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Shoot regeneration of mesophyll protoplasts transformed by *Agrobacterium tumefaciens*, not achievable with untransformed protoplasts*

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Summary. Alternative methods for shoot regeneration in protoplast derived cultures were developed in *Nicotiana paniculata* and *Physalis minima*. In both species protoplast derived callus is not regeneratable to shoots by conventional methods, e.g. hormone treatment. Leaf discs and stem segments of *N. paniculata* and *P. minima* were incubated with *Agrobacterium tumefaciens* "shooter" strains harbouring pGV 2215 or pGV 2298 or wild-type strain B6S3. After 36 h of co-incubation protoplasts were prepared. (Leaf disc and stem segment cloning). Co-cultivation experiments were also undertaken with protoplasts of both species. Transformed clones, characterized by their hormone independent growth and octopine production, could be isolated after about two months. Transformation frequencies of "leaf disc and stem segment cloning" and co-cultivation experiments varied from 5×10^{-3} to 5×10^{-5}. After about one year of cultivation on hormone-free culture medium, shoots could be recovered from colonies of *N. paniculata*, transformed by the strain harbouring pGV 2298. In protoplast derived colonies of *P. minima*, shoot induction was obtained only after transformation by bacteria carrying pGV 2215. This demonstrates the importance of the particular "shooter" mutant, as well as the response of the host plant. Transformed shoots of *P. minima* produced octopine, whereas octopine production in transformed shoots and callus of *N. paniculata* was undetectable after one year of cultivation, though T-DNA was still present in the plant genome. Transformed shoots of *N. paniculata* and *P. minima* do not produce any roots. Shoots of *N. paniculata* have an especially tumerous phenotype. Shoots of both species were successfully grafted to normal donor plants of *N. tabacum*.

Key words: *Agrobacterium tumefaciens* "shooter" mutants – Transformation – Shoot regeneration – Protoplasts

* Dedicated to Professor Dr. G. Melchers in occasion of his 80th birthday

Abbreviations: B5-h = Gamborg medium without hormones (Gamborg 1968), V47 = protoplast medium (Binding 1974), D2a = protoplast medium (Li et al. 1980) MS-h = Murashige and Skoog medium without hormones (Murashige and Skoog 1962)

Introduction

In T-DNAs of *Agrobacterium tumefaciens*, three genes, designated gene 1, 2 and 4, play a major role in the onset, maintenance and morphology of plant tumors. Genes 1 and 2 are involved in the biosynthesis of auxins, while gene 4 codes for an enzyme responsible for cytokinin biosynthesis (Leemans et al. 1982; Barry et al. 1984; Schroeder et al. 1984). Inactivation of either an auxin-gene (1, 2) or cytokinin-gene (4) leads to a shooting ("shooter") or rooting phenotype respectively on tumors of *Nicotiana tabacum* (Ooms et al. 1981; Willmitzer et al. 1982). The expression of the T-DNA allows furthon transformed plant cells to grow without plant hormones and to produce specific compounds, called opines, via T-DNA encoded enzymes (Petit et al. 1978).

Since the number of plant species from which protoplasts can be regenerated to shoots is limited (Davey and Kumar 1983), we decided to ask if "shooter" mutants of *A. tumefaciens* could help to induce shoots from undifferentiated tissue of *N. paniculata* and *P. minima*.

Differentiation experiments via hormone treatment with protoplast derived colonies of both species were undertaken intensively. Bourgin et al. (1979) occasionally observed buds on calli originating from protoplasts of *N. paniculata*. Shoot formation in protoplast derived wildtype callus cultures of *P. minima* failed to occur in spite of growth under a variety of phytohormones and nutrient supplements (Bapat and Schieder 1981).

To obtain a sufficiently high number of transformed clones in vitro, effective cell transformation methods are necessary. Two in vitro transformation methods have been well established which lead to single cell derived clones: co-cultivation of developing plant protoplasts with *A. tumefaciens* (Márton et al. 1979; Wullems et al. 1981) and the fusion of protoplasts with *Agrobacterium* spheroplasts (Hasezawa et al. 1981; Hain et al. 1984). In this paper we present an alternative method leading to single cell derived clones, namely the production of protoplasts from leaf discs and stem segments shortly after treatment with *A. tumefaciens* (Leaf disc and stem segment cloning).

