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RESEARCH ARTICLE

AN IMPROVED PROTOCOL FOR RAPID AND EFFICIENT AGROBACTERIUM MEDIATED TRANSFORMATION OF TOMATO FOR SALT TOLERANCE (*SOLANUM LYCOPERSICUM L.*)

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ABSTRACT

Transformation of tomato with heterologous genes requires rapid and efficient transformation protocols. *Agrobacterium* mediated transformation protocol of tomato (*Solanum lycopersicum* L.) cv. 'Pusa Ruby' using Pg *NHX1*, *AVP1* and co-expression of Pg *NHX1+AVP1* gene under 35S promoter and poly A signal of CaMV in pBI121 binary vector was optimized by varying parameters such as type of explant, combination of hormones, concentration of antibiotic, bacterial culture and co-cultivation period. Cotyledons were found to be the best explants for shoot regeneration in tomato compared to Hypocotyls with 96.95% and 95.90% shoot regeneration, respectively. The hormonal concentration Zeatin (1mg/l), IAA (0.1mg/l), Adenine sulphate (25 mg/l) produced higher shoot regeneration. The selection of transformants on antibiotic kanamycin was standardized at 100mg/l and the transformation efficiency was optimized at Acetosyringone concentration of 375 µM and bacterial culture of 1 x 10^8 at 72 h incubation was standardized to obtain the transformation efficiency of 38.81%. With this modified protocol it was possible to obtain transformed plants within a period of 65 to 75 days with a high regeneration and transformation efficiency.

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INTRODUCTION

Tomato (*Solanum lycopersicum*) belongs to the family *Solanaceace* and is the second most important vegetable crop next to potato. Crop is grown for its edible fruits, which can be consumed either fresh or in the form of various processed products such as paste, powder, ketchup, sauce, soup and canned whole fruits, and it is also a good source of vitamins A, B and C (Khoso, 1994). The present world production is about 161.8 million tonnes of fresh fruits produced on 3.7 million hectares. Tomato production has been reported from 144 countries. In India 18.22 million tonnes on 0.88 million hectares fruits have been produced (FAOSTAT, 2013). In Karnataka Area 57.80 ('000HA), production 19.60 ('000MT) and productivity 20.00 MT/HA (Director of Horticulture, 2013-14). The progressive salinization of soil is estimated at 20% of irrigated land (Ashraf, 2011) and it limits future agriculture, as most crop species are glycophytes, which are usually salt sensitive. An estimate from the Food and Agriculture Organization suggested that 6% of the world's total land area and 20% of irrigated land is affected by high salinity (FAO).

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In India, nearly 8 million hectares is affected by salinity out of 329 million hectares of total geographical area. In Karnataka, it is estimated to be 179 thousand hectares. This immense occurrence of salt affected lands in arid and semi-arid regions of India is because, the annual rainfall is not sufficient to leach down salts to deeper layers. Salinity and drought are major abiotic factors reducing plant productivity and quality (Boyer, 1982). To cope with salt stress, plants have developed multifarious adaptation strategies, one of those is the compartmentalization of Na^+ into the vacuole, which might reduce the deleterious effects of excess Na^+ in the cytosol and to maintain osmotic balance using Na^+ as a cheap osmoregulation substance, thus to enhance water uptake and salt tolerance of plant (Apse, 1999).

Over-expression of *Arabidopsis thaliana* At*NHX1* conferred enhanced salt tolerance in *Arabidopsis* (Apse *et al.,* 1999), *Oryza sativa* Os*NHX1* in rice plants (Fukuda *et al.,* 1999, 2004) and transfer of *Gossypium hirsutum* Gh*NHX1* in Tobacco (Wu *et al.,* 2004), *Hordeum brevisubulatum*, Hb*NHX1* in Tobacco rendered transgenic plants tolerant to both salt and drought stress (Lu *et al.,* 2005). Genes encoding for Na^+/H^+ antiporter have also been isolated from halophytes such as *Mesembryanthemum crystallinum L.* (Chauhan *et al.,* 2000), *Atriplex gmelini* (Hamada *et al.,* 2001), *Sueda salsa* (Ma *et al.,* 2004) and *Beta vulgaris* (Xia *et al.,* 2002).

