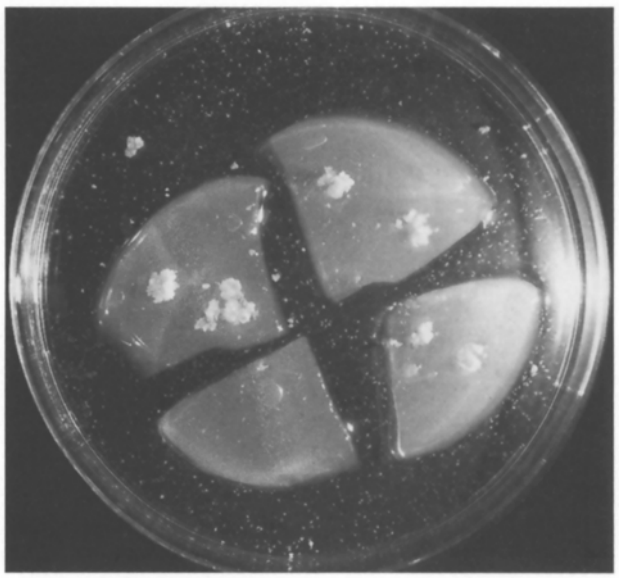

Fig. 1. Green, rapidly growing colonies of *Vigna aconitifolia* on selection medium (75 mg/l kanamycin) after co-cultivation of protoplasts with *A. tumefaciens*

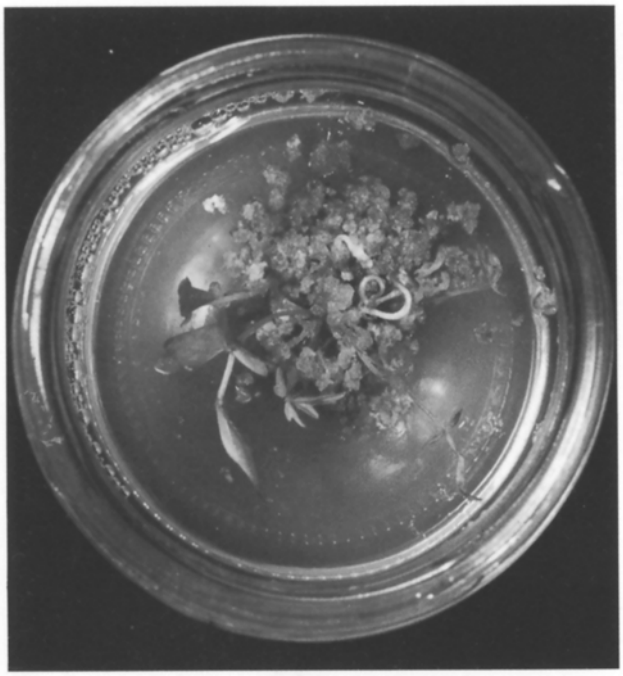

Fig. 2. Differentiation of kanamycin-resistant colonies on MS basal medium

Table 1. Transformation rates in *Vigna aconitifolia* obtained by co-cultivation with *A. tumefaciens* (C58C1 pGV 3850:1103 neo). Resistant colonies were determined 9 weeks after co-cultivation and 6 weeks after starting selection with 75 mg/l kanamycin. Mean values of three independent experiments

Cultivar	No. of protoplasts	No. of colonies	Resistant colonies	Frequency[a]	% Resistant colonies[b]
"560"	10×10^6	12,396	414	4.14×10^{-5}	3.40
"909"	10×10^6	12,500	5	5.0×10^{-7}	0.04

[a] Number of resistant colonies/number of protoplasts treated
[b] Related to total number of colonies regenerated without selection pressure

and finally transferred to the MS differentiation medium where shoot buds and plantlets could be regenerated (Fig. 2).

Analysis of transformed lines

For Southern blot analysis, 5 µg of total DNA from selected lines were double-digested with Eco R1/Hind III. Fragments were separated on 1% agarose gel, blotted onto nitrocellulose filters and hybridized with biotin-labelled probes (pLGV neo 2103). Transformation was confirmed with eight kanamycin-resistant lines tested (Fig. 3). Four nopaline-positive (Fig. 3; lanes 7–10) and four nopaline-negative clones (Fig. 3; lanes 3–6) were examined. In all cases the excepted 3 kb fragment (Hain et al. 1985) containing a functional copy of the NPT II gene, could be detected. The nopaline synthase assay showed that 23% of the transformed lines showed the presence of nopaline synthase (Fig. 4). Out of 31 transformants tested, 7 lines showed the enzyme activity of the non-selectable marker.

