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Effective Protocol for *Agrobacterium*–mediated Leaf Disc Transformation in Tomato (*Lycopersicon esculetum* Mill.)[ψ]

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An efficient transformation system for Indian tomato cultivar, 'Pusa Ruby' was developed by identifying and optimizing various parameters affecting transformation rates, non-transformed growth and bacterial over growth. A protocol consisting leaf explants from 14-day-old *in vitro* seedlings, 2 day pre-conditioning on culture medium, 2 day co-cultivation period for inoculated explants, 100 µm l^{-1} Kanamycin Sulphate and culture medium containing MS salt with 0.1 mg l^{-1} IAA, 1.0 mg l^{-1} Zeatin, 2% (w/v) sucrose, *p*H 5.8 resulted in significant higher transformation rate (66.7%) within 4-6 weeks period. Confirmation of transformation was carried out in protoplast, cell, tissue and shoot cultures by Kanamycin resistance; nopaline assay, NPT-II assay and PCR analysis with primers specific to *npt-II* gene. This system will be certainly exploitable for transferring agronomically useful genes in tomato cultivars.

Keywords: *Agrobacterium*, genetic transformation, tomato, *Lycopersicon esculentum*

Introduction

Transformation is the process by which genetic material from one organism is introduced and incorporated into the genome of another. Genetic transformation has two distinct advantages over classical breeding, the first being selectivity in which single gene for desired trait can be introduced into the concerned cultivar without disturbing plant's genetic make up and thus isogenic lines can be developed. Secondly with genetic transformation it is possible to transfer any desirable gene from any living organism to plants and *vice versa*. Thus making immediate genetic improvement possible. Koorneef *et al* (1987) emphasized the use of *Agrobacterium*-mediated transformation in tomato because of relative simplicity of the technique and reproductive biology of tomato, its ease of culture with higher totipotency and stability of the transplants. Therefore, tomato proved a classical example in genetic transformation, and large number of transgenic plants were reported for various traits such as herbicide resistance, virus resistance, resistance to fungal diseases, insects, and shelf-life (Naik *et al*, 1999).

However, the limitations with this system includes genetic manipulation, which depends on shoot regeneration capacity from leaf explant, in which tomato is poor and genotypically dependent (McCormick *et al*, 1986). Similarly transformation rates with fresh explants are relatively low i.e. 17% (Fillatti *et al*, 1987). Therefore, in present investigation an attempt was made to improve transformation rates in Indian tomato cultivar; various parameters such as plant age, preconditioning of explants, culture media were evaluated in order to develop protocol for efficient *Agrobacterium*-mediated leaf disc transformation in tomato.

Material and Methods

Plant Material and Growth Conditions

The seeds of tomato cultivar, 'Pusa Ruby' (PR) were obtained from Tomato Improvement Project, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra. Axenic leaf explants (1.8 cm^2) were prepared from *in vitro* seedlings (MS medium 25±2°C, 16 hrs photoperiod at light intensity 40 µ mol $m^{-2}s^{-1}$). Leaf explants were pre-conditioned by culturing them on agar-solidified culture medium (MS3 or MS6) with their lower (adaxial) surface in contact with the medium for 2-4 days prior to inoculation with bacteria.

Transformation Procedure

A disarmed strain of *A. tumefaciens* harbouring the cointegrate plasmid; pMON200 was utilized for plant

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transformation. The actual transformation work was carried out according to the procedure described by Davey *et al* (1995) for transformation of tomato by *A. tumefaciens*.

Basically, two separate experiments were carried out to obtain higher transformation frequencies by identifying the effects of various parameters on the regeneration capacity of transformed cells. These two experiments were broadly based on plant age and length of the pre-conditioning period of the explants. In the first experiment, leaf explants were excised from 21-day-old seedlings and pre conditioned for 4 days, whilst in the second experiment, leaf explants were prepared from 14-day-old seedlings and pre conditioned for 2 days. In the first experiment, the effect of different co-cultivation periods was also studied. Both experiments were carried out with a 2^3 (2×2×2) factorial experimental design and the effects of three independent factors each with two levels (leaf explant types: fresh and pre-conditioned; Kanamycin Sulphate concentrations: 50 and 100 μg ml^{-1}; culture media: MS3 & MS6) were analysed on the regeneration potential of transformed cells. Thus, in both experiments total 8 treatment combinations in both experiments were replicated three times and each treatment consisted of 10 explants.