Introduction of *Atriplex gmelinii* Ag*NHX1* conferred only limited salinity tolerance to salt sensitive rice plants (Ohta *et al.,* 2002). The above studies have demonstrated the potential use of specific vacuolar antiporters as a candidate gene in imparting salt tolerance capabilities. Previous studies have also indicated the over-expression of H^+ pyrophosphatase genes have enhanced tolerance to salinity and or drought stresses in plants. Enhanced salt and drought tolerance have been achieved in model plant *Arabidopsis* by over-expressing the *Arabidopsis* H^+ pyrophosphatase *AVP1* (Gaxiola *et al.,* 2001). *Suaeda salsa* H^+ pyrophosphatase *SsVP* (Guo *et al*., 2006) and H^+ pyrophosphatase *TVP1* from wheat (Brini *et al*., 2007). Moreover, Dyakova *et al*., 2006, reported that over-expression of an H^+ pyrophosphatase from the bacterium *Rhodospirillum rubrum* conferred salt tolerance in transgenic tobacco. Similarly salt tolerant phenotype was observed in tobacco plants engineered with the *Thellungiella halophila* H^+ pyrophosphatase *TsVP* (Gao *et al.*, 2006).

From these results, it was also predicted that the vacuolar Na^+/H^+ antiporter and H^+ pyrophosphatase co-expression would confer even higher salt tolerance to transgenic plants than that of the single gene (Shimna Bhaskaran and Savithramma, 2011). Though Tomato (*Solanum lycopersicum*), is an important crop in the world, is sensitive to moderate levels of salt in the soil. However, good yields are limited to warm and dry areas such as the mediterranean. The regions affected with salinity and drought are overcome by the cultivation of some cultivars like 'Money maker' and 'Edkawi', perform well under moderately saline treatments. In the last few years, significant advances have been made in identification and isolation of several genes that could potentially be involved in salt tolerance. According to literature published from 1993 till date, various authors have claimed enhancement of salt tolerance through either overexpression of endogenous genes or expression of genes that evidently act on mechanisms involved in the process of tolerance (Borsani *et al.*, 2003, Flowers, 2004). Overall, the result obtained by previous researchers suggests that the expression of individual and Co-expression of genes in transgenic plants can increase salinity tolerance to some extent, which would be sufficient from breeding point of view.

The advance development of transgenic plants is an effective approach for improving tolerance to stresses. Hence, establishment of an efficient transformation system is essential. The different agronomically useful traits have been incorporated into tomato using *Agrobacterium* mediated transformation (Raj *et al*., 2005; 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 (Sharma *et al*., 2009), type of explants (Bhatia *et al*., 2005), plant growth regulators (Gubis *et al*., 2003) and antibiotics used (Briza *et al*., 2008) 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). In present study tomato plants of "Pusa ruby" over-expressing Pg*NHX1* gene a vacular Na^+/H^+ antiporter gene from *Pennisetum glaucum*, *AVP1* and Co-expression of Pg*NHX1+AVP1* were regenerated after transformation with *Agrobacterium tumefaciens.*

MATERIALS AND METHODS

Plant material

Seeds of Tomato variety 'Pusa Ruby' (*Solanum lycopersicum* L.).

Genes

The cassettes used for transformation were pBI121/Pg*NHX1,* pBI121/*AVP1* and (Co-expressing) pBI121/Pg*NHX1+AVP1*.

Explant preparation

Seeds of tomato cv. 'Pusa Ruby' were surface sterilized for 5 min in 70% v/v. ethanol and washed with sterile distilled water followed by immersing in 4% sodium hypochlorite solution for 15 min then washed with sterile distilled water four times. The surface sterilized seeds were blot dried on sterilized tissue paper. About 25 to 30 surface sterilized seeds were sown in a 300 mL wide mouth glass bottle with polypropylene screw cap containing ½ strength MS medium (Murashige and Skoog, 1962) with 1.5% (w/v) sucrose and gelled with 0.7% (w/v) agar. The pH of the media was adjusted to 5.8 before autoclaving. Media were steam sterilized at 121°C for 15 min.

Culture medium and conditions

Seeds were germinated at 25°C with 16 hours light period and 8 hours dark period. The *in-vitro* grown seedlings were used as source of explants. Explants (cotyledons/Hypocotyls) were taken from 8-10 days old plants before the first true leaves (Madhu, S.V and Savithramma, D.L., 2014). The explants were inoculated directly on MS nutrient medium with cytokinins and auxin in various combinations as shown below (Table 1). All the media mentioned below were supplemented with 3% sucrose and were semi solidified by the addition of 0.7% agar. The pH of the media was adjusted to 5.8 before autoclaving (121°C and 15 lbs per sq. inch). Ten explants were used per bottle and there were ten replications for each treatment. All the cultures were maintained at 25°C with 16 hours light period and 8 hours dark period of illumination with a light intensity of 40-60 umol/sec.

