INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
15–565/2016/18–3–471–482
DOI: 10.17957/IJAB/15.0107
http://www.fspublishers.org

Full Length Article

Genetic Improvement of Tomato (*Solanum lycopersicum*) with *AtDREB1A* Gene for Cold Stress Tolerance using Optimized *Agrobacterium*-mediated Transformation System

Sabir Hussain Shah[1*], Shaukat Ali[2], Zahid Hussain[3], Sohail Ahmad Jan[4], Jalal-ud–Din[5] and Ghulam Muhammad Ali[2]
[1]*Department of Agricultural Sciences, Allama Iqbal Open University, Islamabad, Pakistan*
[2]*National Institute for Genomics & Advanced Biotechnology (NIGAB), National Agricultural Research Centre (NARC), Islamabad, Pakistan*
[3]*Centre for Biotechnology and Microbiology, University of Swat, KPK, Pakistan*
[4]*PARC Institute of Advanced Studies in Agriculture (PIASA), National Agricultural Research Centre (NARC), Islamabad, Pakistan*
[5]*Plant Physiology Program, National Agricultural Research Centre (NARC) Islamabad-Pakistan*
[*]For correspondence: sabir.hussain@aiou.edu.pk

Abstract

Cold is a serious threat that limits the productivity of tomato as it is a cold-sensitive crop. The cold tolerant gene *DREB1A* plays crucial roles in the survival of plants under cold stress. We have developed cold stress tolerant transgenic plants of tomato by the overexpression of an *Arabidopsis DREB1A* gene. They were obtained by using EHA-105 strain of *Agrobacterium* harboring pBIH binary vector that contained *DREB1A* controlled by an inducible promoter Lip9 and hygromycin phosphotransferase gene (*hpt*) as a plant selectable marker. Sequential 15 day old *in vitro* seedlings, 48 h pre-culture period, *Agrobacterium* density ($OD_{600\ nm}$ = 0.2), 3 min infection period and 60 µM acetosyringone yielded 15–18% TE. T_0 transformants were identified by PCR and then they were self-pollinated to generate T_1 progeny that was evaluated by gene expression and cold tolerance analyses. *AtDREB1A* transcript expression was detected by RT-PCR and stable integration of *AtDREB1A* gene was confirmed by Southern blotting. Morphological analyses of T_2 generations demonstrated that there were no significant differences among transgenic and NT plants under normal growth conditions. The T_2 transgenic lines exhibited more cold stress tolerance when exposed to 4ºC as compared to their NT counterparts. These findings clearly indicate that transgenic tomato plants over-expressing *Arabidopsis DREB1A* gene enhanced protection and provided cold tolerance under controlled conditions in transgenic containment. © 2016 Friends Science Publishers

Keywords: Cold stress; *DREB1A* gene; Hygromycin phosphotransferase gene; Lip9 promoter; *Solanum lycopersicum*

Introduction

Cold has a giant blow on agriculture since there are a small number of areas liberated of abiotic stresses in which crops can attain their utmost potential. The damage caused by freezing is a major limiting factor on crop production and geographical distribution (Li *et al.,* 2004). These unfavorable environmental stresses can cause the death of plants and restricts the agricultural yield (Jan *et al.,* 2009). The chilling temperature between 0 and 12°C produces serious harm to tropical and subtropical plants including tomato (Zhang *et al.,* 2009). Tomato is very sensitive to low temperature (Shah *et al.,* 2015a). Plant acts in response to cold stress to stay alive and various sorts of changes occur in the cellular, molecular and physiological levels of their growth and development (Porta *et al.,* 2014). Gene expression is induced by various abiotic stresses in different plants (Lee *et al.,* 2002). Cold tolerance is controlled by multiple genes and it is difficult to engineer multigenic traits for resistance like that of monogenic traits of resistance for herbicides and pests (Roy *et al.,* 2006). Therefore, the transformation of transcription factors (regulatory genes) in place of one or a few genes has been well-liked among researchers (Singh *et al.,* 2011). Cold acclimatization is directly associated with signal transduction pathways that ensure the regulation of genes under cold stress (Thomashow, 1998). Dehydration-responsive-element-binding protein or C-repeat-binding protein (DREBs/CBFs) is a well-known set of transcriptional factors that are up-regulated in response to cold (Liu *et al.,* 1998). These proteins contain a sole chain of DNA binding AP2 domain of 60 amino acids that allows them to recognize and attach as a single molecule to the DRE/CRT sequences of cold tolerance genes promoters (Khan, 2011).