Culture Media

Both culture media (MS3 & MS6) contained MS salt but MS3 medium was supplemented with 0.1 mg l^{-1} IAA, 1.0 mg l^{-1} Zeatin with 2% (w/v) sucrose, and *p*H 5.8 (Wijbrandi *et al*, 1990). While, MS6 medium was supplemented with 0.5 mg l^{-1} IAA, 5.0 mg l^{-1} BAP, 3% (w/v) sucrose, *p*H 5.8 (Gracia-Reina & Luque, 1988).

Transformed Growth (rate), Non-transformed Growth and Bacterial over growth

Following co-cultivation with bacteria (*Agrobacterium*) the percentage leaf explants showing shoot regeneration via specific chlorophyllus callus (i.e. Kanamycin resistant) on selection media was defined as the "transformation rate". By contrast, the percentage of explants showing Kanamycin sensitive, non-chlorophyllous and non-morphogenic callus, which was indued all over the edge of leaf expants within 14 days' of culture and subsequently turned brown was characterized as "non-transformed growth". The percentage leaf explants contaminated with bacterial over-growth were designated as "bacterial over-growth".

Confirmative Tests for Transformation

1 *Kanamycin resistance for shoot cultures.* The transformed shoots were subcultured on agar-solidified MS medium containing 100-1000 µg ml^{-1} Kanamycin Sulphate.

2 *Kanamycin resistance for organogenesis.* The leaf explants of all putative transformants were cultured on MS3 medium containing 100 µg ml^{-1} Kanamycin Sulphate.

3 *Kanamycin resistance for protoplast culture.* The mesophyll protoplast was isolated and cultured according to protocol described by Patil *et al* (1994). After 6th and 9th day culture. 25 and 50 µg ml^{-1} Kanamycin Sulphate was added to the protoplast culture medium. Furthermore, the development of protoplast-derived calli and shoot regeneration was assessed in presence of Kanamycin Sulphate (100 µg ml^{-1}).

4. *Nopaline assay.* The presence of nopaline in transformed plants was assayed by paper electrophoresis (Aerts *et al*, 1979).

5 *NPT II assay.* The assay was carried out as per procedure described by Tomes *et al* (1990).

6 *PCR amplification of a segment of the npt II gene using specific primer.* The PCR amplification was performed according to protocol of Davey *et al* (1995). For PCR analysis two primers specific to *npt II* gene were used which designed by Prof Hall, Texas A and M University, USA; the former primer had a sequence of *5' GTC GCT TGG TCG GTC ATT TCG 3'* ; while reverse primer had a sequence of *3' GTC ATC TCA CCT TGC TCC TGC C 5'*.

Results and Discussion

Effect of Various Parameters on Transformation

The results of both experiments were tabulated in Table 1. In the first experiment, where leaf explants were prepared from 21-day-old axenic seedlings and pre-conditioned for 4 days in respective culture media (Table 1A), the data revealed that no significant differences were observed for the percentage bacterial over-growth due to the various treatment-combinations of three factors. However, non-transformed growth was significantly influenced by explant type (E), Kanamycin concentration (K) and their interaction (Ex K). Four-day pre-conditioned leaf explants produced the highest non-transformed growth (30.8%) compared to the fresh leaf explants (2.5%). A higher level of Kanamycin concentration

(100 μg ml^{-1}) produced significantly lower non-transformed growth (10.8%) than did a concentration of 50 μg ml^{-1} Kanamycin Sulphate (22.5%). The transformation rate was significantly improved only by the explant type. Pre-conditioned leaf explants showed 19.2% shoot regeneration, while fresh leaf explants expressed only 6.7%. However, no significant effects of Kanamycin concentration and culture media were observed on shoot regeneration from transformed cells.

In the second experiment, where leaf explants were prepared from 14-day-old axenic seedlings and pre conditioned for 2 days in respective culture media (Table 1B), data revealed that with such modifications, the bacterial over-growth and non-transformed growth was under control and non-significant due to various factors; only exception was Kanamycin concentration, where higher concentration (100 μg ml^{-1}) completely checked non-transformed growth (0%). However, transformation rate was significantly improved by two factors i.e. explant type (E) and culture medium (M). For explant type, the two days pre-conditioned explants recorded the highest transformation frequency (61.7%), which was significantly superior than the fresh explants (24.2%). Among two culture media, medium MS3 produced significantly more transformed shoots (45.8%) than the medium MS6 (40.0%). In addition, the factor Kanamycin concentration remained non-significant for transformation rate, which indicated that both the concentrations of Kanamycin (50 & 100 μg ml^{-1}) were effective in transformation work.

