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Punctured-hypocotyl method for high-efficient transformation and adventitious shoot regeneration of tomato

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ABSTRACT

To produce transgenic tomato (*Solanum lycopersicum* L.), an important vegetable crop worldwide, we need an efficient transformation method to thwart multiple adverse growth conditions. In this study, for the first time we compared the efficiency of the punctured-hypocotyl and normal immersion methods of *Agrobacterium*-mediated transformation in Indian tomato cultivars using hypocotyl explants. The transformation factors such as bacterial density of 0.4 OD_{600}, 40-min infection, and 48-h co-cultivation were optimal to obtain maximum transformation efficiency in the tomato cv. PKM1 among four Indian cultivars tested. The transgene integration of the tomato genome was confirmed by PCR and Southern hybridization. Transformation efficiency was greater with the punctured-hypocotyl method (55.8 ± 0.01%) than immersion method (48.3 ± 0.06%) and comparatively higher than the previously reported transformation efficiency in tomato. The developed method is simple, efficient and could be used to transfer agronomically important genes into the tomato genome for the potential improvement in terms of quality and quantity.

1. Introduction

Tomato (*Solanum lycopersicum* L. 2*n* = 2X = 24), a major vegetable crop widely cultivated throughout the world, is rich in vitamins (A and C), carotenoids, and lycopene (Bhatia et al., 2004). Nutritional studies have well reported the beneficiary effect of lycopene against cancer, cardiovascular and neurodegenerative diseases and its antioxidant properties (Fuhrman et al., 1997; Giovannucci, 1999; Suganuma et al., 2004). Even though tomato is cultivated in different seasons, productivity of tomato is constrained because of the adverse effects of abiotic stresses (Luengwilai et al., 2012; Zhang and Blumwald, 2001; Zhu, 2002).

Genetic transformation is an appropriate option for developing abiotic stress tolerant crops (Bhatnagar-Mathur et al., 2008). The type of explant used for the regeneration plays an important role in the production of transformed plants (Bhatia et al., 2004; Pozueta-Romero et al., 2001). The hypocotyl was found the most responsive explant for high regeneration frequency than cotyledon, and leaf explants of tomato (Abu-El-Heba et al., 2008; Asakura et al., 1995; Gubiš et al., 2004; Gunay and Rao, 1980).

Agrobacterium-mediated transformation remains the most successful gene transfer method in plants because of its advantages are associated with natural gene transfer, low copy number, defined and preferential integration of transgene into transcriptionally active regions on the chromosome, and production of fertile transformed plants (Hiei et al., 2000; Kumar et al., 2011; Ling et al., 1998). Since the first report of *Agrobacterium*-mediated transformation and regeneration in leaf-disc explants of cv. UC 82b (McCormick et al., 1986), myriad reports have described tomato genetic engineering to improve crop quality and yield. Variable transformation efficiency, from 1.8% to 57%, has been achieved in different tomato cultivars (Cruz-Mendívil et al., 2011; Dan et al., 2006; Devi et al., 2012; Frary and Earle, 1996; Hamza and Chupeau, 1993; Ling et al., 1998; Park et al., 2003; Sun et al., 2006; van Roekel et al., 1993). However, most of the available procedures were time-consuming or relied on feeder layers or low transformation efficiency and/or limited to few cultivars (Kaur and Bansal, 2010). Mainly, non-availability of sufficient *Agrobacterium* interaction sites in the target plant tissue limits the transformation efficiency, as well *Agrobacterium* density, infection time and co-cultivation duration are the major factors known to limits the transformation efficiency of tomato (Devi et al., 2012; Kumar et al., 2011; Wu et al., 2006). A recent review on use of *Agrobacterium*-mediated transformation methodology in tomato emphasized the low transformation efficiency as a major challenge, and suggested to modify the different

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genetic and environmental aspects of transformation method to enhance the transformation efficiency (Kumar et al., 2011). Hence, we still need an appropriate simple and general procedure to all cultivars to overcome the existing above limitations on tomato transformation and to increase the transformation efficiency of tomato. Recently, wounding methods has drawn much attention to overcome problems associated with host/tissue specificity and to increase the *Agrobacterium* infection in the target plant tissue. Different wounding methods such as puncturing with a syringe needle and a sonication-assisted transformation have been used to increase the *Agrobacterium* mediated transformation efficiency (Chee et al., 1989; Dutta et al., 2013; Trick and Finer, 1997). A punctured cotyledon transformation procedure for transforming cultivated tomato produced phenotypically normal fertile plants (Shahin et al., 1986). However, no study has compared punctured transformation and immersion methods (without puncture) in different cultivars of tomato.

In this study, we compared the efficiency of puncture transformation and normal immersion methods (without puncture) in hypocotyl explants of different tomato cultivars to improve the transformation efficiency and to develop a stable universal method to transform different cultivars of tomato for further crop improvement. The factors influencing transformation efficiency such as *Agrobacterium* density, infection time and co-cultivation period were also evaluated using the fusion gene *w*heat *co*ld-*r*egulated 15-green fluorescent protein (*Wcor15-GFP)* as a reporter.