Materials and methods

1 Leaf disc and stem segment preparation

Leaf discs (0.4 cm diameter) were excised with a corkborer and stem segments were obtained by cutting stems from aseptic shoot cultures into small pieces of 0.5–1 cm length with a razor blade. The shoot material of *N. paniculata* was cultured on B5-h medium (0.8% agar) and shoot material of *P. minima* on MS-h (0.8% agar), at a temperature of 26 ± 1 °C, and with a 16 h photoperiod of 3,000 lux (Cool white, Power Grove, General Electric). Leaf discs or stem segments were transferred to a plastic Petri dish (9 cm diameter) with 10 ml liquid B5-h (MS-h).

2 Agrobacterial strains

Wildtype strain B6S3 and "shooter" mutants C58Cl (pGV 2215) and C58Cl (pGV 2298) of *A. tumefaciens* were used. C58Cl (pGV 2215) is a substitution mutant in the T-region of pTi B6S3; gene 2 was deleted (Leemans et al. 1982). C58Cl (pGV 2298) is a mutant in the T-region of pTi B6S3 with the deletion of genes 1, 2, 5, 7, 6a and 6b. (R. Deblaere, personal communication). Gene 3 of the T-DNA, coding for LpDH, the enzyme responsible for octopine synthesis, is present in all three Agrobacterial strains.

Bacteria were grown in 30 ml of AB medium (Chilton et al. 1974) to the late log phase on a gyratory shaker under continuous dim light at 150 rpm.

3 Leaf disc and stem segment infection

Leaf discs and stem segments were incubated with 100 µl of the late log-phase bacterial culture at 27 °C under 1,000 lux continuous illumination. After 2 days, leaf discs and stem segments were washed twice with B5-h (*N. paniculata*) or MS-h (*P. minima*) containing 0.05% carbenicillin. Protoplasts were isolated overnight and purified as described by Schieder (1984).

Protoplasts from leaf discs and stem pieces of *N. paniculata* were cultivated in V47 medium and those of *P. minima* in D2a medium, containing 0.025% carbenicillin, at a density of 5×10^4 protoplasts per ml.

4 Co-cultivation

Two-day-old protoplasts, isolated from leaves of aseptic grown shoot material, cultured in D2a-red (*P. minima*) or V47-red (*N. paniculata*) (red = half hormone concentration) at a density of 1×10^5 protoplasts per ml, were incubated with bacteria of the late log phase at 27 °C for 36 h at 1000 lux. In the case of *P. minima,* the mixture was centrifuged and washed once with the same protoplast medium supplemented with 0.05% carbenicillin. The cells were then cultured at 5×10^4 cells per ml in D2a-red, containing 0.025% carbenicillin. In the case of *N. paniculata,* the mixture was diluted 1 : 2 with V47-red containing 0.025% carbenicillin, and cultured as described above.

5 Selection for hormone independent growth and lysopine dehydrogenase (LpDH) assay

Three-week-old colonies of *N. paniculata* were diluted 1 : 2 with V47 containing 0.025% carbenicillin, 0.4% agar, 0.35 M mannitol. Three-week-old colonies of *P. minima* were diluted 1 : 2 with D2a containing 0.025% carbenicillin, 0.4 M glucose. Both media lacked hormones. In addition, aliquots of the cultures were diluted with hormone-containing medium to test viability. After another 3 weeks of cultivation, dishes were split and colonies were transferred to V47 (*N. paniculata*) or D2a (*P. minima*) agar medium (0.8% agar, 0.09 M sucrose, 0.025% carbenicillin, lacking mannitol or glucose) either with hormones (as control) or without hormones (for selection). Surviving colonies were transferred to fresh culture medium. For the induction of plantlets, different combinations of auxins and cytokinins were tested in the medium.