To cite this paper: Shah, S.H., S. Ali, Z. Hussain, S.A. Jan, J.U. Din and G.M. Ali, 2016. Genetic improvement of tomato (*Solanum lycopersicum*) with *AtDREB1A* gene for cold stress tolerance using optimized *Agrobacterium*-mediated transformation system. *Int. J. Agric. Biol.,* 18: 471–482

The overexpression of cDNA encoding *DREB1A* activates many other stress tolerance genes and improves tolerance against chilling stress (Kasuga *et al.,* 1999).

Tomato (*Lycopersicon esculentum* Mill) has been used as a representative plant for *Agrobacterium* mediated transformation (Romero *et al.,* 2001) due to its distinctive uniqueness that build it a suitable form of plant species (Carvalho *et al.,* 2011), like its comparatively compact genome (950 Mb, n = 12) (Wang *et al.,* 2006; Yarra *et al.,* 2012), for fruit maturity (Giovannoni, 2004), plant protection (Pedley and Martin, 2003) and improving other dicot plants (Paramesh *et al.,* 2010). Recently, tomato utilization has increased that could defend against tumor and heart diseases for its antioxidant characters (Rein *et al.,* 2006). Modern techniques of biotechnology and genetic engineering can generate gorgeous agronomic characters more efficiently for recombinant proteins (Arokiaraj *et al.,* 2002).

This study was conducted to optimize *in vitro* seedling age, pre-culture period, *Agrobacterium* density, infection duration and acetosyringone concentration that have direct effect on transformation efficiency in tomato, screen three tomato transgenic lines namely Rio Grande, Moneymaker and Roma by various molecular analyses, and to evaluate chilling tolerance efficiency in three tomato transgenic lines.

Materials and Methods

Cloning Vector

A binary vector pBIH was provided by NIGAB, NARC, Islamabad, Pakistan. It contained *AtDREB1A* gene under the influence of an inducible lip9 promoter (Fig. 1).

Transformation of *Escherichia coli* (DH5α)

The *E. coli* strain DH5α was transformed with the plasmid (pBIH) under aseptic conditions in order to get the multiple copies of the desired plasmid. For this purpose, the competent cells of DH5 alpha (without its plasmid) were prepared by following protocol devised by Sambrook and Russel (2001). The bacterial culture (*E. coli*; DH5α) was grown overnight in Luria Broth medium (3 mL) by inserting single colony from selection plates. The LB medium was also fortified with kanamycin sulphate (50 mg L^{-1}). The recombinant plasmid DNA from DH5α was isolated and purified from the culture using a Miniprep method (Birnboim and Doly, 1979).

Agrobacterium tumefaciens Transformation with Recombinant Plasmid

The plasmid pBIH containing *DREB1A* gene was transformed in a super virulent *A. tumefaciens* strain, EHA105 under aseptic conditions. The electro-competent cells of *A. tumefaciens* strain (EHA105) were made ready by the procedure followed by Sambrook and Russel (2001) and transformation of *Agrobacterium* was done through electroporation.

Genetic Transformation of Tomato via *A. tumefaciens*

The genetic transformation of three tomato genotypes through *Agrobacterium*-mediated method was performed by optimizing the following parameters for improving overall genetic transformation efficiency in tomato.

Bacterial Culture Preparation

A. tumefaciens culture was prepared by scrapping a single colony from selection plate with the help of a flame-sterilized metal loop and put in 5 mL liquid YEP medium having kanamycin sulphate (50 mg L^{-1}). The bacterial suspension was incubated at 160 revolutions per minute with constant temperature (28°C) for 24 h. The suspension was spun at 8000 revolutions per minute for ten minutes in a large centrifuge tube. The pellet was dissolved in sterilized water and effects of various cell densities of *Agrobacterium* ($OD_{600\ nm}$ = 0.1, 0.2, 0.3, 0.4 and 0.5) were evaluated on transformation efficiency. Acetosyringone (AS) solution was prepared by dissolving in DMSO or ethanol to requisite stock concentration, sieved through filter and then preserved at – 20°C. Different concentrations of AS (0, 20, 40, 60, 80 and 100 µM) were added in two experiments for the improvement of transformation efficiency. In one experiment, acetosyringone (0-100 µM) was added in inoculation medium (Table 1; IM) and its effect on transformation efficiency was investigated. The hypocotyls and leaf discs were excised to about 0.5 cm segments from different ages of *in vitro* seedling (5, 10, 15, 20 and 25 days) to check the influence of seedling age on transformation efficiency. Before co-cultivation, the explants were pre-cultured on pre-culturing media (Table 1; PCM) for various time periods (0, 24, 48, 72 and 96 h) and their impact on transformation efficiency was examined.