Regeneration

The explants were tested for their morphogenic ability and all the cultures were sub-cultured every 15 days once. After 3 to 4 sub-cultures the regenerated shoots. Well regenerated shoots were inculcated in to rooting medium; the plantlets with vigorous roots were directly transferred to soil rite for hardening and profuse rooting, in small plastic pots which were covered with polythene bags for 6-7 days. When grown to 8-10 cm plant height, the plants were transplanted into soil and grown in the green house until harvest.

Development of transgenic Tomato plants

Agrobacterium tumefaciens mediated gene transfer in Tomato

Seeds of tomato var. Pusa Ruby were used for the transformation experiments.

Table 1. Media components used in tomato transformation

Components	Seed germination	Pre culture medium (PCM)	Inoculation medium (IM)	Co-cultivation Medium (CCM)	Washing medium (WM)	Selective Regeneration Medium (SRM)	Shoot Elongation Medium (SEM)	Rooting Medium (RM)
MS salts	0.5x	1.0x	0.5x	1.0x	0.5x	1.0x	1.0x	0.5x
Sucrose (%/L)	1.5%	3.0%	0	3.0%	0	3.0%	3.0%	1.5%
Agar (% w/v)	0.7	0.7	0	0.7	0	0.7	0.7	0.7
ZA (mg/L)	0	0.5	0	0.5	0	1.0	0.5	0
IAA (mg/L)	0	0	0	0	0	0.1	0.1	0.5
Adinine sulphate (mg/L)	0	0	0	0	0	25	25	0
Acetosyringone (µM)	0	375	0	0	0	0	0	0
Cefotoxine (mg/L)	0	0	0	0	300	300	300	300
Kanamycin (mg/L)	0	0	0	0	0	100	100	100
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8

*ZN-Zeatin, MS-Murashige & Skoog, IAA-Indole-3-Acetic acid.

Table 2. Effect of kanamycin treatment on explants (Cotyledon/Hypocotyl) surveillance in tomato variety "Pusaruby".

Treatments	Kanamycin (mg/l)	Number of explants inoculated	Cotyledon	Hypocotyl
T_0	0	30	30.00	30.00
T_1	25	30	14.50	13.25
T_2	50	30	20.50	18.50
T_3	75	30	17.50	16.50
T_4	100	30	23.50	20.50
T_5	125	30	11.50	10.50
Mean SEm ± CD at 1% CV (%)			19.58	18.20

Table 3. Effect of different concentrations of Acetosyringone on *Agrobacterium* mediated transformation for cotyledon and Hypocotyl explants

Acetosyringone Concentration (µM)	Number of explants	Number of Kan resistant cotyledon line		Number of Kan resistant Hypocotyl line	
		I Selection	II Selection	I Selection	II Selection
T_0 (0)	30	08.57	06.90	08.25	07.10
T_1 (75)	30	17.20	16.80	16.30	16.00
T_2 (150)	30	18.80	18.55	17.50	17.25
T_3 (225)	30	20.27	19.60	20.35	19.70
T_4 (300)	30	21.55	20.75	20.50	20.05
T_5 (375)	30	22.80	22.69	21.80	21.45
Mean		18.20	17.80	17.45	16.92
SEm ±		02.33	02.20	2.02	2.12
CD at 1%		01.21	01.49	01.11	01.35
CV (%)		02.55	03.86	02.28	02.38

The *Agrobacterium tumefaciens* strain LBA 4404 harboring the binary vector pBI121 was used for fine tuning the transformation protocol in tomato (Shimna Bhaskaran and Savithramma, 2011). The pBI121 vector contains kanamycin resistance gene (*nptII*) as bacterial and plant selection marker. The *Agrobacterium tumefaciens* strain LBA 4404 was plated by streaking one loop of bacterial glycerol stock kept at -80°C into LB medium supplemented with 50mg/L kanamycin and 10mg/L rifampicin and incubated at 28°C for 48 hours. A single, isolated bacterial colony was used to prepare the starter culture (1mL) in liquid LB medium (with 50mg/L kanamycin and 10mg/L Rifampicin). Further, a larger *Agrobacterium* culture (25mL) was prepared using 200µL of the starter culture.

The bacterial cultures were suspended in inoculation medium (IM, Table 1), adjusted to the required optical density (λ = 600 nm) of 0.6 to 0.8 using spectrophotometer (UV- Min- 1240) and used for inoculation within 30 min.