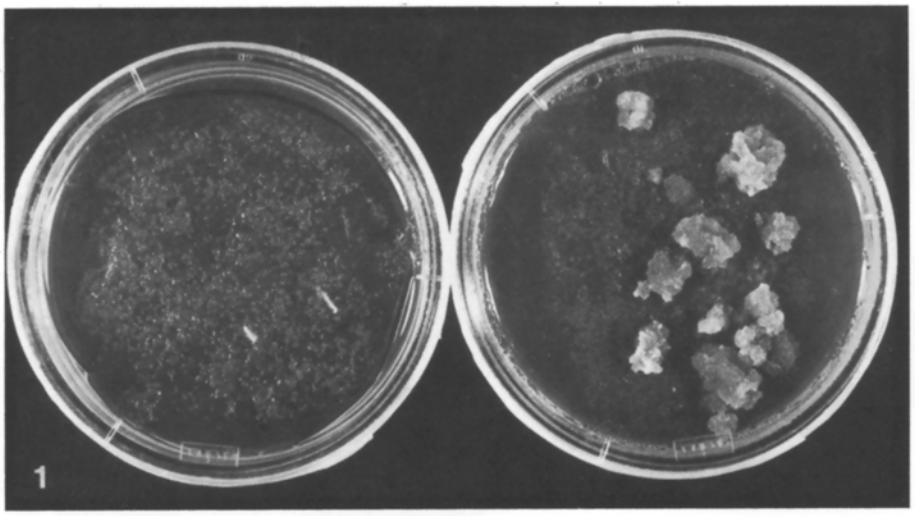
The LpDH assay was carried out as described by Otten and Schilperoort (1978).

Results

1 "Leaf disc and stem segment cloning"

The initial division rate (the number of protoplasts which started to divide after 2–3 days of cultivation) of protoplasts isolated from *Agrobacterium*-treated leaf discs of *N. paniculata* was about 50%. Protoplasts prepared from discs of *P. minima* divided at a rate of about 10% to 20%. Leaf discs of *P. minima* were found to be susceptible to any form of harsh treatment, especially to a lack of sufficient moisture. From 80% dividing stem-segment-derived protoplasts of *P. minima,* about 60% continued growing to callus without the application of any selection pressure. The division rate of protoplasts isolated from stem pieces of *N. paniculata* was lower by a factor of 2 in comparison to *P. minima.*

Three to four weeks after protoplast preparation, colonies were transferred to hormone-free medium. After about four weeks of culture on this medium, dark, pale-green and also white calli of *N. paniculata* grew against a background of brown dying cell colonies.



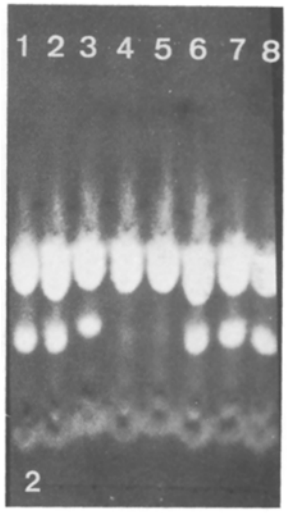


Fig. 1. Colonies of *N. paniculata* (8 weeks), which derived from protoplasts of *Agrobacterium*-treated leaf discs, grown on hormone-free medium (*right*). Control plate (*left*)

Fig. 2. Electropherogram of transformed tissue of *N. paniculata,* derived from protoplasts of *Agrobacterium*-treated leaf discs. *Lane 1:* octopine positive control tissue, *lane 2, 3, 6, 7, 8:* octopine-positive, hormone-independently growing colonies, *lane 4, 5:* octopine-negative, hormone-independently growing colonies

Cultures of *P. minima* had to be transferred twice to hormone-free medium before dark green and compactly growing colonies could be recovered in *Agrobacterium*-treated cultures. Control cultures on selective medium never gave rise to such calli (Fig. 1).

Octopine production could be detected in 70% of the hormone-independent and therefore putatively transformed colonies of *N. paniculata* and 90% of those of *P. minima* (Fig. 2).