Co-infection and Pre-selection

The pre-cultured explants were immersed in *Agrobacterium* culture for 2, 3, 5, 8 and 10 min in order to optimize the co-infection duration. After co-infection, the hypocotyls and leaf discs were blotted dry on sterilized filter papers in order to avoid surplus bacteria. The explants were then transferred to co-cultivation media (Table 1; CCM) having various AS concentrations (0, 20, 40, 60, 80 and 100 µM) were also optimized in transformation experiments. The co-cultivation media plates were covered with filter papers to control the bacterial overgrowth, and incubated in dark at 28°C. The explants without co-infected were also treated with the same growth conditions and used as control. During co-cultivation period, the material was monitored regularly for any contamination. After exposure to co-cultivation duration, hypocotyls and

Table 1: Different types of culture media used for regeneration and transformation of three genotypes of tomato

Culture media	Composition
Germination medium (GM)	MS basal medium (Murashige and Skoog, 1962), 30 g/L sucrose, 3.0 g/L phytagel, pH 5.7
YEP medium	10 g/L yeast extract, 10 g/L bacto-peptone, 5 g/L NaCl, pH 7.0, ± 7.0 g/L phytagel (for solid and liquid medium) (Sambrook and Russell, 2001)
LB medium	5 g/L yeast extract, 10 g/L bacto-tryptone, 10 g/L NaCl, pH 7.0, ± 7.0 g/L phytagel (for solid and liquid medium) (Sambrook and Russell, 2001)
Inoculation medium (IM)	MS basal medium, 30 g/L sucrose, 1.0 g/L casein hydrolysate, 60 µM acetosyringone, 3.0 g/L phytagel, pH 5.7
Pre-culture medium (PCM)	MS basal medium, 30 g/L sucrose, 2.0 mg/L IAA, 2.5 mg/L BAP, 1.0 g/L myo-inositol, 3.0 g/L phytagel, pH 5.7
Co-cultivation medium (CCM)	N6 basal medium, 30 g/L sucrose, 2.0 mg/L IAA, 2.5 mg/L BAP, 0.5 g/L cysteine, 60 µM acetosyringone 3.0 g/L phytagel, pH 5.7
Washing medium (WM)	N6 basal medium, 30 g/L sucrose, 1.0 mg/L IAA, 1.5 mg/L BAP, 0.5 mg/L cysteine, 500 mg/L cf, 3.0 g/L phytagel, pH 5.7
Pre-selection medium (PSM)	N6 basal medium, 30 g/L sucrose, 1.0 mg/L IAA, 2.0 mg/L BAP, 0.5 mg/L cysteine, 1.0 g/L myo-inositol, 30 g/L sorbitol, 500 mg/L cf, 3.0 g/L phytagel, pH 5.7
Selection medium (SM)	N6 basal medium, 30 g/L sucrose, 1.0 mg/L IAA, 2.0 mg/L BAP, 0.5 g/L cysteine, 1.0 g/L myo-inositol, 30 g/L sorbitol, 500 mg/L cf, 35 mg/L hygromycin, 3.0 g/L phytagel, pH 5.7
Shoot induction medium (SIM)	N6 basal medium, 30 g/L sucrose, 0.1 mg/L IAA, 1.0 mg/L ZEA, 2.0 mg/L BAP, 0.5 g/L cysteine, 1.0 g/L myo-inositol, 30 g/L sorbitol, 500 mg/L cf, 35 mg/L hygromycin, 3.0 g/L phytagel, pH 5.7
Shoot elongation medium (SEM)	N6 basal medium, 30 g/L sucrose, 0.5 g/L cysteine, 1.0 mg/L glycine, 1.0 g/L myo-inositol, 30 g/L sorbitol, 500 mg/L cf, 35 mg/L hygromycin, 3.0 g/L phytagel, pH 5.7
Root induction medium (RIM)	MS basal medium, 30 g/L sucrose, 0.4 mg/L IBA, 500 mg/L cf, 35 mg/L hygromycin, 3.0 g/L phytagel, pH 5.7

N6; 4.0 g/L N6 salts and vitamins (Chu, 1978), IAA; Indole-3-acetic acid, BAP; 6-benzylaminopurine, cf; cefotaxime

leaf discs were washed with ddH_2O four to five times followed by four times washing with washing medium (Table 1; WM) fortified with 500 mg/L claforan® (cefotaxime sodium) (Sanofi-aventis, Pakistan) for about 40 min to limit the *Agrobacterium* growth in co-infected explants during callus induction and morphogenesis phases. The explants were then blotted on autoclaved filter papers and transferred to pre-selection medium (Table 1; PSM). In this medium, hygromycin was not added in order to prevent the direct shock of antibiotics. The explants after washing were also shifted directly to selection medium (Table 1; SM) and used as control.