The cotyledons and hypocotyls obtained from 8-10 days old seedlings were cut at the tip and base and cultured on the pre-culture medium for 48 hours, 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 and transferred to each petri dish containing pre-culture medium (Table 5) and incubated for 48 hours and then soaked in Inoculation medium (IM, Table 5) containing the *Agrobacterium* cells for 45 min, blot dried on tissue paper to remove excess bacteria and plated on co-cultivation media (CCM, Table 5). Co-cultivation was allowed to proceed for 48 to 60 hours at 25°C at dark, after which the explants were washed in washing medium (WM, Table 5) containing cephotaxime (300mg/L) to kill the *Agrobacterium*, and sub cultured on regeneration medium (RM, Table 5) for shoot bud initiation. The regenerated shoot buds were transferred to the shoot elongation medium (SEM, Table 5). When the plantlets reached about 8-10 cm in length they were

transferred to rooting media (RM, Table 5). All regeneration steps were carried out at 25°C under fluorescent light (150–200μEm^{-2}s^{-1}). 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 5mL of 0.25 x MS salts, for hardening in the transgenic facility. After two to three weeks the acclimatized plants were transferred to net house into 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 *NHX1, AVP1* gene specific primers, Confirmed plants were allowed to self pollinate and fruit maturation.

RESULTS AND DISCUSSION

Tomato is one of the model plant because of its importance as a crop species, ease of manipulation at genome level followed by physiological experiments (McCormick *et al*., 1986). The *in-vitro* morphogenic response of cultured plants tissues of tomato are affected by the different components of the culture medium, especially concentration of growth hormones, antibiotic (kanamycin) treatment on explant, concentration of Acetosyringone, optical density of bacterial culture and co-cultivation period on transformation. Development of an efficient protocol for tomato transformation and its subsequent regeneration is a prerequisite for the production of transgenic plants. Previous data on tomato regeneration have been reported using cotyledon explants (Shimna Bhaskaran and Savithramma, D.L., 2011, Raj *et al*., 2005, vanRoekel *et al*., 1993 and leaf explants (McCormick *et al*., 1986 and Gaffer, 1997 and Oktem *et al*., 1999). In the present study, we have developed rapid shoot regeneration system in tomato cultivar 'Pusa Ruby' *via* direct shoot organogenesis using cotyledonory explant followed by transformation using *Agrobacterium*-mediated gene transfer was adopted.

Effect of explant type and culture media on shoot organogenesis efficiency

The most frequently used explants were cotyledons and hypocotyls for tomato regeneration and transformation (Gubis *et al.,* 2003 and Madhu *et al.,* 2014). The cytokinins and auxins ratio influence the regenerability of tomato explants to a large extent were optimized in tomato cultivar 'Pusa Ruby'. Cotyledons were found to be the best explants for shoot regeneration compared to hypocotyl (96.95% and 95.90%) followed by maximum number of shoot primordia per explant (16.65) and highest number of shoots per explant (17.35) and Shoot length (8.45 cm). The hormonal combination (1.0 mg/L Zeatin, 0.1 mg/L IAA and 25mg/L Adenine Sulphate) gave higher (75%) shoot regeneration than IAA alone. The number of days required for shoot bud formation was 12 days and to produce a shoot length of 8-10 mm was 22 days in cotyledons compared to hypocotyls. In the present study the cotyledons produced highest shoot regeneration percentage in four to five weeks. Whereas earlier researchers observed the time frame for transformation of tomato was about three to four months, while Dan *et al.,* (2006) have cut down the time frame required for regeneration of a tomato cultivar "Micro-Tom" by 2-3 months compared to other tomato cultivars. Similarly in tomato cultivar "Rio Grande" the period required for regeneration was two months and ten days (Khoudi *et al.,* 2009). The present modified protocol produced transformed plants within a period of 70 to 75 days with a high regeneration efficiency (96.95%) compared to over 120 days using earlier published protocols.

Tomato transformation

Transformation was carried out according to McCormick *et al.,* (1986) with minor modifications. Cotyledon and hypocotyl explants were wounded and infected with *A. tumefaciens* strain LBA 4404 harboring the binary vector pBI121/Pg*NHX1*, pBI121/*AVP1* (Shimna Bhaskaran and Savithramma, D.L., 2011). The *nptII* locus proved to be a good selectable marker for tomato (Vries *et al*., 1997).