Transformation frequencies varied from experiment to experiment, ranging between 5×10^{-3} and 5×10^{-5}, calculated by counting octopine positive colonies on all plates of one experiment on the basis of the initial protoplast density.

2 Co-cultivation

Both species were found to be useful for co-cultivation experiments with *A. tumefaciens.* After two days of culture, when most protoplasts were elongated and some of them already dividing, the bacteria were added. Protoplasts and bacteria were incubated for 32 h. In the case of *N. paniculata,* the dilution of the mixture with fresh carbenicillin-containing medium was less harmful to the cells than centrifugation. After this treatment protoplasts were plated with a density of about 5×10^4 protoplasts per ml.

The growth rate of protoplasts to callus after co-cultivation with bacteria was reduced (about 20%) for both *N. paniculata* and *P. minima,* in comparison to untreated control cultures.

After several weeks of cultivation on hormone-free medium some colonies continued growing while most of the surrounding, probably untransformed ones were dying. The data on transformation frequencies do not show significant differences between the "leaf disc and stem segment" method on one hand and the co-cultivation method on the other hand.

3 Shoot regeneration

Fifty transformed colonies of *P. minima* and 55 of *N. paniculata* were cultured on hormone-free medium and observed for more than one and a half years after protoplast isolation.

Three months after protoplast preparation, the phenotype of the colonies transformed by B6S3, C58Cl (pGV 2215) and C58Cl (pGV 2298) ressembled that of the wildtype colonies on hormone-containing medium. No morphogenic capacity was observed in either of the transformed calli during that time. After several transfers to hormone-free medium (about 6 months after protoplast isolation) some of the "shooter"-transformed colonies became morphogenic. They produced buds and finally formed shoots.

It was found that the regeneration of shoots for a given species was strictly dependent on the bacterial strain, used for transformation. Colonies transformed by the wildtype strain B6S3 never showed morphogenic capacity neither in *P. minima* nor in *N. paniculata.* In experiments with *P. minima,* regeneration to shoots could only be achieved after transformation with C58Cl

Fig. 3. Comparison between wildtype shoots (*right*) and transformed shoots (*left*) of *N. paniculata*

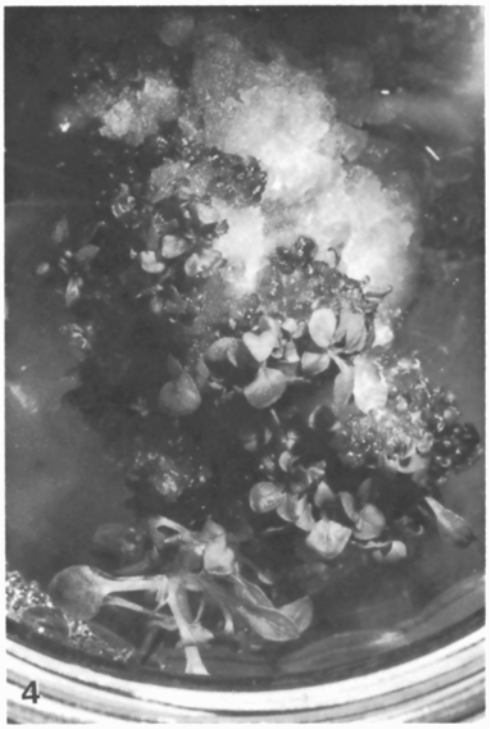


Fig. 4. Shoot producing colony of *P. minima*, transformed by C58Cl (pGV2215) on hormone-free medium

Table 1. Shoot regeneration in protoplast derived transformed colonies

	Nicotiana paniculata		
	pTi B6S3	pGV 2215	pGV 2298
"Leaf disc cloning"	–	–	+
"Stem segment cloning"	–	–	*
Co-cultivation	–	–	+
	Physalis minima		
	pTi B6S3	pGV 2215	pGV 2298
"Leaf disc cloning"	–	*	*
"Stem segment cloning"	–	+	–
Co-cultivation	–	+	–

+: Shoot regeneration in more than 1 transformed colony per experiment
* : Not carried out

(pGV 2215) whereas shoot production from *N. paniculata* was only induced by C58Cl (pGV 2298). An overview is presented in Table 1.

