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An improved protocol for rapid and efficient *Agrobacterium* mediated transformation of tomato (*Solanum lycopersicum* L.)

M. Manamohan*, M.N. Prakash, G. Sharath Chandra, R. Asokan and S.N. Nagesha

**Corresponding author : Division of Biotechnology, Indian Institute of Horticultural Research, Hesaraghatta Lake PO. Bangalore-560089, Karnataka, India. *E-mail. manamohan.s.m@gmail.com, manmohan@iihr.ernet.in*

Abstract

Transformation of tomato with heterologous genes requires rapid and efficient transformation protocols. *Agrobacterium* mediated transformation protocol of tomato (*Solanum lycopersicum* L.) *cv.* 'Arka Vikas' using dreb1A gene under Rd29A promoter in pCAMBIA 2301 binary vector was optimized by varying parameters such as type of explant, type and concentrations of hormones. Hypocotyls were found to be the best explants for shoot regeneration in tomato compared to cotyledons with 53.2 and 22.8% shoot regeneration, respectively. In the shoot regeneration medium, 0.1 mgL^{-1} IBA as a source of auxin gave nearly 50% higher shoot regeneration than IAA at similar concentration. With this protocol it was possible to obtain transformed plants within a period of 77 days with a high regeneration and transformation efficiency (34%) compared to over 120 days using earlier published protocols. The T_1 generation plants segregated in a 3:1 ratio for the transgene and Southern blot analysis of the selected plants had shown the transgene integration was at a single locus. With this method it is possible to rapidly and efficiently generate transgenic tomato plants.

Key words: *Agrobacterium* mediated transformation, tomato transformation, hypocotyls, regeneration, transgenic tomato.

Introduction

Tomato is an important vegetable crop with one of the highest production and consumption worldwide. Tomato has been widely used as a model crop in genetic manipulation experiments due to its ease of transformation, comparatively small genome and its agro-economic importance (Arumugathan and Earle, 1991). Crop yield is adversely affected by several stresses such as drought, nutrient deficiencies, and various diseases (Bhatnagar-Mathur *et al*., 2008). Developing transgenic plants is an effective approach for improving tolerance to stresses. Hence, establishment of an efficient transformation system is essential.

Different agronomically useful traits have been incorporated into tomato using *Agrobacterium* mediated transformation (Raj *et al*., 2005; Reed *et al*., 1996; Roy *et al*., 2006). After first report on tomato leaf disc transformation by McCormick (1986) there have been a number of publications on optimization of different factors involved in tomato transformation such as genotype (Koornneef *et al*., 1986; Sharma *et al*., 2009; Ultzen *et al*., 1995), type of explants (Bhatia *et al*., 2005; Fillati *et al*., 1987; Pfitzner, 1998; McCormick *et al*., 1986; Ohki *et al*., 1978), plant growth regulators (Gubiš *et al*., 2003) and antibiotics used (Briza *et al*., 2008; Ling *et al*., 1998) in regeneration of tomato. However, transformation of tomato is still far from routine, and it can show widely variable rates of success, depending on the cultivar and other factors (Park *et al*., 2003). The shoot regeneration efficiency in the selective antibiotic containing media ranged from 5% for cv ATV847 (Ultzen *et al*., 1995) to 32.9% for cv Moneymaker (Ling *et al*., 1998) with a transformation efficiency of 8 to 48% (Cortina *et al*., 2004).

In our experiment 'Arka Vikas' an open pollinated tomato variety developed from Tip Top by pure line selection (Sharma *et al*., 2009) was used, as it is a good general combiner (Bhatt *et al*., 2001) in breeding program. However the transformation and regeneration efficiency for this cultivar were reported to be very low, 22 to 28% in comparison with other commonly used tomato genotypes such as Ailsa Craig (45%), Money Maker, Microtom (53%), Pusa Ruby (41%) (Pozueta-Romero *et al*., 2001; Sharma *et al*., 2009). Information on parameters influencing tomato transformation is limited, except for the few cultivars of tomato such as, Micro Tom (Dan *et al*., 2006), Rio Grande, etc. but for other commonly used cultivars, negligible information is available on transformation and time period required. In view of this, we established a rapid regeneration and efficient transformation protocol by optimizing different parameters such as explant type, growth hormone concentration and duration of transformation protocol.

