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Transformation of *Solanum nigrum* L. protoplasts by *Agrobacterium rhizogenes*

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Summary

Solanum nigrum protoplasts were co-cultivated with Agrobacterium rhizogenes harboring agropine-type Ri plasmid (pRi15834). A large number of transformed calli were obtained on Murashige and Skoog's (MS) medium lacking plant growth regulators. Frequency of transformation was about 3.5×10^{-3}. In most of the calli, hairy roots appeared on MS medium without plant growth regulator. When the hairy roots were cut into segments and subcultured on MS medium lacking plant growth regulators, calli were readily formed. Plantlets were regenerated by transferring those calli to MS medium supplemented with 1 mg/l zeatin and 0.2 mg/l naphthaleneacetic acid. Frequency of plant regeneration was about 70 %.

Introduction

Agrobacterium rhizogenes causes hairy root disease in many dicotyledonous plants (Elliot, 1951). A. rhizogenes is closely related to the members of biotype 2 group of A. tumefaciens by physiological and DNA homology criteria (White and Nester, 1980). However, A. rhizogenes is distinguished from A. tumefaciens because it induces hairy root disease. Interestingly, when spheroplasts of A. tumefaciens strain harboring the virulence Ri plasmid that originated from A. rhizogenes strain 15834 (Willmitzer et al.1982) were incorporated into SR-1 mesophyll protoplasts of Nicotiana tabacum cv. Petit Havana, several calli were formed and typical hairy roots were formed on the calli (Hain et al. 1984). The transformation of normal cells to cells showing non-selflimiting growth is induced by the presence of A. rhizogenes large plasmids (Ri plasmids) (Currier and Nester, 1976). Most natural isolates of A. rhizogenes have been found to carry three large plasmids (White and Nester, 1980; Costantino et al. 1981; Petit et al. 1983). A. rhizogenes strain 15834, which incites hairy root disease in dicotyledonous plants, also harbors three large plasmids: pRi15834a (107×10^6 daltons), pRi15834b (154×10^6 daltons), and pRi15834c (258×10^6 daltons). The largest constituent plasmid is a cointegrate of the two smaller ones (White and Nester, 1980).

In vitro transformation of plant cells by co-cultivation and transfer of foreign genes into plant cells using A. tumefaciens Ti plasmid as a vector are now becoming routine in many plants, including some crop plants, such as potato and tomato. However, the in vitro transformation of protoplasts and introduction of foreign genes into the plant genome using A. rhizogenes Ri plasmid as a vector have not yet been established. We report here stable transformation of Solanum nigrum L. protoplasts by co-cultivation with A. rhizogenes strain 15834.

Material and Methods

Bacterial strains: A. rhizogenes wild type strain 15834 harboring pRi15834 was used in this work. The bacteria were grown overnight in 20 ml of YEB liquid medium (Vervliet et al. 1975) at 28°C with shaking in dark. The bacteria were harvested by centrifugation followed by two washings with 0.4 M sucrose solution, then suspended in the 0.4 M sucrose solution.

Isolation of plant protoplasts: Mesophyll protoplasts of Solanum nigrum L. were isolated from sterile shoot cultures grown on hormone-free MS medium. Leaves were cut into 2 mm strips, then treated with an enzyme solution consisting of 2 % Cellulase Onozuka R-10, 0.4 % Macerozyme R-10 and 0.4 M sucrose, the pH being adjusted to 5.7 with 1N NaOH. During a 16 h incubation in the dark at 26°C with occasional gentle swirling, mesophyll cells were converted into protoplasts. Isolated protoplasts were separated from non-digested cells and cell debris by filtering through Miracloth with 50 µm pore size. Protoplasts were washed twice by density floatation with repeated centrifugation (4 min at ca. 100 xg) using 0.4 M sucrose solution in a 10 ml test tube. Protoplasts were cultured at a density of 4×10^5 per ml in MS liquid medium (Murashige and Skoog, 1962) supplemented with 1 µg/ml 2,4-dichloro-

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phenoxyacetic acid (2,4-D), 0.5 µg/ml benzyladenine (BA) and 0.4 M sucrose, in 4 ml aliquots in plastic Petri dishes (60 mm x 15 mm). The dishes were sealed with parafilm and kept at 26°C in the dark for three days.

