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Fusion of *Agrobacterium* and *E. coli* spheroplasts with *Nicotiana tabacum* protoplasts – Direct gene transfer from microorganism to higher plant

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ABSTRACT

Spheroplasts of Agrobacterium tumefaciens strains and E. coli were fused with protoplasts of Nicotiana tabacum. Fusion products were cultured in the presence of antibiotics to eliminate remaining bacterial spheroplasts. On hormone free medium, tobacco protoplasts treated with wild type Agrobacterium-strains formed colonies with an average frequency of 10^{-4}. Opine synthesis was detected in the tissues. Some calli derived from protoplasts treated with A. tumefaciens C58C1pRi15834 formed typical hairy roots. Kanamycin resistant calli were obtained after fusion with A. tumefaciens containing pLGVTi23 neo (frequency = 10^{-3}). Fusion of E. coli spheroplasts containing a virulent pTiB6S3::RP4 co-integrate with tobacco protoplasts yielded two hormone independent growing calli producing octopine out of 10^{5} microcalli.

ABBREVIATIONS: PEG, Polyethylene glycol; PVA, Polyvinyl alcohol

INTRODUCTION

A. tumefaciens and A. rhizogenes are phyto-pathogenic gram-negative soil bacteria causing crown gall and hairy root disease respectively on various dicotyledonous plants. Both bacteria engineer their host plants by transferring a part of a large plasmid (Ti-plasmid or Ri-plasmid) into the host nuclear genome (Tomashow et al. 1980; Lemmers et al. 1980; Willmitzer et al. 1980; Chilton et al. 1982; Willmitzer et al. 1982). The transferred part is maintained and expressed in transformed cells (Drummond et al. 1977; Willmitzer et al. 1982). Recently, altered Ti-plasmids containing chimeric drug resistant genes (consisting of the promoter of the nopaline synthase gene linked to different structural genes coding for chloramphenicol acetyltransferase, amino-glycoside phosphotransferase or methotrexate-insensitive dihydrofolate reductase) have been constructed and expressed in plant cells (Herrera-Estrella et al. 1983a, b and Fraley et al. 1983). Deletion of all oncogenic parts of the T-DNA and replacement by foreign DNA do not interfere with the transfer functions of the Ti-plasmid and the transferred DNA segment (borders and nopaline synthase gene) does not reduce the regeneration ability of the engineered cell (Zambryski et al. 1983).

In vitro transformation procedures for economically important plants (e.g. cereals) have not yet been established (Steinbiß and Broughton 1983). Because cereals are not susceptible to A. tumefaciens infection and can only be regenerated from tissue culture with difficulty, no transformation system for such plants has yet been developed. Since attachment of A. tumefaciens cells to the cell wall of cereals might be a limiting factor, in vitro transformation systems such as uptake of isolated Ti-plasmids (Davey et al. 1980; Krens et al. 1982), micro-injection of DNA (Steinbiß and Stabel 1983) or fusion of Agrobacterium spheroplasts with plant protoplasts (Hasezawa et al. 1981) may help overcome the host-range limitations. In this communication we have examined the last possibility.

MATERIAL AND METHODS

Isolation of plant protoplasts: Nicotiana tabacum cv. petit havanna SR1 mesophyll protoplasts were isolated from sterile shoot cultures grown on hormone free Linsmaier and Skoog medium according to Nagy and Maliga (1976), Martón et al. (1979) and Wullems et al. (1981).

Preparation of spheroplasts: Spheroplasts were prepared from A. tumefaciens wild type strains B6S3, C58 and from the mutants C58C1pRi15834 (Willmitzer et al. 1982) (containing the plasmid from A. rhizogenes) and C58C1pLGVTi23 neo. pLGVTi23 neo is a Ti-plasmid with a chimeric drug resistent gene (aminoglycoside phosphotransferase of Tn5) in its T-DNA (Herrera-Estrella et al. 1983b). The method of Hasezawa et al. (1981) was modified for isolation of Agrobacterium spheroplasts: 10 ml of AB minimal medium (Chilton et al. 1974) were inoculated with Agrobacterium and incubated for 24 h at 28°C and 250 rpm. At late log phase, 5 mg of carbenicillin were added and the shaking frequency was reduced to 160 rpm. Two hours later, bacteria were harvested by centrifugation followed by two washings with 20 mM Tris-HCl (pH 8.2), 10 mM Na_2 EDTA, and 0.4M mannitol. The pellet was suspended in the same buffer (4×10^{8} bacteria/ml^{-1}). Freshly prepared lysozyme solution was added to a final concentration of 500 µg/ml^{-1}. After 1.5 h incubation at 30°C with stirring, more than 80% of the bacteria were converted to spheroplasts as shown by interference microscopy (Fig 1D). Clumps were removed by filtration over sterile cotton wool. Finally, spheroplasts were resuspended in K_3 culture medium (4×10^{9} cells/ml^{-1}). E. coli spheroplasts were prepared from E.coli N100 containing a pTiB6S3::RP4 co-integrate (GV5001, Holsters et al. 1978a, b) according to Weiss et al. (1976).