Materials and methods

Plant material and culture conditions: Seeds of tomato *cv.* 'Arka Vikas' were obtained from the Division of Vegetable Crops, Indian Institute of Horticultural Research, Bangalore. Seeds were surface sterilized for 5 min in 70% v/v. ethanol and washed with sterile water followed by immersing in 4% sodium hypochlorite solution for 10 min then washed with sterile distilled water three times. The surface sterilized seeds were blot dried on sterilized tissue towel. About 35 to 40 surface sterilized seeds were sown in a 300 mL wide mouth glass bottle with polypropylene screw cap containing MS medium and sucrose, gelled with 0.7% agar after the pH of the media was adjusted to 5.8 and steam sterilized at 121°C for 20 min. Culture bottles were kept initially for two days in dark at 25°C then were exposed to a photoperiod of 16 hours illumination with a light intensity of 40-60 $\mu mol\ sec^{-1}$.

Table 1. Media components used in tomato transformation

Components	Seed germination	Preculture medium	Co-culture medium	*Agrobacterium* elimination medium (AEM)	Selective Regeneration medium (SRM)	Rooting medium
MS salts	0.5x	1x	1x	1x	1x	1x
Sucrose (gL^{-1})	1.5%	3%	3%	3%	3%	1.80%
Agar (% w/v)	0.70%	0	0	0	0	0
Gelrite™	0	0.30%	0.30%	0.30%	0.30%	0
BAP (mgL^{-1})	0	2.0	2.0	2.0	2.0	0
IBA	0	0.1	0.1	0.1	0.1	0.5
Kanamycin (mgL^{-1})	0	0	0	0	100 mgL^{-1}	0
Augmentin	0	0	0	300 mgL^{-1}	0	300 mgL^{-1}

Culture media, hormones and antibiotics: MS-medium (Murshige and Skoog, 1962) with components listed in Table 1 was used. Benzylaminopurine (BAP), Indole butyric acid (IBA) procured from sigma were used for the shoot and root regeneration, respectively. Stocks of antibiotics kanamycin sulphate (Macleods), augmentin (Glaxo SmithKline) and phytohormones BAP, IBA were prepared in sterilized distilled water and filter sterilized serially before storing at -20ºC. The concentrations of the stocks were: BAP 1 mgL^{-1}, IBA 1 mgL^{-1}, kanamycin 100 mgL^{-1} and augmentin 300 $mgmL^{-1}$. The media components were adjusted to pH 5.8 either with 0.1N NaOH or with 0.1N HCl, and autoclaved for 20 min at 121ºC. Prior to dispense into the Petri dish, antibiotics were added to medium after it cooled to 50 ºC.

Transformation protocol: Cotyledons and hypocotyls from ten days old seedlings were used as explants. Cotyledons were cut at the tip and base and hypocotyls proximal, central, distal pieces were cultured on the preculture medium for 48 h, where cotyledons with the abaxial surface were in contact with the medium. Explants were incubated in the *Agrobacterium* cell suspension for 15 min with three intermittent shakings. After incubation, explants were blot dried on sterilized tissue towel and transferred to each petri dish containing preculture medium and incubated for 48 h. Explants were shifted to AEM containing augmentin 300 mgL^{-1} for 48 h. and then on to (SRM) selection medium containing kanamycin 100 mgL^{-1}. Plates were incubated at 25°C under 16/8 light and dark cycle. Explants that responded for SRM were subcultured onto fresh SRM medium every three weeks, regenerated shoots of about one cm length were excised and shifted to the rooting medium and well rooted shoots were acclimatized by transferring to plastic bags (12 x 6.5 cm) containing steam sterilized Soilrite™ (Keltech Energies Ltd. Bangalore, India) supplemented with 5 mL of 0.25 x MS salts, the top open end of the polythene bag was stapled. After seven days, 10 mL of 0.25 x (strength) salts was applied (Fig. 2c). After about two-three weeks the acclimatized plants were transferred to net house in to pots containing potting mix and watered, fertigated and plant protection measures were carried out as required. The flowers were bagged for selfing and fruits were harvested when red ripe for seed extraction.