Co-cultivation of protoplasts with A. rhizogenes: Three-day-old cell wall regenerating protoplasts were co-cultivated with bacteria for 36-48 h at a ratio of ca. 250-1,000 bacteria per protoplast in the dark at 26°C. Following co-cultivation, free bacteria were removed by repeated centrifugation (4 min at ca. 100 xg) with the above medium, and the plant cells (5×10^4 per ml) were cultured in MS medium supplemented with 0.5 µg/ml 2,4-D, 0.25 µg/ml BA, 250-1,000 µg/ml carbenicillin, and 0.3 M sucrose at 26°C. The light intensity during this incubation and following treatments was ca. 500 lx.

Selection for hormone-independent growth: After 20 days of culture, microcolonies were collected by centrifugation, washed and plated on selection medium solidified with 0.2 % gelrite (MS medium supplemented with 0.3 M sucrose and 500 µg/ml carbenicillin, pH 5.7) lacking phytohormones. Within 15 days, transformants which showed hormone independent growth could be readily distinguished. After 35 days, surviving microcalli were transferred to and maintained on hormone-free MS medium solidified with 0.8 % agar.

Detection of opines: Agropine and mannopine were identified by the methods of Tepfer et al. (1981).

Estimation of transformation frequencies: The frequency of transformation was estimated either as the number of surviving calli producing opines or as the number of calli surviving on hormone-free MS solidified medium, based on the initial number of protoplasts.

Results

Effect of carbenicillin on survival of bacteria: The majority of bacteria were removed by repeated centrifugation after co-cultivation. The growth of the remaining bacteria was completely inhibited by the addition of 1,000 µg/ml carbenicillin to the culture medium. This medium allowed normal multiplication of protoplasts. However, addition of 250 µg/ml carbenicillin had only a moderate effect on the growth of the remaining bacteria and the growth of the bacteria resulted in protoplast death.

Frequency of transformation: Frequencies of transformation of protoplasts with A. rhizogenes are summarized in Table 1. Transformed cells were expected to grow on a hormone deprived medium and actually microcalli showing hormone-independent growth were selected. Some of the colonies grown on MS agar medium lacking hormones developed into microcalli (Fig. 1A), which were counted to determine the frequency of microcalli formation. Although the frequency fluctuated, usually about 150-220 microcalli were found in each Petri dish. From these results, the frequency of transformation was calculated to be about $3\text{-}4 \times 10^{-3}$.

Subculture of putative transformants: Subsequently these putative transformants were transferred and maintained on solidified MS medium lacking hormones where ca. 5 % of microcalli were lost during these manipulations. When these remaining calli were subcultured on hormone-free MS agar medium, typical hairy roots were formed after 4 to 5 weeks of the transfer (Fig. 1B).

Tissue culture of hairy roots and plant regeneration: When segments of the hairy roots were transferred to hormone-free MS agar medium, calli were formed from the root segments. When those calli were transferred to MS agar medium supplemented with 1 mg/l zeatin and 0.2 mg/l naphthaleneacetic acid, about 70 % of calli produced shoots (Fig. 1C), which gave rise to roots after the transfer to hormone-free MS agar medium. Therefore, those shoots readily developed to phenotypically normal plants (Fig. 1D).

Detection of opines: Agropine and mannopine were detected in the initial calli, the hairy roots which formed from the calli, and regenerated plants (Fig. 1E). Neither compounds was present in control (untransformed) callus.