Fusion of spheroplasts with plant protoplasts: Approximately 10^6 tobacco protoplasts in 250 µl K_3 medium (Martón et al. 1979) were carefully mixed with 10^9 Agrobacterium or 2 x 10^9 E. coli spheroplasts in 250 µl of the same medium. 500 µl of 20% (w/w) polyvinylalcohol (PVA, Nagata 1978, kindly supplied by Dr. Nagata) or 40% polyethylene glycol 4000 (PEG, Fluka) were slowly added. After 15 min at room temperature 5 ml of 50 mM glycine-OH buffer (pH 10) containing 50 mM $CaCl_2$ and 0.4 M mannitol (Keller and Melchers 1973) were pipetted stepwise into the viscous mixture. After 20 min at 30°C protoplasts were washed three times with isotonic seawater followed by one hour incubation in K_3 medium. The floating protoplasts were collected and cultured in K_3 medium supplemented with 1 mg/ml^{-1} carbenicillin or 0.5 mg/ml^{-1} cefotaxim (Hoechst, FRG) at a density of 5 x 10^4 cells/ml^{-1}.

Selection for hormone independent growth: One week after fusion, protoplasts were diluted by addition of K_3 medium with 0.3 sucrose (5 x 10^3 protoplasts/ml^{-1}). Three weeks later the hormone concentration was reduced tenfold. After two weeks microcalli were washed with hormone free medium and plated on hormone free K_3 agar medium (0.2 M sucrose). Surviving colonies were transferred to and maintained on hormone free Linsmaier and Skoog agar.

Selection of kanamycin-resistant calli: Three weeks after fusion, 50 µg/ml^{-1} kanamycin was added to the microcalli suspension and fourteen days later the hormone concentration was lowered by dilution with K_3 medium lacking hormones. After two weeks, microcalli were plated on Linsmaier and Skoog agar (without hormones) containing 50 µg/ml^{-1} kanamycin and lowered osmotic pressure (0.2 M sucrose). Controls, consisting of non-transformed SR1 tobacco microcalli and C58 transformed SR1 tissues derived from protoplasts, were completely inhibited by 25 µg/ml^{-1} of kanamycin.

Detection of opines: Lysopine dehydrogenase and nopaline dehydrogenase activity were assayed according to Otten and Schilperoort (1978), agropine and mannopine were identified by the methods of Dahl et al.(1983).

Estimation of transformation frequencies: The frequency of transformation was estimated either as the number of surviving calli producing opines or, for kanamycin resistant calli, as the number of calli surviving on kanamycin containing hormone free agar, relative to the number of microcalli plated on selection medium.

RESULTS AND DISCUSSION

Spheroplast preparation: Using the original method of Hasezawa et al. (1981) as many as one hundred Agrobacterium spheroplasts clumped together. We tried to avoid this by stirring the cells during lysozyme treatment and removed the remaining clumps by filtration through cotton wool. Fig. 1C, D shows intact B6S3 Agrobacterium cells and freshly prepared spheroplasts. Not all of the bacteria can be converted to rounded spheroplasts. Up to 20% retained their original shape. Such preparations were still able to induce tumors on Kalanchoe leaves, albeit at much lower frequencies.

Hasezawa et al. (1981) showed that uptake of untreated Agrobacteria gave rise to transformed calli, but with a tenfold reduced frequency compared to uptake of Agrobacterium spheroplasts. In contrast to Hasezawa et al. (1981) the introduction of untreated Agrobacteria into SR1 tobacco resulted in the drastic loss of viable plant protoplasts and we were unable to recover transformants. Since it is known that exopolysaccharides of Agrobacterium and other bacteria can be toxic to plant cells (Beiderbeck 1977) we assume that uptake of intact Agrobacteria into plant protoplasts is more toxic than fusion with Agrobacterium spheroplasts.