Confirmation of transgene integration: The putative transgenic plants of T_0 generation were subjected for PCR screening using NPTII and dreb1A gene specific primers, about 80% of the plants confirmed positive. Confirmed plants were allowed to self pollinate and after fruit set and fruit maturation, the T_1 generation seedlings were grown in portrays and the segregation of the transgene in the seedlings was analyzed by PCR analysis. 3:1 ratio of gene was observed in the population. Randomly selected T_1 putative plants were screened for the copy number by Southern hybridization using Digoxigenin labeled probes (Fig. 2e). The gene integration was found to be of single copy in the screened plants.

Statistical analysis: For each treatment 25 plates containing about 15 explants each in three replications were used for ANOVA. Shoot regeneration efficiency was calculated by the ratio of number of shoots regenerated to the explants used. Transformation efficiency was based on the number of confirmed transgenic plants to the number of explants used in the experiment.

Results and discussion

Some of the parameters involved in transformation of tomato were optimized for the cultivar 'Arka Viaks'. Explant type determines the regeneration potential, the hormone sources mainly cytokinin and auxins in tomato influence the regenerability of tomato explants to a large extent.

Effect of explant type on shoot regeneration efficiency: Cotyledons and hypocotyls are the most frequently used explants among others such as leaf, epicotyls, petiole and internode for tomato transformation and regeneration (Gubiš *et al*., 2003). In this study, cotyledon and hypocotyl explants were compared for their shoot regeneration percentage (Fig. 1). After co-cultivation and elimination of *Agrobacterium*, in SRM medium the explants showed regeneration response by swelling and producing callus within ten days. Hypocotyl explants exhibited higher (53.2%) shoot regeneration than cotyledons (22.8%) on the SRM. This difference in the shoot regeneration response of explants is due to a low realization of shoot buds in to elongated shoots in cotyledons, even though cotyledons had higher number of shoot buds initially, in hypocotyls the realization of shoot buds in to elongating healthy shoots was higher, whereas, most of the

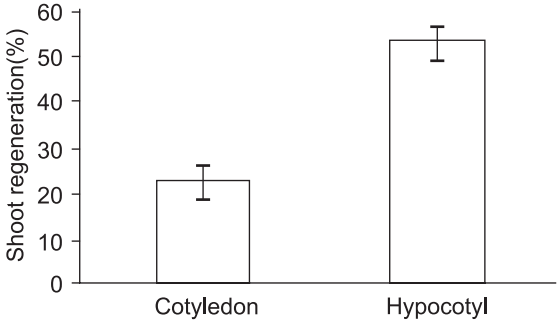

Fig. 1. Shoot regeneration percentage from cotyledon and hypocotyl explants. (Values are percent mean ± SE, from three replications of 25 plates each)

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shoots formed on cotyledons were rosette-like leafy structures, which showed difficulty in elongation. Similar observations were recorded by Khoudi *et al*. (2009) though, the shoot primordial initiation on cotyledons was more, only few (four out of 170 cotyledon explants) normal plants could be recovered. Gubiš *et al*. (2003) obtained a significantly higher shoot primordial initiation in hypocotyls than in cotyledons, in 13 tomato cultivars, however no significant differences in realization of number of shoots among both the explants was noticed after six weeks of culture in all 13 tomato cultivars used. Mathews *et al*. (2003) have also reported that, hypocotyl explants are the explants of choice due to their ease of manipulation and consistently high transformation rate in tomato cultivar Micro-Tom. Similarly, in different tomato cultivars hypocotyl explants were reported to be superior to cotyledon explants (Yasmeen, 2009).