Table 1—Optimisation of various parameters to obtain a higher transformation rate [Leaf explants giving shoot regeneration on selection plates, following co-cultivation with bacteria] with lower rates for non-transformed growth and bacterial over-growth

Treatments	Table 1A 21-day-old seedling and 4-day-old leaf explant pre-conditioning			Table 1B 14 day-old seedling and 2-day-old leaf explant pre-conditioning		
Medium and Kanamycin concentration	% Bacterial over-growth	% Non-transformed growth	% Trans. rate	% Bacterial over-growth	% Non-transformed growth	% Trans. rate
		Fresh explants (F)			Fresh explants (F)	
MS6-K50	10.0	3.3	3.3	0.0	3.3	20.0
MS6-K100	3.3	0.0	3.3	3.3	0.0	20.0
MS3-K50	6.7	3.3	13.3	3.3	0.0	30.0
MS3-K100	3.3	3.3	6.7	3.3	0.0	26.7
		Pre-conditioned explants (P)			Pre-conditioned explants (P)	
MS6-K50	3.3	43.3	16.7	3.3	6.7	63.3
MS6-K100	6.7	20.0	20.0	3.3	0.0	56.7
MS3-K50	10.0	40.0	20.0	0.0	3.3	60.0
MS3-K100	3.3	20.0	20.0	3.3	0.0	66.7
		Factor mean			Factor mean	
		[1] Explants (E)			[1] Explants [E]	
F	5.8	2.5	6.7	2.5	0.8	24.2
P	5.8	30.8	19.2	2.5	2.5	61.7
		[2] Media (M)			[2] Media (M)	
MS6	7.3	16.7	10.8	2.5	2.5	40.0
MS3	5.8	16.7	15.0	2.5	0.8	45.8
		[3] Kan conc μ g/ml (K)			[3] Kan conc μ g/ml (K)	
K50	7.5	22.5	13.3	1.7	3.3	43.3
K100	4.1	10.8	12.5	3.3	0.0	42.5
		C D at 5% level			C D at 5% level	
E	NS	6.7	9.1	NS	NS	5.8
M	NS	NS	NS	NS	NS	5.8
K	NS	6.7	NS	NS	3.2	NS
ExK	NS	9.5	NS	NS	NS	NS

Kan Conc=Kanamycin concentration, K50 and K100=Kanamycin Sulphate of 50 and 100 μg/ml, MS3 and MS6=culture media, Trans rate=transformation rate, NS=F test nonsignificant.

In both the experiments, higher transformation rates were observed in pre-conditioned explants and MS3 culture medium than the fresh explants and MS6 medium. Similarly, Kanamycin concentration gave satisfactory results at both the levels. However, it was important to note that marked increase in transformation frequency was observed in experiment with 14-day-seedling age and 2 day pre conditioning than in the other one. In this case the transformation rate was increased nearly 4 times (i.e. 6.7 to 24.2%) with fresh explants and about 3 times (19.2 to 61.7%) with preconditioned leaves. Besides, the transformation frequency was increased about 10 times with 2 day pre-conditioning of 14-day-old explants (61.7%) over the fresh 21-day-old explants (6.7%). Thus, it showed solid impact of plant age and preconditioning of explants in transformation of tomato.

Confirmation / Verification of Transformation

Efficient shoot and root growth was observed from putative transformed shoots at all the higher levels (up to 1000 μg ml^{-1}) of Kanamycin Sulphate as compared to the Kanamycin sensitivity of non-transformed shoot at lower level (50 μg ml^{-1}). Similarly, cultured leaf explants of all putative transformants showed shoot regeneration on regeneration medium containing 100 μg ml^{-1} Kanamycin Sulphate. Besides, no detrimental effects of Kanamycin were observed upon protoplast cultures of putative transformants and equally viable micro-colonies were recovered as compared to control. Also protoplast-derived calli were also obtained on selection medium (Unpublished data).

The electropherogram of leaf samples of putative transformants for nopaline assay (Fig. 1A) showed nopaline band similar to a nopaline standard. Thus, the identification of nopaline suggested that stable integration of T-DNA from pMON200 of *A. tumefaciens* into the *L. esculentum* genome had occurred. The autoradiogram of leaf proteins of putative transformants for the NPT II assay (Fig. 1B) confirmed the activity of *npt-II* gene. Nevertheless, the presence of *npt-II* gene was demonstrated in putative transformants by amplification of the DNA segment of *npt-II* gene with PCR analyses using specific primers (Fig. 1C). On electropherogram, a 540 bp PCR amplified DNA fragment of *npt-II* gene was present in plasmid (pCaMVNEO) control and in putative transgenic plants while it was absent in non-transformed plant.