We are aware of the fact that it is difficult to exclude a modified type of cocultivation in experiments using impure Agrobacterium spheroplast preparation. We would stress however that all Agrobacterium strains were pretreated with 500 µg of carbenicillin for 2 hours before preparation of spheroplasts. Such pretreated Agrobacteria (e.g. B6S3) are in the presence of carbenicillin unable to induce lysopine dehydrogenase activity in the sensitive Kalanchoe internode assay (Otten 1982).

Nevertheless at the beginning of the experiments there were about 200 non-spheroplast Agrobacteria per protoplast. After the fusion, washing and floating steps, the number of bacterial cells present, however, were reduced at least about 10^6 fold. That means that about 1000 spheroplasts remained in the cultured protoplast suspension. In other words in the worst case (70% loss of initial protoplasts) there would be 1 spheroplast per 300 protoplasts.

Alternative experiments carried out omitting PEG or PVA and high pH Ca^{2+} treatments following the same washing, cleaning and culture steps failed to generate transformed tissues.

E. coli spheroplasts prepared from E. coli N100 according to Weiss (1976) are completely spherical (Fig. 1A, B) and transformation via cocultivation can be excluded since this particular E. coli strain (containing a Ti:RP4 cointegrate) is completely avirulent (Holsters et al. 1978).

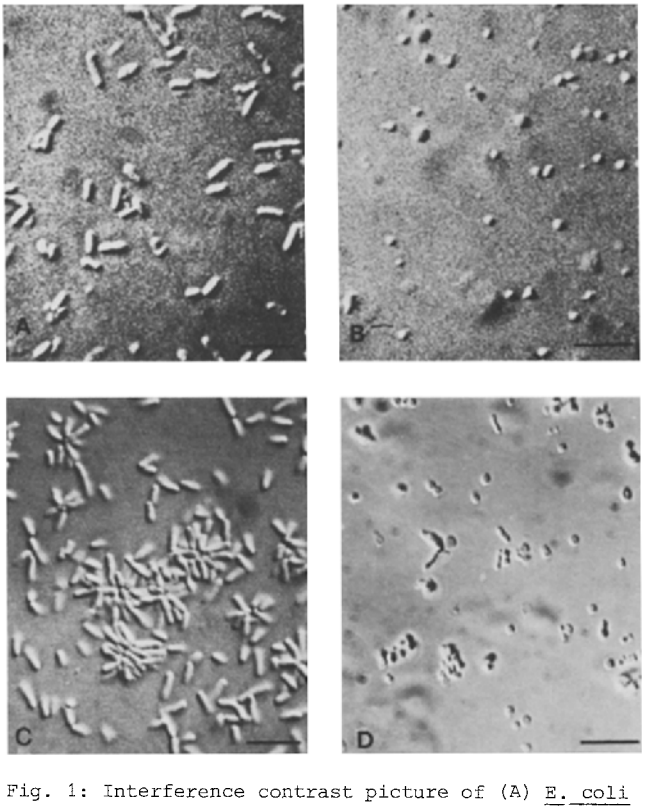

Fig. 1: Interference contrast picture of (A) E. coli N100, (B) E. coli N100 spheroplasts, (C) A. tumefaciens B6S3 and (D) A. tumefaciens B6S3 spheroplasts. bar = 5 µm

Fusion of bacterial spheroplasts with tobacco protoplasts: after addition of PEG or PVA the spheroplasts stuck to the tobacco plasmalemma (Fig. 2). Some fused plant protoplasts were also visible. At the end of the fusion procedure, 30 - 70% of the initial protoplasts were lost. Differences between PEG and PVA treatments were not observed and negative effects of the antibi-

otic treatments were not noticed. Cefotaxim and carbenicillin seemed to be most effective for the elimination of the remaining bacterial spheroplasts. The fate of the spheroplasts after fusion into plant protoplasts has not yet been investigated in detail (Hasezawa et al. 1981, 1983; Matsui et al. 1983), but we assume that they would be digested within the cytoplasm in a few hours.