Our study showed that cotyledon explants curled up after induction period thus it was difficult to blot away the *Agrobacterium* cell suspension after incubation in *Agrobacterium* culture, which rendered the elimination of *Agrobacterium* tough, leading to the browning and tissue death. Even though, a number of researchers have opined that cotyledons yield more number of shoots in tomato than the hypocotyl explants (Sharma *et al*., 2009; Wu *et al*., 2006), we have observed that hypocotyl explants are the preferred explants for *Agrobacterium* mediated tomato transformation. We also observed that hypocotyls gave rise to more shoots than that of cotyledon explants. The observed differences in the shoot regeneration response among the explants in our experiment may be partly due to the type of auxin *i.e.* IBA instead of IAA. IBA at concentration of 0.1 mgL^{-1} in the shoot induction/regeneration medium consistently resulted in higher (38%) shoot regeneration response than in IAA (24.16%) in both hypocotyl and cotyledon combined on antibiotic (100 mgL^{-1} kanamycin) selection medium.

Effect of auxin types and cytokinin concentrations on shoot regeneration efficiency: The effects of different concentrations of cytokinin, BAP and the type of auxin were compared, *i.e.* IAA or IBA at 0.1 mgL^{-1}. Explants cultured on IBA containing media showed higher (38%) shoot regeneration than those cultured on IAA (24.16%). IAA is generally used in tomato transformation (Cortina *et al*., 2004). Most of the earlier reports mention the use of IAA as auxin source in the shoot regeneration experiments in tomato (Dan *et al*., 2006; Qui *et al*., 2007; Shivakumar *et al*., 2007). In our experiment IBA was observed to be a better source of auxin than IAA in shoot regeneration of tomato. The comparison of IBA with other commonly used auxin type (NAA) has also shown that IBA elicited higher shoot regeneration response than NAA. Yasmeen (2009) could obtain a shoot regeneration of 20.5% in tomato *cv.* Rio Grande when NAA was used at 0.1 mgL^{-1}. Similarly, Khoudi *et al*. (2009) had employed NAA at 0.1 mgL^{-1} in the shoot regeneration of leaf explants and a transformation efficiency of 10-14% was obtained. Whereas, in our experiment, we could obtain a much higher transformation efficiency of 34% which may in part be attributed to the use of IBA as auxin source in the shoot regeneration medium. The effect of IBA was similar in both hypocotyl and cotyledon explants on shoot regeneration.

It was observed that the MS medium containing 2 mgL^{-1} BAP and 0.1 mgL^{-1} IBA (Fig. 2a) gave the highest shoot regeneration (Table 2), compared to that of BAP at 3 or 5 mgL^{-1} along with 0.1 mgL^{-1} IBA where the former level of hormone combination showed the marginal difference in the % shoot regeneration. Similarly, Yasmeen, (2009) observed that highest regeneration percentage was obtained at 2.0 mgL^{-1} of BAP compared to 1.0 and 1.5 mgL^{-1}. BAP is commonly available and a less expensive cytokinin than zeatin in *in vitro* regeneration and transformation of tomato.