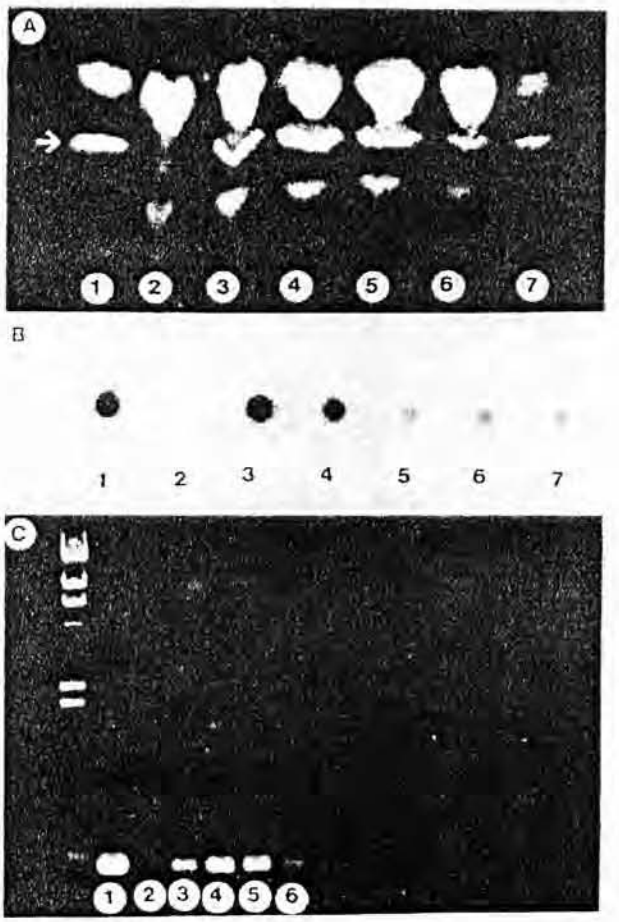

Fig. 1—Confirmative tests for stable transformation of tomato cv PR.

A. Electropherogram of leaf samples of putative transformants for nopaline assay [× 1.1]
1 and 7. Nopaline standard as a positive control.
2. Leaf sample of non-transformed tomato cv PR as a negative control.
3-6.Leaf samples of putatively transformed plants: T-23, T-11, T-7 and T-5.

Presence of the nopaline band is indicated by the arrow.

B. Autoradiogram of leaf proteins of putative transformants for the NPTII assay [× 0.8].
1. *A. tumefaciens* bacterial strain resistant to kanamycin sulphate.
2. Non-transformed tomato cv PR as a negative control.
3-7. Putative transformed plants: T-23, T-11, T-7, T-5 and T-1 respectively.

C. Electropherogram for *npt*II gene segment amplified by polymerase chain reaction (PCR) with specifi primers [× 1.4].
1. Plasmid DNA (pCaMVNEO) containing the *npt*II gene as a positive control.
2. Plant DNA extracted from non-transformed tomato cv PR as a negative control.
3-6. Plant DNA extracted from putative transformants: T-23, T-11, T-7 and T-5 respectively.

After optimizing the parameters such as plant age, co-cultivation period, pre-conditioning of explants, culture media and Kanamycin concentration; an efficient transformation protocol was developed for the tomato cultivar, 'Pusa Ruby', which produced transformed shoots with the highest frequency (66.7%) within short period (4-6 weeks) on selection media. The plant age and explant pre-conditioning period were proved dominating factors which influenced the transformation rate. The results were in agreement with the findings of the earlier workers such as Fillati *et al* (1987) and van Roekel *et al* (1993), who reported increased transformation rate in tomato i. e. 17% from fresh explant to 52% in pre-incubated plants but in those experiments, leaf explants were pre-incubated in tobacco and petunia suspension cells, respectively. However, in present studies the explants were pre-conditioned only on culture medium, which is the most simple and practicable approach with more efficient transformation rate (61.7%).

The enhancement of transformation rate because of preconditioning of explants may be due to exposing more surface area as a result of plant cell division for integration of T-DNA of *Agrobacterium* into plant genome and accumulation of phenolic substances (acetosyringone) that activates virulence genes in *A. tumefaciens* (Lipp Joao & Brown, 1993). The younger *in vitro* plant tissues (14-day-old) proved to be more responsive for shoot regeneration from transformed cells. Fillatti *et al* (1987) also demonstrated that size and age of explants were important factors, which influenced the regeneration and transformation rate in tomato.

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