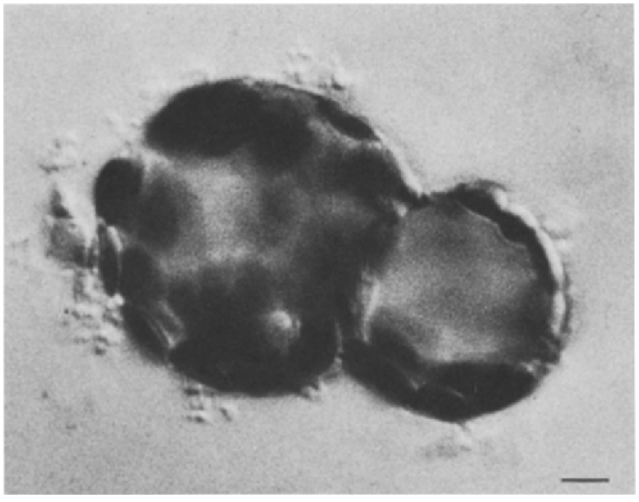

Fig. 2: Interference contrast microscopic picture of E. coli N100 sticking to the plasmalemma of SR1 tobacco protoplasts during treatment with polyethylene glycol. bar = 5 µm

In this sense, spheroplasts are comparable to liposomes as gene transfer systems. So far, it has not been possible (with reasonable degrees of efficiency) to encapsulate macro-molecules as large as Ti-plasmids in liposomes and thus transfer the entrapped DNA to plant cells (Steinbiß and Broughton 1983). It is assumed that bacterial spheroplasts will protect the Ti-plasmids from degradation by nuclease activity and damage caused by experimental conditions (Davey and Kumar 1983). Furthermore, the ability to amplify plasmid DNA in E. coli might increase the frequency of transformation.

Frequency of transformation: The frequencies of transformation of tobacco protoplasts with different bacterial spheroplasts are summerized in Table 1.
The lowest frequency was obtained with E. coli strain pGV5001, but this could be due to the fact that the Ti-plasmid was delivered as an RP4 co-integrate (A. tumefaciens C58 containing Ti::RP4 co-integrates showed reduced transformation frequencies compared with wild type strains - Holsters et al. 1978).

Transformation efficiencies using Agrobacterium spheroplasts are significantly higher than those obtained in experiments with naked Ti-plasmids (Davey et al. 1980; Krens et al. 1982). Superior to both is the so-called co-cultivation system (Martón et al. 1979; Wullems et al. 1981; Jia et al. 1983; Herrera-Estrella et al. 1983b; Fraley et al. 1983). In our hands transformation frequencies up to 15% of the selected microcalli were obtained by cocultivation of regenerating SR1 tobacco protoplasts with Agrobacterium. Yet this method has limitations too: (a) only a few species can be transformed successfully (which might be due to an unsuitable culture medium for both partners - Jia et al. 1983; and for example soybean protoplasts survived fusion with Agrobacterium spheroplasts but not cocultivation with Agrobacterium - Ozias-Akins and

Table 1: Transformation frequencies of tobacco protoplasts by fusion with Agrobacterium and E. coli spheroplasts

Bacterial strains	Initial protoplasts	Antibiotic used[a]	Microcalli selected	Putative transformants	Frequency	Opine positive	Transformation frequency
a) A. tumefaciens SB6S3	10^6	Cb	5×10^4	51	1.02×10^{-3}	17	3.4×10^{-4}
spheroplasts	10^6	Cla	10^5	42	4.2×10^{-4}	12	1.2×10^{-4}
b) A. tumefaciens C58	10^6	Cb	5×10^4	26	5.2×10^{-4}	13	2.6×10^{-4}
spheroplasts	10^6	Cla	5×10^4	22	4.5×10^{-4}	9	1.8×10^{-4}
c) A. tumefaciens C58CIpRi15834 spheroplasts	10^6	Cb	10^5	25	2.5×10^{-4}	5	5.0×10^{-5}
d) A. tumefaciens C58CIpLGVTi23neo spheroplasts	10^6	Cb	10^5	214	2.1×10^{-3}	n.d.[b]	2.1×10^{-3}[c]
e) E. coli N100 pTiB6S3::RP4 spheroplasts	10^6	Cla	10^5	n.d.[b]	---	2	2.0×10^{-5}

[a] Cb, Carbenicillin; Cla, Claforan (Cefotaxim)
[b] n.d., not determined
[c] determined by resistance to 50 µg/ml kanamycin

Hain, unpublished observation) and (b) the insusceptibility of monocotyledonous plants to tumor induction (which is presumably due to the absence of bacterial attachment sites on the cell wall - Lippincott and Lippincott 1980; Schilperoort and Wullems 1983).