Table 2. Shoot regeneration percentages at different BAP concentrations in shoot induction media

BAP concentrations	Shoot regeneration (%)
2 mg L^{-1}	53.2
3 mg L^{-1}	42.0
5 mg L^{-1}	41.6

Effect of auxins on rooting of the regenerated shoots: The regenerated and elongated shoots of tomato root readily in presence of auxin. In this experiment we have evaluated the influence of the auxins, IAA or IBA on rooting of the regenerated tomato shoots. Highest rooting of 66.6% was observed in 0.5 mgL^{-1} IBA compared to 29.16% in IAA within 21 days in rooting medium (Fig. 2b). IAA has been generally used by many researchers for improving the rooting of the elongated shoots of tomato with concentrations varying from 0.1 to 1.0 mgL^{-1} IAA (Dan *et al*., 2006; Gao *et al*., 2009; Sharma *et al*., 2009). Shivakumar *et al*. (2007) observed 100% rooting in three tomato cultivars in media supplemented with 0.1 mgL^{-1} of IAA and a rooting of 32-80% in MS basal medium devoid of auxin, however, the time period required to obtain the given rooting was not specified. Raj *et al*. (2005) obtained a rooting percentage of 14.2 in media containing 0.05 mgL^{-1} IBA for the cultivar Pusa Ruby. Maximum rooting response was observed in the first 10 days in the rooting medium than in 15 and 20 days. The shoots, which were not rooted, were sub cultured. After 3 weeks well rooted plants were acclimatized in Soilrite™, later they were shifted to the pots and maintained in green house.

Table 3. Comparison of time duration of different tomato different transformation

Stages of protocol	Standard tomato protocol Dan *et al*. (2006)	Micro Tom protocol Dan *et al.* (2006)	Khoudi *et al.* (2009).	Present experiment
Preparation (d)	6	7	21	8
Preculture (d)	1	0	2	2
Co-cultivation (d)	2	2	2	2
Agrobacterium elimination(d)	0	0		2
Shoot induction (d)	28-35	21-28	14	15-24
Shoot elongation (d)	28-42	14-21	13	14-21
Rooting (d)	21-28	21-28	18	21-25
Transformation period (d)	3-4 months	2-3 months	2 months 10 days	2-2.5 months

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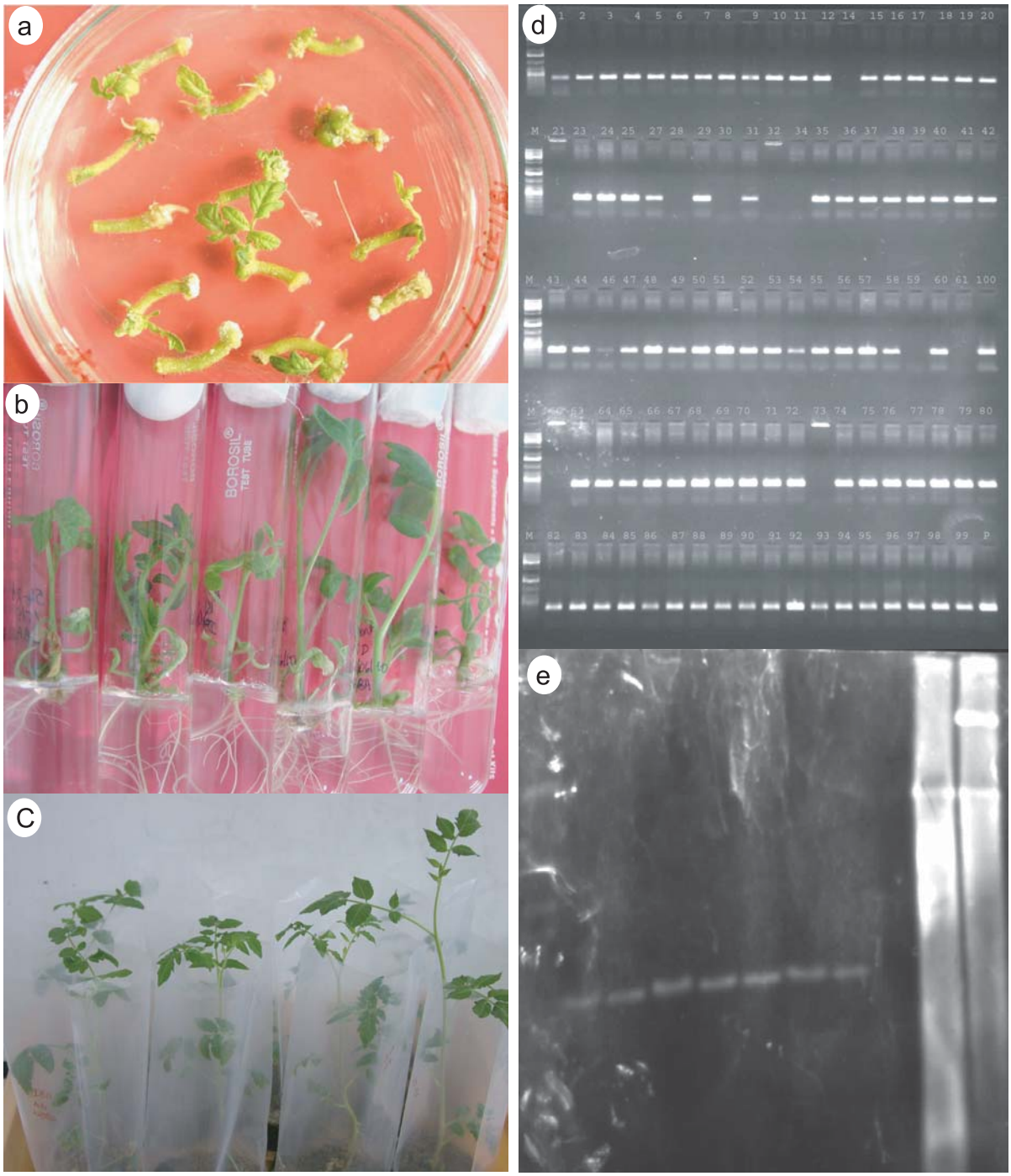