Discussion

Solanum nigrum protoplasts were transformed by co-cultivation with A. rhizogenes harboring pRi15834. Our work indicates that an engineered Ri plasmid may be used as a natural vehicle in transformation of plant protoplasts similar to that found for the Ti plasmid. Marton et al. (1979) reported the transformation of streptomycin resistant tobacco (SR-1) cells by co-cultivation with A. tumefaciens harboring the Ti plasmid. Davey et al. (1980) reported the transformation of Petunia protoplasts with Ti plasmid in the presence of poly-L-ornithine. Hasezawa et al. (1981) reported the transformation of Vinca protoplasts with spheroplasts of A. tumefaciens harboring the Ti plasmid. Compared with data reported in these preceding papers, the frequency of transformation in our experiment is much higher (about 10 fold). The difference was possibly due to the fact that, in our system, Ri plasmid was more readily introduced into S. nigrum protoplasts. Recently, Fraley et al. (1984) reported that Petunia protoplasts were transformed with A. tumefaciens at a high frequency (ca. 10^{-1}) by an improved co-cultivation method using a feeder plate. By using this method, it is expected to improve the transformation frequency in our system with S. nigrum protoplasts. As tobacco SR-1, Petunia and Vinca protoplasts, S. nigrum protoplasts can also be an appropriate experimental system for the introduction of foreign genes. The division frequency of S. nigrum protoplasts transformed with A. rhizogenes was also higher than that of tobacco SR-1 protoplasts transformed with A. rhizogenes in our experiments (data not shown here).

The toxicity of the Ri plasmid to transformed tissues is generally less than that of Ti plasmid, and thus, roots formed due to Ri plasmid transformation can readily form calli, followed by easy regeneration of phenotypically normal plants. In addition, opine (agropine and mannopine) could be detected in calli, hairy roots and regenerated plants. Therefore, engineered Ri plasmid will certainly constitute appropriate vehicles for the introduction of foreign genes into protoplasts and the transformation of plants.

Table 1. Transformation frequencies by co-cultivation of protoplasts with A. rhizogenes

Bacterial strain	Initial cell number	Number of putative transformants	Number of transformants exibiting hormone independent growth	Number of colonies producing opine per number of colonies examined	Transformation frequency (Tansformants/ initial cells)
A. rhizogenes strain 15834 (pRi15834)	$5x10^4$	187	178	9/9	$3.6x10^{-3}$
	$5x10^4$	155	146	7/7	$2.9x10^{-3}$
	$5x10^4$	216	203	12/14	$4.1x10^{-3}$