Properties of transformed calli: Similar to the observations of Wullems et al. (1981) using the co-cultivation method we obtained a variety of phenotypes after fusion with B6S3 spheroplasts (Fig. 3). Tumor calli derived from these experiments grew as undifferentiated white, octopine positive calli (Fig. 3A, F) or as teratoma like tissues (Fig. 3B, C, E). Separated teratoma shoots developed on hormone free medium into teratomous tissue. On the other hand, some octopine positive calli gave rise to normal looking shoots. They were octopine negative and formed roots when subcultured on hormone free medium. We assume that these shoots were derived from untransformed cells present in the material.

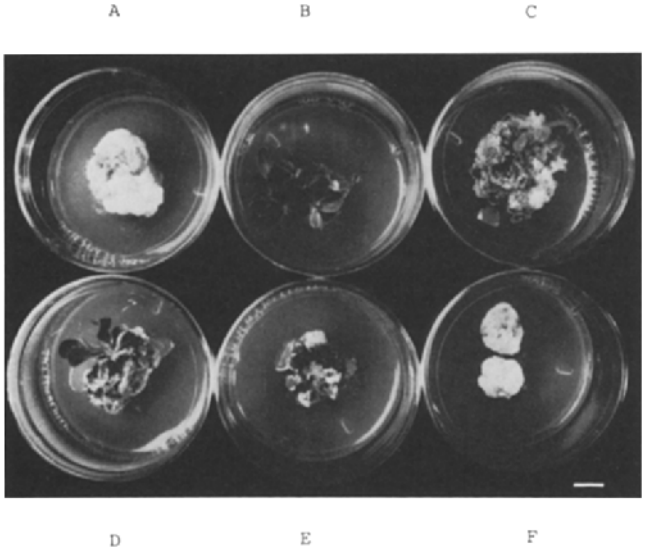

Fig. 3: Properties of transformed calli derived from fusion of A. tumefaciens B6S3 spheroplasts with tobacco protoplasts (A-F). bar = 1 cm

Calli derived from C58C1pRi15834 treated tobacco protoplasts formed typical "hairy roots" after selection on hormone free medium (Fig. 4). One contained mannopine, four mannopine and agropine and the rest (20) none of these opines.

Surprisingly, when E. coli spheroplasts were used many leaves , shoots and embryos developed as early as 3 weeks after fusion. This effect cannot be due to the presence of the Ti-plasmid, since it was also observed using E. coli spheroplasts containing only pBR322 derivatives. It is known that bacterial extracts contain cytokinins (Beiderbeck 1977). It is therefore possible that the effect might have been caused by the release of cytokinin-like substances from the E. coli spheroplast after uptake into the protoplasts.

On the other hand, it has been recently demonstrated that microinjection of pBR322 into mammalian cells stimulates DNA synthesis and alters the cell cycle in these cells (Hyland et al. 1984). It could be possible that such phenomena were involved in our findings.

We checked more than one hundred shoots derived from the E. coli N100 spheroplast experiment for octopine synthesis, but all were negative. Our on hormone independence based selection was difficult due to the cross feeding by the shoots and leaves, but by transferring all developing calli to solid hormone-free medium we succeeded in selecting two hormone independent calli which produced octopine.

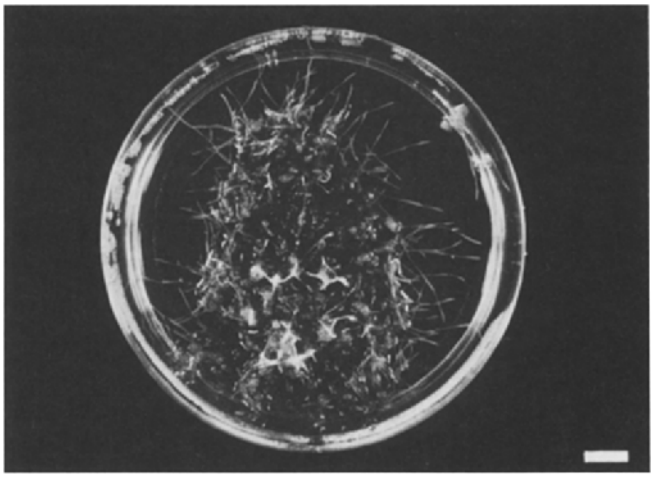

Fig. 4: Calli derived from fusion of A. tumefaciens C58C1pRi15834 spheroplasts with tobacco protoplasts formed typical hairy roots on hormone free medium. bar = 1 cm.

In general hormone independence and opine production are used for identification of transformed tissues (Jia et al. 1983). From this point of view our lines are real transformants. The presence of T-DNA in B6S3 derived tissues was confirmed by Southern hybridization (Southern 1975) (data not shown).

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