Fig. 2. a. Shoot regeneration from hypocotyl explants, b. Rooting of the elongated shoots in rooting media, c. Acclimatization of rooted tomato plants in polythene bags, d. PCR of T_1 tomato plants using dreb1A gene specific primers, e. Southern blot of selected T_1 tomato plants using DIG labeled dreb1a gene as probe.

Duration of transformation and regeneration: The period required for the shoot regeneration and rooting in the transformation of tomato was recorded (Table 3). The shoot bud formation started at 12 days and the shoot regeneration response to obtain a shoot length of 8-10 mm was around 24 days in hypocotyls. In hypocotyl explant, 82.44% of shoot regeneration was obtained within four to five weeks. The time frame for transformation of tomato was about three to four months, while Dan *et al*. (2006) have cut the time frame required for transformation of a tomato cultivar Micro-Tom by 2-3 months compared to other tomato cultivars. Whereas, in Rio Grande the period required for transformation was two months and ten days (Khoudi *et al*., 2009) however, the regeneration was in the antibiotic free medium. In our protocol the time frame for tomato transformation was about two and half months, which is comparable to that of Rio Grande by Khoudi *et al*. (2009), but even though the antibiotic selection medium drastically reduces the transformation rate and efficiency, the present experiment achieved high transformation (34%) and regeneration rates (up to 53.2%) in a short period of 62 to 77 days, in the antibiotic selection medium without the use of complicated tobacco or tomato feeder layer or acetosyringone. The reduction in the time frame for tomato transformation protocol in our experiment may be attributed to the choice of the hypocotyl explants, use of IBA as auxin source in the shoot regeneration

medium and fewer subcultures for elongation, as we used only one medium for shoot regeneration and elongation.

In this experiment, we have improved the tomato transformation protocol for rapid and efficient transformation of tomato cultivar 'Arka Vikas' by studying different parameters involved in transformation and regeneration. It is possible to obtain rooted tomato transformants within a period of 62-77 days with a high efficiency of 53.2% regeneration and 34% transformation efficiency by using this protocol. We have also observed that hypocotyl explants were superior to cotyledon explants and IBA as a source of auxin is better than IAA in tomato transformation.

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