Transgenic tomato overexpressing Pg*NHX1*, *AVP1* and Co-expressing Pg*NHX1* and *AVP1*

In basic and practical studies for tomato improvement, successful transformation is essential. However, tomato transformation is still not routine nor reliable (Van Roekel *et al.,* 1993, Frary and Earle 1996; Ling *et al.,* 1998). Therefore, the development of an efficient and genotype independent tomato transformation method is crucial. The first report of tomato transformation was by McCormick *et al.,* (1986). Since then there have been numerous publications of transformation in various species and cultivars of tomato (Chyi and Philips 1987; Fillatti *et al*., 1987; Fischhoff *et al.,* 1987; Delannay *et al.,* 1989; Van Roekel *et al.,* 1993; Agharbaoui *et al*., 1995; Frary and Earle 1996; Ling *et al.,* 1998; Tabaeizadeh *et al.,* 1999; Vidya *et al.,* 2000; Hu and Phillips 2001; Abu-El-Heba *et al.,* 2008 and Paramesh *et al.,* 2010), Though the success was achieved in producing transgenic tomato lines, but couldn't reduce these steps involving obtaining the transgenics, as it required addition of irradiated cell lines of tomato, petunia or tobacco, time consuming media formulations or successive subcultures that produced significant variability between genotypes. No simple general procedure for tomato transformation exists. In the view of above insufficient protocol or standardization we are focusing and subjected some parameters for highly efficient transformation protocol in cv. Pusa ruby. The following parameters were standardized for highly efficient transformation protocol.

Effect of kanamycin treatment on explants (Cotyledon/Hypocotyl) surveillance in tomato variety "Pusa ruby"

The antibiotic concentration plays a very important role in selecting the transformed events from the untransformed events. In the present experiment 100mg/l kanamycin was found to be the best concentration to distinguish the transformed with untransformed. The highest surveillance transformed events was obtained from the cotyledonory explants (23.50) compared to hypocotyl explant (20.50). The

overall mean of surveillance of cotyledon and hypocotyl explants was 19.58 and 18.20 respectively (Table 2). The results obtained were in congruence with the results observed by Shimna Bhaskaran and Savithramma, D.L., 2011, for cotyledonary explants. Ultzen *et al*., 1995, obtained the regeneration efficiency of 5% for *cv.* ATV847, Ling *et al*., 1998 obtained 32.9% for cv. Money maker and Cortina *et al.,* 2004 produced to 48%. The results depicted the shoot regeneration efficiency on the selective antibiotic containing media varied for genotype to genotype.

Effect of different concentrations of Acetosyringone on *Agrobacterium* mediated transformation for cotyledon and Hypocotyl explants

Acetosyringone plays a major role in the transformation of crop plants as it is the main source for the activation of virulent genes in the *Agrobacterium* Ti plasmid. In the present study an attempt was made for the standardization of acteosyringone concentration to produce highest number of transformants, the highest number of kanamycin resistance was obtained at a concentration of 375 µM Acetosyringone for both cotyledonory and hypocotyl explants. Overall mean for number of kanamycin resistant lines in cotyledon explants were 18.20 and 17.80, whereas in hypocotyl explants 17.45 and 16.92 in I and II selections respectively. The best treatment was observed in T5 for both explants in the concentration of 375 µM, the number of kanamycin resistant lines of cotyledon (22.80) and in hypocotyl (21.80) explants (Table 3).

The cotyledonory explants were the best explants in both the selections at the concentration of 375 µM (Treatment 5) and it's significantly differed among the treatments, same results were also observed in cotyledon explants by Shimna Bhaskaran and Savithramma, 2011.

Effect of bacterial density and co-cultivation period on transformation of tomato cv. Pusa Ruby

The transformation efficiency was evaluated for cotyledonory and hypocotyl explants using *Agrobacterium* mediated transformation. Both explants were wounded and infected with *A.tumefaciens* strain LBA 4404 harboring the binary vector pBI121. Bacterial density or concentration of the culture is the most effective in co-cultivation period. The standardization for bacterial density or concentration cells/ml was most important to calculate transformation efficiency in time interval of co-cultivation period. Five different bacterial density or concentrations of cells/ml were used in the range of $0.5x10^8$ to $6.0x10^{8.}$ The co-cultivation time period at 72 h and 96 h for both cotyledon and hypocotyl explants (Table 4). In this experiment 50 explants were used for each treatment in both explants type. The co-cultivation time and the transformation efficiency varied in different concentrations of bacteria. The best concentration and co-cultivation period was observed at $1.0x10^8$ at 72 h incubation of bacterial culture and the transformation efficiency was recorded highest in cotyledon and hypocotyl at 45.6, 42.3 respectively.