Shoots of both species do not produce any roots. The tumerous phenotype of shoots of *N. paniculata* is conspicuous (Fig. 3), whereas shoots of *P. minima* appear rather normal (Fig. 4). Shoot material of both species could be grafted to *N. tabacum*.

Several shoots were tested for LpDH-activity. It was found that shoots of *P. minima* were octopine-positive, while octopine could not be detected in the shoots or in callus of *N. paniculata* after one year of cultivation. By Southern hybridization experiments it could be shown that T-DNA of pGV 2298 is present in the plant genome (data not shown).

It was not possible to induce shoot formation in protoplast derived wildtype or B6S3-transformed colonies of both species by conventional regeneration methods. No hormone combination caused bud or shoot formation. Even cytokinin shock treatment for three days with a subsequent culture on hormone-free medium did not induce morphogenesis. The morphogenic capacity of the "shooter"-transformed colonies of both species could not be increased by cytokinin-treatment.

Discussion

In this report two main results are presented:
1. Shoot regeneration of mesophyll protoplasts is possible with the help of "shooter" strains of *A. tumefaciens*.
2. The "leaf disc and stem segment cloning" method can be effectively used as an alternative to co-cultivation for the transformation of protoplasts of *N. paniculata* and *P. minima*.

We agree with Ooms et al. (1981), who postulate from their results with *N. tabacum,* that the tumor phenotype depends on the relative concentration of various phytohormones present in the cells. They propose that genes 1 and 2 of the T-DNA control an auxin-like effect. Consequently, inactivation of either of these genes would result in lower auxin-activity. On the other hand, gene 4 is thought to cause a cytokinin-like effect. The "shooter" mutants of *A. tumefaciens,* like C58Cl (pGV 2215) or C58Cl (pGV 2298) could, therefore, change the internal auxin/cytokinin ratio so that shoots are induced. Without external influence shoots could be recovered in transformed material after one year of culture. One may speculate that a long culture period is necessary to achieve a suitable internal hormone balance to induce morphogenesis.

The choice of the "shooter" mutant is important for the shoot development of different species. Only C58Cl (pGV 2298) could induce shoots in protoplast derived cultures of *N. paniculata.* Shoot development in colonies of *P. minima* was only obtained after transformation with C58Cl (pGV 2215).

The type of response after transformation with *A. tumefaciens* "shooter" strains is not only "shooter" dependent, but also host-specific. This result was also found by others. In *N. tabacum* and *Kalanchoe daigremontiana* the inactivation of genes 1 or 2 leads to a "shooting" tumor. On *Nicotiana rustica,* tumor induction with "shooter" strains is not very different from tumors induced by wildtype strains of *A. tumefaciens* (Ooms et al. 1981). Therefore, we recommend the testing of several "shooter" mutants with different Ti-plasmids in order to find the most effective combination of host-plant and *Agrobacterium.*

The transformed shoots do not produce roots. One can overcome this disadvantage in plant development by grafting. After selfing one might obtain stably transformed and untransformed seedlings as described for *N. tabacum* (Woestemeyer et al. 1984).

We have compared co-cultivation experiments with the "leaf disc and stem segment cloning" method. Shoot regeneration in all cases is correlated to the bacterial strain in combination with the host, but not to the transformation method (see Table 1). The data of the transformation frequencies do not show significant differences between the "leaf disc and stem segment" method on one hand and co-cultivation method on the other hand.

In both methods hormone independent octopine negative lines were found. One cannot, therefore, exclude the existence of partial or abortive transformants and it is possible that crossfeeding by transformed calli caused the independent growth of untransformed clones. Habituation to hormone-free medium in control experiments did not occur.

The loss of octopine activity in colonies of *N. paniculata,* which continue to produce shoots gives rise to a number of speculations. As we know from Southern hybridization experiments the T-DNA is integrated into the plant genome. The absence of octopine in the tissue may be due to methylation of the LpDH-gene (Van Slogteren et al. 1984). We cannot at this time exclude small deletions or point mutations as the cause of the loss of octopine.

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