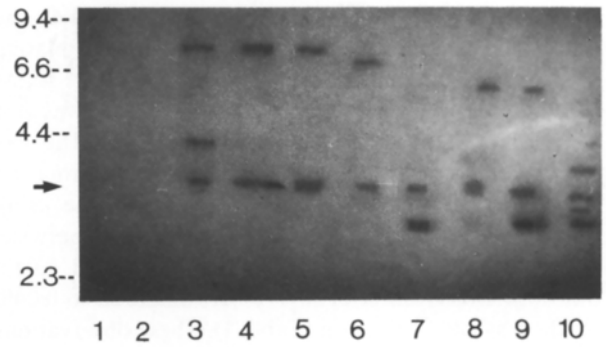

Fig. 3. Southern blot analysis: 5 µg genomic DNA were double digested with Eco R1/Hind III. Hybridization was carried out with biotin-labelled probe DNA. All kanamycin-resistant clones analysed (*lanes 3–10*) contained the expected 3 kb fragment (*arrow*) with a functional copy of the NPT II gene (Hain et al. 1985). *Lanes 1, 2:* wild type DNA of *Vigna aconitifolia*. *Ordinate values* are lambda/Hind III size standards (kb)

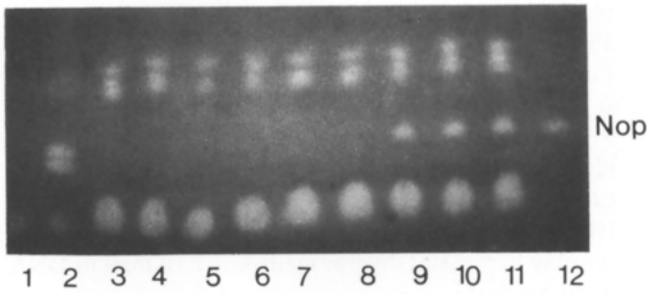

Fig. 4. Nopaline test according to Otten and Schilperoort (1978) with kanamycin-resistant clones of *Vigna aconitifolia*. *Lane 1:* arginin standard; *lane 2:* nopaline (nop)-octopine-arginine standard; *lanes 3–8:* nop-negative clones; *lanes 9–11:* nop-positive clones; *lane 12:* nopaline standard

Discussion

The successful transfer and expression of an antibiotic resistance gene in the moth bean cultivars "560" and "909" using the co-cultivation method with *Agrobacterium tumefaciens* could be demonstrated. In the only other report on a chimaeric gene transfer to a grain legume (*Vigna unguiculata*), Garcia et al. (1986) used the leaf disc transformation technique, which yielded transformed calli.

The present method has shown that the cultivar (genotype?) of the plant used is an important factor in determining the transformation rate. Line "560"

showed an 85-fold higher transformation rate than the other cultivar "909", although both had similar plating efficiencies. The results conform with direct gene transfer studies in the same species where line "560" was also superior to line "909" (Köhler et al. 1987b). The frequency of *A. tumefaciens* mediated transformation (3.4%) is much higher than transformation rate using direct gene transfer (0.58%) in IPCMO-560. Otherwise, direct gene transfer using IPCMO-909 yielded 0.08% transformants (Köhler et al. 1987b) compared to 0.04% reached by co- cultivation (Table 1). These observations demonstrate that the plant cultivar is of eminent importance not only in direct gene transfer experiments but also when *A. tumefaciens* is used as a vector. Plant cultivar was also recognized as an factor influencing transformation rates in tobacco transformed via direct gene transfer (Köhler et al. 1987b).

In cell and tissue culture it is well known that the plant genotype has a great influence upon the regeneration potential. Unfortunately, conclusive evidence for the differential ability of plant genotypes to regenerate is not available (see Halperin 1986). Here, the two *Vigna aconitifolia* lines "560" and "909" possess an identical "morphogenetic capacity" to regenerate but exhibit different transformation rates. This may be correlated to different integration and/or expression levels possibly because of diverse methylation of foreign DNA in the host cells.

We found expression of co-transferred nopaline synthase gene in only 23% of the kanamycin-resistant cultures. Four nopaline-negative clones were examined by Southern blot analysis for the presence of a functional copy of the NPT II gene. The expected 3 kb fragment could be detected in these clones. These observations are in agreement with the results of Czernilofsky et al. (1986), who noted that the co-transferred nopaline synthase gene was inactivated in two out of nine cases using the same *A. tumefaciens* strain for transformation of tobacco. Though nopaline synthase sequences were at least partially present in nopaline-negative lines, expression of the gene could not be detected possibly because of the deletion of promoter specific sequences or the presence of methylation.

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