Table. 4. Effect of bacterial density and co-cultivation period on transformation of Tomato cv. Pusa Ruby

Bacterial concentration Cells/ml	Number of explants	Average per cent transformation efficiency ± SE			
		Cotyledons		Hypocotyls	
		72 h Co-cultivation	96 h Co-cultivation	72 h Co-cultivation	96 h Co-cultivation
0.5×10^8	50	21.8±1.56	22.3±1.05	20.8±1.60	21.4±1.25
1.0×10^8	50	45.6±1.30	23.5±2.36	42.3±1.20	22.4±2.48
2.0×10^8	50	39.3±2.21	18.6±2.91	38.2±1.32	18.3±2.85
5.0×10^8	50	35.6±1.33	17.0±1.85	33.8±1.20	15.6±2.15
1.0×10^8	50	12.5±2.13	--------	12.2±2.85	--------

Transformation efficiency was calculated as the per cent co-cultivated explants producing independent transformation events, leading to regeneration of plantlets on antibiotic-containing medium. Transformation efficiency values represent the average ±SE of values from three independent experiments. The binary vector used for transformation was pBI121.

Table. 5. Effect of different growth regulators and their combinations on transformation of tomato cv. Pusa Ruby

Growth regulators (mg/l)	Number of explants		Average transformation efficiency (% ± SE)	
	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
BAP (2mg/l)	50	50	32.45±1.20	30.25±1.05
Zeatin (1mg/l)	50	50	41.65±1.02	39.45±2.12
BAP (2mg/l), IAA (1mg/l), Adenine sulphate (25 mg/l)	50	50	33.85±2.05	30.68±1.98
Zeatin (1mg/l) , IAA (0.1mg/l), Adenine sulphate (25 mg/l)	50	50	45.70±1.25	42.21±0.25
Zeatin (1mg/l) , IAA (1mg/l)	50	50	38.56±2.95	32.75±2.65

Transformation efficiency was calculated as described in table 4. The vector used for transformation was pB121. Standard error is mentioned for the values of transformation efficiency. BAP, 6-benzylaminopurine.

Table. 6. Transformation of tomato variety Pusa Ruby with pBI121/Pg*NHX1*, pBI121/*AVP1* and pBI121/Pg*NHX1+AVP1* by *Agrobacterium*-mediated method

Media	pBI121/Pg*NHX1*		pBI121/*AVP1*		pBI121/Pg*NHX1+AVP1*	
Experiment	1	2	1	2	1	2
Number of explants	80	90	80	90	80	90
RGM (Regeneration media)	50	54	45	49	52	58
SLM(Shoot elongation media)	39	45	35	38	40	48
RM (Rooting media)	26	30	25	28	31	35
Transformation efficiency (%)	32.50	33.33	31.25	31.11	38.75	38.88
Mean	32.91		31.18		38.81	

Table 7. Comparison of time duration of different tomato transformation protocols

Stages of protocol	Standard tomato protocol Dan *et al.* (2006)	Micro Tom protocol Dan *et al.* (2006)	Khoudi *et al.* (2009)	Present Experiment
Explant Preparation (d)	6	7	21	15
Preculture (d)	1	0	2	2
Co-cultivation (d)	2	2	2	2
Agrobacterium Elimination (d)	0	0		1
Shoot Induction (d)	28-35	21-28	14	14
Shoot Elongation (d)	28-42	14-21	13	15
Rooting (d)	21-28	21-28	18	15
Transformation Period (d)	3-4 months	2-3 months	2 months 10 days	2 months 5 days

The transformation efficiency was calculated as the per cent co-cultivated explants producing independent transformation events, leading to regeneration of plantlets on antibiotic-containing medium. Fillatti *et al.* (1987) found that when the concentration of the bacteria increased or decreased from 5.1 bacteria per ml, the rate of transformation was reduced by at least 20%, when cotyledons and hypocotyls were used as explants. In our experiment binary vector pBI121 was used for transformation and the results showing highest transformation efficiency was observed in cotyledon explants at 72 h incubation and bacterial concentration of $1.0x10^8$ was the best for co-cultivation.

Effect of different growth regulators and their combinations on transformation of tomato cv. Pusa Ruby

The different growth regulators and combinations plays important role on explant regeneration and transformation efficiency. In the present experiment we have used best combinations of growth regulators which were already used and standardized for cv. Pusa ruby (Table 5). The best transformation efficiency was observed in the hormonal combination of Zeatin (1mg/l), IAA (0.1mg/l), Adenine sulphate (25 mg/l) compared to 2 mg/l of BAP (6-benzylaminopurine) alone, the average transformation efficiency was ranging from 32.45% to 45.70%, 30.25% to 42.21% in cotyledon and hypocotyl respectively, the results suggest that the combination of Zeatin, IAA and Adenine sulphate is better than without combination of Zeatin alone than BAP. The best and highest transformation efficiency was recorded in Zeatin (1mg/l), IAA (0.1mg/l), Adenine sulphate (25 mg/l) is 45.70%, 42.21% in cotyledon and hypocotyl explants respectively. We are the first to report the use of these growth regulators and combinations influencing the increase in number of transformants and transformation efficiency.