Transformants Selection

After pre-selection, the explants were transferred to selection medium supplemented with hygromycin and cefotaxime (Table 1; SM) and incubated at 25ºC in dark in order to select the transformants. After 4–5 weeks, the resistant calli were initiated. These calli were transferred to shoot induction medium (Table 1; SIM) and were sub-cultured twice after every two weeks. The shoots were regenerated from resistant calli clumps on shoot induction medium within 3–4 weeks (Shah *et al.*, 2013; 2014a, b; 2015b). The regenerated shoots (2–3 cm) were separated from calli clumps, washed with tap water and shifted to root induction medium (Table 1; RIM). However, the smaller shoots were excised and cultured on shoot elongation medium (Table 1; SEM). Transformation efficiency was measured by the number of hygromycin resistant explants showing regeneration divided by the total number of explants cultured on selection medium, expressed in percentage. The plantlets with efficient roots were acclimatized in culture tubes having tap water for five days at 25ºC. Subsequently, the plants were shifted to optimized growth medium such as soil and vermiculite (1:1) and maintained under glasshouse conditions. The plantlets survival rate was recorded periodically for each experiment and they were allowed to grow until maturity to bear fruit and seeds.

Molecular Analyses

The three tomato transgenic lines $T_0 - T_2$ were confirmed by following molecular analyses.

Genomic DNA Extraction and PCR Analysis

Genomic DNA was isolated from leaf samples by CTAB method based on the procedure given by Murray and Thomson (1980). The desired incorporated genes namely *DREB1A* and *hpt* were identified by PCR analysis. The primers pairs sequence, optimized PCR reagents and optimized PCR profile have been shown in Table 2–4.

RNA Isolation and Expression Analysis

Total RNA from transgenic and NT tomato plants was extracted from leaf samples using NucleoSpin RNA kit (MACHEREY-NAGEL GmbH, Germany) following manufacturer's instructions. The isolated RNA by this method was quantified using spectrophotometer (IMPLEN, Germany) at 260 nm. Semi-quantitative RT-PCR detected differential expression pattern of *AtDREB1A* gene. Two steps were followed to run RT-PCR. In step one; cDNA was prepared from pure RNA following the instruction of RevertAid Reverse Transcriptase kit, Catalog # EP0442 (Thermo Scientific). In step two, cDNA was used as a template for running PCR by using *DREB1A* specified primer pairs. The primers pairs sequence, optimized PCR reagents and optimized PCR profile have been shown in Table 2–4.

Table 2: List of primer pair sequences used for confirmation and expression analyses of *AtDREB1A* and *hpt* genes

S. No.	Gene amplified	Product size	Sequence (5' to 3')
1	*DREB1A*	649 bp	TGAACTCATTTTCTGCTTT
			TAATAACTCCATAACGATA
2	*hpt*	399 bp	TCGTGCTTTCAGCTTCGATG
			TCCATCACAGTTTGCCAGTG

Table 3: Optimized PCR reagents used for the confirmation of *AtDREB1A* and *hpt* genes by PCR analyses

S. No.	Reagent	Quantity
1	10 × Taq DNA Buffer (Fermentas)	2 µL
2	50 mM $MgCl_2$ (Fermentas)	2.4 µL
3	10 mM dNTPs (Fermentas)	0.4 µL
4	*DREB1A* forward primer	1.0 µL
5	*DREB1A* reverse primer	1.0 µL
6	Taq DNA polymerase (Fermentas)	0.3 µL
7	DEPC-treated water	10.9 µL
8	DNA template	2.0 µL
Total volume in PCR reactions system		20 µL
These reactions were done in PCR machine (Applied Biosystem)		

Table 4: Optimized PCR profile used for PCR analyses

S. No.	Stages	Temperature (°C)	Time (min)	No. of cycles
1	Initial Denaturation	94	5	1
	Final Denaturation	94	1	
2	Annealing	50	1	35
3	Initial Extension	72	1	1
	Final Extension	72	10	