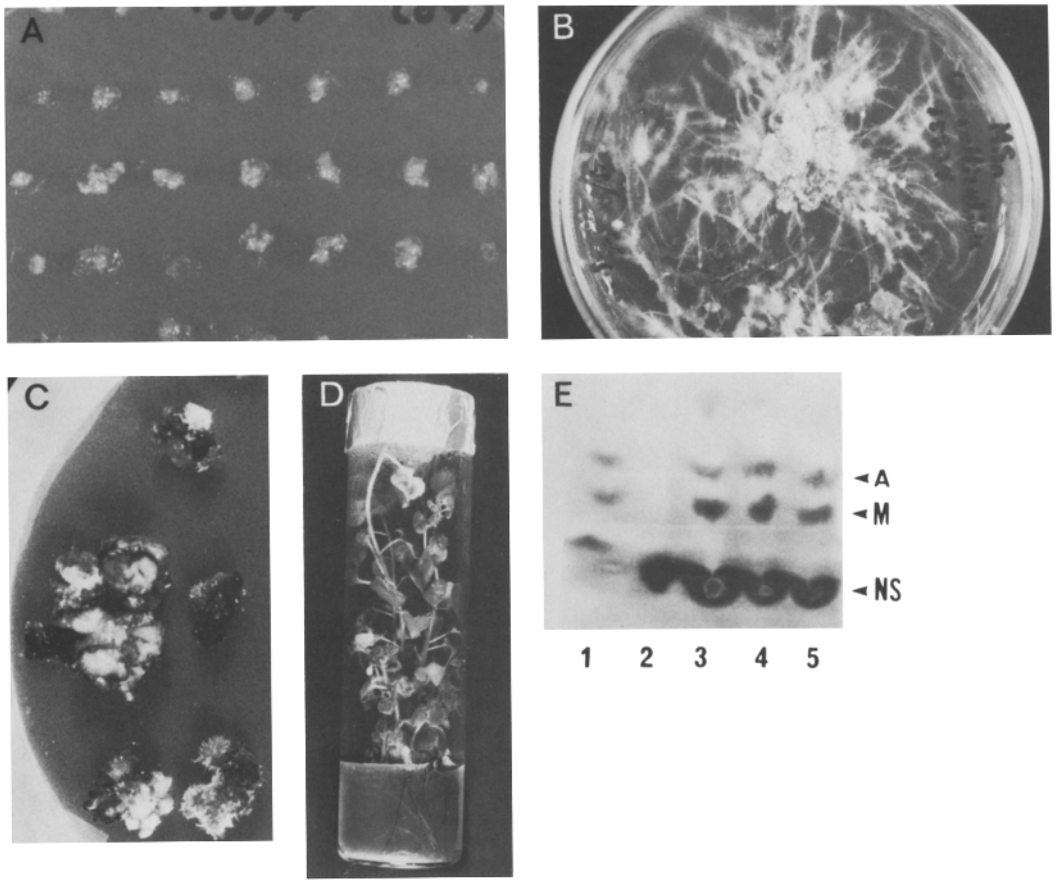

Fig. 1A: Some of microcalli grown on MS agar medium lacking hormones after co-cultivation.
Fig. 1B: Calli derived from co-cultivation of protoplasts with A. rhizogenes on hormone free MS medium. Typical hairy roots were developed from the calli after 4 to 5 weeks of the transfer.
Fig. 1C: Shoot regeneration on calli derived from hairy roots. Calli were transferred to MS medium supplemented with 1 mg/l zeatin and 0.2 mg/l naphthaleneacetic acid.
Fig. 1D: Root development on a shoot placed on hormone-free MS medium to form a phenotypically normal plant.
Fig. 1E: Detection of agropine and mannopine in the extracts of initial callus, hairy root, and a regenerated plantlet, respectively. A; Agropine, M; Mannopine, NS; Neutral Sugar. Lane 1. Standard Marker, 2. Control Callus, 3. Transformed Callus, 4. Hairy Root, 5. Regenerated Plant. The spots were detected by staining with alkaline silver nitrate reagent after the separation by paper electrophoresis.

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References

Costantino P, Mauro ML, Micheli G, Risuleo G, Hooykass PJJ, Schilperoort RA (1981) Plasmid 5:170-182

Currier TC, Nester EW (1976) Anal Biochem 66:431-441

Davey MR, Cocking EC, Freeman J, Pearce N, Tudor I (1980) Pl Sci Lett 18:307-313

Elliot C, (1951) "Manual of Bacterial Plant Pathogens", 2nd rev. ed. Chronica Botanica, Waltham, Mass.

Fraley RT, Horsch RB, Matzke A, Chilton M-D, Chilton WS, Sanders PR (1984) Plant Mol Biol 3:371-378

Hain R, Steinbiß HH, Schell J (1984) Plant Cell Reports 3:60-64

Hasezawa S, Nagata T, Syono K (1981) Mol Gen Genet 182: 206-210

Marton L, Wullems GJ, Molendijk L, Schilperoort RA (1979) Nature 277:129-131

Murashige T, Skoog F (1962) Physiol Plant 15: 473-497

Petit A, David C, Dahl GA, Ellis JG, Guyon P, Cassé-Dolbart F, Tempé J (1983) Mol Gen Genet 190:204-214

Tepfer DA, Tempé J (1981) C R Acad Sci Paris 292: Série III, 153-156

Vervliet G, Holsters M, Teuchy H, Montagu M van, Schell J (1975) J gen Virol 26:33-48

White FF, Nester EW (1980) J Bacteriol 41: 1134-1141

Willmitzer L, Sanchez SJ, Buschfeld E, Shell J (1982) Mol Gen Genet 186:16-22