The above standardized parameters were subjected for development of a highly efficient transformation protocol with optimization of time. The transformation efficiency was calculated based on the number of regenerated explants on kanamycin selection media to total number of explants inoculated. Transformation efficiency of pBI121/Pg*NHX1*, pBI121/*AVP1* and pBI121/*AVP1*+Pg*NHX1* was 32.91% (Fig.1), 31.18% (Fig.2) and 38.81% (Fig.3) respectively (Table 6). The transformation efficiency of double gene construct pBI121/*AVP1*+Pg*NHX1* was significantly higher compared to single gene constructs pBI121/Pg*NHX1* and pBI121/*AVP1*. The results are comparable to earlier reports, where, transformation efficiencies have ranged from 18.57% in pBI121/Pg*NHX1*, 19.55% in pBI121/*AVP1* and 12.40% in co-expression of pBI121/*AVP1*+Pg*NHX1* (Shimna Bhaskaran and Savithramma, 2011), 6% (Vidya *et al.*, 2000), 7 to 37% (Ling *et al.*, 1988), 11% (Frary and Earle 1996), 9% (Van Roekel *et al.*, 1993), 14% (Hamza and Chupeau, 1993), and 25% (Hu and Phillips, 2001) in tomato.

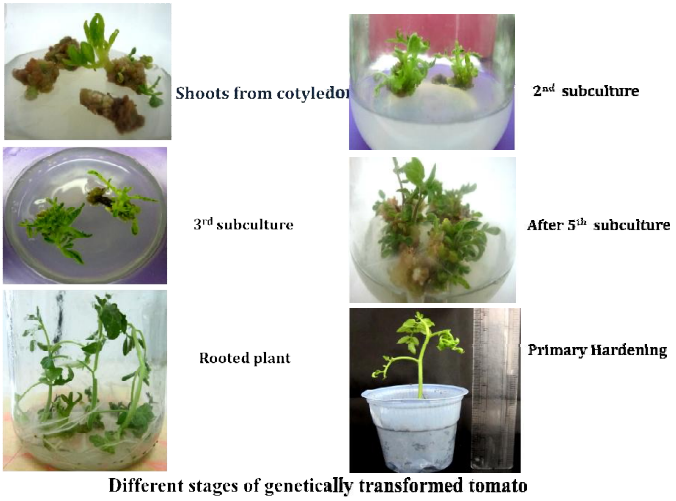


Fig. 1. Development of transgenic tomato over expressing Pg*NHX 1* for salt tolerance

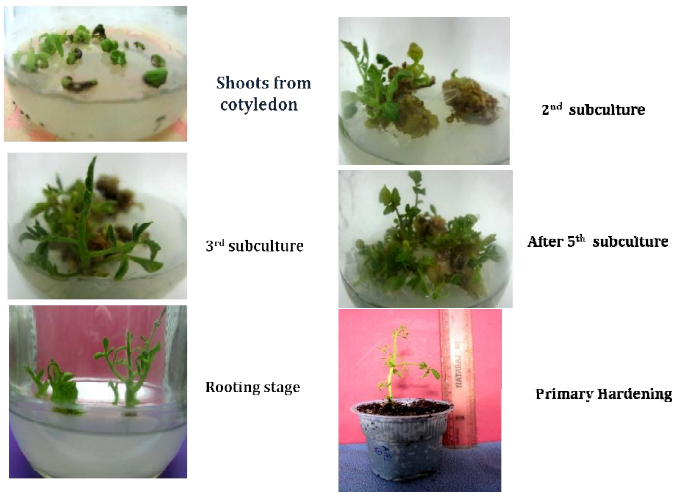


Fig. 2. Development of transgenic tomato over expressing *AVP1* for salt tolerance

Molecular characterization of transgenic plants

PCR analysis of primary transformants of tomato, overexpressing Pg*NHX1*, *AVP1* and co-expressing *PgNHX1* and *AVP1* (10 plants per each construct) were carried out using

appropriate gene specific primers. PCR amplification showed the presence of the expected 1.4kb fragment of *PgNHX1* and 2.3kb of *AVP1* gene, amplified products in the transgenic tomato plants transformed with pBI121/Pg*NHX1*, pBI121/*AVP1* 5 out of 10 plants showed positive amplification for each gene, (Plate. 1 and Plate 2) respectively. Similar kind of PCR analysis of the putative primary transformants of pBI121/Pg*NHX1+AVP1* showed the presence of appropriate amplification products in 6 out of 10 plants (Plate 3) of both the genes, thus confirming the integration of the respective genes.