Southern Blot Analysis

Genomic DNA from T_1 generation was isolated by CTAB method (Murray and Thomson, 1980) and Southern blot was performed by following standard procedure (Southern, 2006). Briefly, 10 µg pure DNA was digested by 2 µL BamHI and transferred to nylon membrane. Subsequently, the nylon membrane loaded with DNA was UV cross linked to avoid DNA partial digestion, dried and incubated at 42°C for 2 h in prehybridization solution to remove non-precise binding of probe to DNA. The Biotin DecaLabelled probe after denaturing at 100°C for 5 min was hybridized with target DNA by incubating in hybridization solution at 44°C for 12 h.

Chilling Tolerance Assessment Test in T_2 Transgenic Lines

The T_2 transgenic plants of three tomato genotypes (Rio Grande, Moneymaker and Roma) that followed Mendelian segregation ratio (3:1) were chosen for cold tolerance analysis. Three weeks old transgenic and NT plants (obtained from normal seeds) of all the genotypes were exposed to low temperature stress (4°C) under controlled environment conditions (16/8 h photoperiod and 50 µmole m^{-2} s^{-1} fluorescence light) for seven days and then returned to 25°C for recovery. The plant survival rate was determined after seven days of stress when plants regained their growth under normal conditions.

Statistical Analysis

Completely randomized design with factorial arrangement was used to perform all experiments. Data for each experiment (repeated thrice) was analyzed by analysis of variance at $p \leq 0.05$. LSD test was used to compare logical differences among means (Steel *et al.*, 1997). All the statistical analyses were performed by statistical software namely The Statistic v. 8.1 (Analytical Software, 2005) at 95% confidence interval.

Results

Assessment of *In Vitro* Seedling Age on Transformation Efficiency

Effect of *in vitro* seedling age on transformation efficiency (TE) was investigated in three tomato genotypes (Rio Grande, Moneymaker and Roma). A significant difference of seedling age was noticed for both hypocotyls and leaf discs in all the genotypes. The TE increased with the increase in seedling age up to fifteen days. It was observed that TE was higher in 15 days old *in vitro* seedlings as compared to 5 and 10 ays old seedlings in both types of explants in all the genotypes (Table 5; Fig. 2a). Similarly, 20 and 25 days old seedlings showed lower transformation efficiencies than that of fifteen days old seedlings. The highest TE was recorded in Roma (15.71%) followed by Rio Grande (14.61%) and Moneymaker (13.74%) respectively. Leaf discs derived maximum transformation efficiency which was 11.62, 10.52 and 9.5% in Roma, Rio Grande and Moneymaker at fifteen days old seedlings (Table 5). These results indicated that fifteen days old *in vitro* seedling was ideal for maximum TE in tomato.

Assessment of Pre-culture Period on Transformation Efficiency

To investigate the effect of different pre-culture periods on TE in tomato, hypocotyls and leaf discs were pre-cultured on optimized *in vitro* shoot regeneration media (Shah *et al.*, 2014a). During this study, continual pre-culture experiments were conducted at regular intervals of 0, 24, 48, 72 and 96 h. Most of the explants increased significantly in size during pre-culture period. The lowest TE (0.57 and 1.27%) was secured using leaf discs and hypocotyls without pre-culturing. The highest TE (8.25, 9.88 and 10.69%) was recorded in Moneymaker, Roma and Rio Grande when hypocotyls were pre-cultured for 48 h (Table 6; Fig. 2b). Similarly, the highest

Table 5: Assessment of *in vitro* seedling age on transformation efficiency in tomato

In vitro seedling age (days)	Transformation efficiency culturing hypocotyls (%)			Transformation efficiency culturing leaf discs (%)		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
5	2.69^{qr}	3.33^{pq}	2.14^{rs}	1.57st	0.53^{u}	1.00^{tu}
10	5.97^{lm}	6.66^{kl}	5.00^{n}	3.1^{q}	3.92^{op}	2.89^{qr}
15	14.61^{b}	13.74^{c}	15.71^{a}	10.52^{fg}	9.5^{hi}	11.62^{de}
20	10.99^{ef}	9.93^{gh}	12.13^{d}	7.11^{k}	6.50^{kl}	7.90^{j}
25	8.07^{j}	8.05^{j}	8.92^{i}	5.25^{mn}	4.09^{o}	4.65^{no}