2. Materials and methods

2.1. Plant material and preparation of explants

Temperate tomato cv. PKM1 and CO3 seeds were obtained from Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India. Tomato varieties Sindhu and Shalimar, growing in higher altitude, were obtained from the Defence Institute of High Altitude Research (DIHAR), Leh, Ladakh, India. Tomato cv. PKM1 was used for the standardization of adventitious shoot regeneration and transformation. Seeds were surface sterilized by treating with 70% ethanol for 30 s followed by 1% (v/v) sodium hypochlorite solution for 20 min, and then stringently washed with sterile distilled water to remove the sterilants. Surface sterilized seeds were inoculated and germinated on half strength (1/2) MS basal medium which consisted of MS salts and vitamins (Murashige and Skoog, 1962) with 1.5% (w/v) sucrose (pH 5.8) and solidified with 0.2% phytagel (Sigma, St Louis, MO, USA). Cultures were incubated for 3 days at 25 ± 2 °C under dark and thereafter maintained under 16 h photoperiod, at a photon flux of 50 μmol m^{-2} s^{-1} for 14 days. About 2 cm long, apical segments of hypocotyls close to the cotyledons were excised and used for transformation studies.

2.2. Agrobacterium-mediated transformation

2.2.1. Agrobacterium strain and binary vector

Agrobacterium tumefaciens LBA4404 (Horsch et al., 1985) harbouring recombinant pBI121:*35S::Wcor15-GFP* was used for transformation. Its T-DNA region contains neomycin phosphotransferase gene (*npt* II) driven by the nopaline synthase (*nos* P) promoter for plant selection, which confers resistance to kanamycin, and *Wcor15-GFP* fusion gene (Shimamura et al., 2006; Takumi et al., 2003) under CaMV 35S promoter which acts as reporter gene.

A single colony of *A. tumefaciens* LBA4404 carrying pBI121:*35S::Wcor15-GFP* was cultured on 10 mL LB broth containing 10 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin for overnight at 28 °C in the dark. The bacterial culture was then diluted 10 folds into 35 mL LB broth containing aforesaid antibiotics and allowed to grow until the culture reach log phase. They were harvested by centrifugation and resuspended in 35 mL of liquid MS medium. The bacterial optical density was adjusted to 0.2, 0.4, 0.6 and 0.8 at OD_{600}.

2.2.2. Transformation by punctured-hypocotyl and immersion method

Prior to *Agrobacterium* infection, the apical hypocotyl explants were precultured on MSB5 medium [MS salts, B5 vitamins (Gamborg et al., 1968), 3% (w/v) sucrose, pH 5.8, 0.2% phytagel] supplemented with 3 mg L^{-1} BAP and 0.5 mg L^{-1} NAA) for 3 days at 25 ± 2 °C under 16 h photoperiod, at a photon flux of 100 μmol m^{-2} s^{-1}.

The punctured-hypocotyl transformation was carried out as per the procedure of Shahin et al. (1986) with modification. In this method, a separate sterile hypodermic needles (27G1/1) (Dispovan, New Delhi, India) were touched in deep in the aforesaid different density of *Agrobacterium* suspension fortified with 100 μM acetosyringone (Himedia, Mumbai, India) and a separate group of precultured explants were punctured approximately 1.5 mm in depth, and randomly throughout the explants (6–8 times) using the respective needles. Then, explants were incubated for different time duration (0, 10, 20, 30, 40, 50 and 60 min) in sterile petri plates.

In the immersion transformation method, intact hypocotyls segments (without puncture) were fully immersed in the aforesaid *Agrobacterium* suspension, and incubated for different time duration (0, 10, 20, 30, 40, 50 and 60 min) with gentle shaking. The explants infected by both the transformation methods were blot dried on sterile filter paper to remove the excess *Agrobacterium* and co-cultivated for 0, 1, 2, and 3 days on co-cultivation medium (MSB5 medium fortified with 3 mg L^{-1} BAP and 0.5 mg L^{-1} NAA, 3% (w/v) sucrose, pH 5.2, 0.2% phytagel and 100 μM acetosyringone). The co-cultivated explants were washed with sterile distilled water followed by sterile liquid MS medium containing 300 mg L^{-1} timentin (Duchefa Co., Haarlem, The Netherlands). The washed explants were blot dried on sterile filter paper and inoculated onto a selection medium (MSB5 medium fortified with 3 mg L^{-1} BAP and 0.5 mg L^{-1} NAA) containing 50 mg L^{-1} kanamycin and 300 mg L^{-1} timentin for two weeks. For each factor, a separate control was maintained by means of LB medium without *Agrobacterium* suspension, zero time incubation, and zero day co-cultivation in the optimization of *Agrobacterium* density, infection time, and co-cultivation period, respectively, for both punctured-hypocotyl and immersion method (without puncture). The cultures were incubated at 25 ± 2 °C under 16 h photoperiod. Each treatment comprises 100 explants in replicates of three.

2.3. Selection and regeneration of transformants

Explants with adventitious shoot buds were subcultured in the shoot regeneration medium (MSB5 medium fortified with 1 mg L^{-1} zeatin and 0.1 mg L^{-1} IAA) containing timentin (300 mg L^{-1}) and kanamycin (100 mg L^{-1}) (Costa et al., 2000) for two weeks. After shoot regeneration, shoots longer than 20 mm were individualized and transferred into a fresh elongation medium containing the aforesaid constituent with kanamycin (150 mg L^{-1}). One-week-old elongated shoots were allowed to initiate roots on 1/2 MS containing B5 vitamins and 0.5 mg L^{-1} IAA supplemented with 50 μg L^{-1} kanamycin. After 4 weeks, the well-rooted plantlets were transferred to earthen pots containing soil:sand:vermiculite (1:1:1 ratio). Hardened transformants were maintained in the green house conditions and evaluated for the presence of transgene in vegetative tissues.