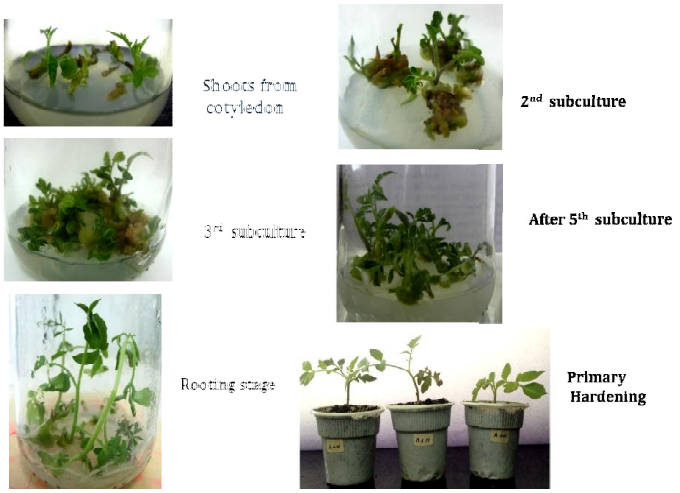
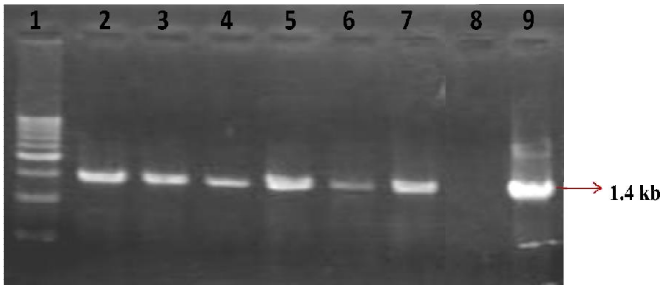


Fig. 3. Development of transgenic tomato over expressing Co- expressing Pg*NHX1* and *AVP1* for salt tolerance

Fig. 4. Secondary hardening of putative transformants plants

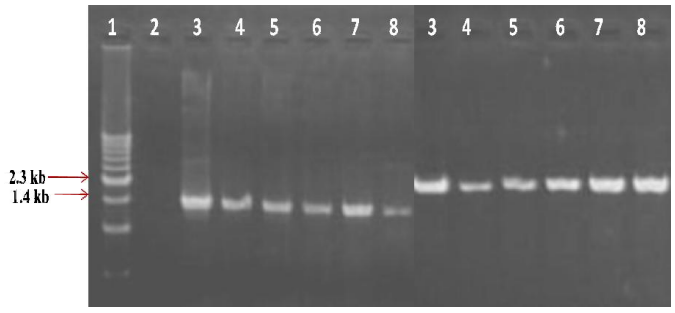


1.500bp ladder	2.N1	3. N2	4. N3	5. N4	6. N5	7. N6	8. –Ve control	9.+Ve control

Plate. 1. PCR analysis of transgenic (T_0) plants of tomato transformed with pBI121/Pg*NHX1*

1.A1	2.A2	3. A3	4. A4	5. A5	6.+Ve control	7.–Ve control	8.500bp ladder

Plate 2. PCR analysis of transgenic (T_0) plants of tomato transformed with pBI121/*AVP1*



1.500bp ladder	2. –Ve control	3. AN1	4. AN2	5. AN3	6. AN4	7. AN5	8. AN6

Plate 3. PCR analysis of transgenic (T_0) plants of tomato transformed with pBI121/*AVP1*+Pg*NHX1*

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 primordia formation started at 10 days and to obtain a shoot length of 9-10cm, 8-10cm was around 22 days in cotyledons and 25 days in hypocotyls. In cotyledon and hypocotyl explant, 96.95% and 95.90% 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), even though the antibiotic selection medium drastically reduces the transformation rate and efficiency, the present experiment achieved highest transformation (32.91% (pBI121/Pg*NHX1,* Fig. 1), 31.18% (pBI121/*AVP*1, Fig. 2) and 38.81% (pBI121/Pg*NHX1+AVP1,* Fig. 3) and regeneration rates (80-90%) in a short period of 55 to 65 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 response of variety,

cotyledon explant, use of Zeatin, IAA and Adenine sulphate as auxin and cytokinins sources in the regeneration medium and fewer subcultures for elongation, as we have 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 'Pusa Ruby' by studying different parameters involved in transformation and regeneration compared to other researchers on this variety. It is possible to obtain rooted tomato transformants within a period of 60-75 days with an efficiency of 95.65% regeneration and 38.81% transformation efficiency by using this protocol. We have also observed that cotyledon explants were superior to hypocotyl explants.

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