The values are means and percentages of three separate experiments. Mean values following by the different letters show significant differences ($p \leq 0.05$). LSD value is 0.75 at $p \leq 0.05$

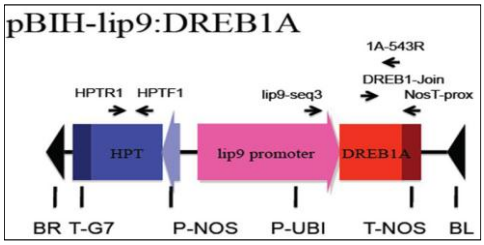

Fig. 1: Schematic diagram of the structure of the T-DNA region in the plasmid pBIH-lip9: *DREB1A*. LB; Left border, RB; Right border, T-G7; Terminator, *hpt*; hygromycin phosphotransferase gene, T-NOS and P-NOS (terminator and promoter sequences of nopaline synthase gene, Lip9; Lipase 9 promoter

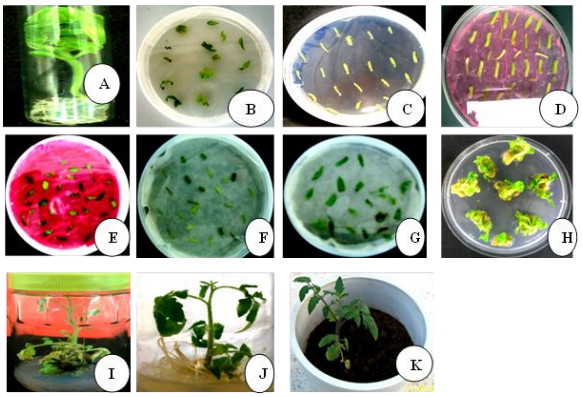

Fig. 2: Development of tomato transgenic plants in a sequential manner via *Agrobacterium*-mediated transformation method **(a)** Two-three week old *in vitro* seedlings for hypocotyls and leaf discs explants (**b)** Pre-culturing stage of hypocotyls for 48-h **(c)** Pre-culturing stage of leaf discs for 48-h **(d)** Co-cultivation stage of hypocotyls after infecting them with *Agrobacterium* ($OD_{600\ nm}$ = 0.2) for 3 min and incubated on autoclaved filter paper to avoid access bacterial growth **(e)** Co-cultivation stage of leaf discs after infecting them with *Agrobacterium* ($OD_{600\ nm}$ = 0.2) for 3 min and incubated on autoclaved filter paper to avoid access bacterial growth **(f)** Pre-selection stage of hypocotyls after incubating them on co-cultivation media supplemented with 60 µM acetosyringone **(g)** Pre-selection of leaf discs after incubating them on co-cultivation media supplemented with 60 µM acetosyringone **(h)** Hygromycin resistant calli showing root and shoot development **(i)** Hygromycin resistant plants produced on MS medium fortified with 10 mg/l $AgNO_3$, 0.1 mg/l IAA, 1.0 mg/l ZEA, 2.0 mg/l BAP and 35 mg/l hygromycin **(j)** Hygromycin resistant plants produced on MS medium enriched with sucrose: sorbitol (30:30 g/L) and 35 mg/L hygromycin **(k)** Acclimatization of hygromycin resistant plants on soil: vermiculite (1:1) growth medium for 4-week in growth room

TE (6.4, 9.18 and 9.88%) was recorded in Moneymaker, Rio Grande and Roma culturing leaf discs for 48 h (Table 6; Fig. 2c). The pre-culture period beyond 48 h significantly decreased TE in all the genotypes (Table 6).

Assessment of *Agrobacterium* Densities on Transformation Efficiency

Bacterial densities significantly affected TE in all the genotypes. Different optical densities (0.1, 0.2, 0.3, 0.4 and 0.5) at 600 nm were scrutinized their influence on TE giving infection to explants. Statistically, significant results for genotype, explants type and OD were recorded in all the genotypes that showed maximum TE at $OD_{600\ nm}$ = 0.2. The highest TE (8.83%) at $OD_{600\ nm}$ = 0.2 was secured in Rio Grande culturing hypocotyls as a source of explants, followed by Moneymaker (7.33%) and Roma (6.66%) (Table 7; Fig. 2d). When OD was increased from 0.2, TE gradually decreased due to excessive growth of bacteria causing necrosis in both types of explants in all genotypes tested (Table 7). There was a significant linear relationship between bacterial concentration and percentage of necrotic explants and also a significant interaction of cultivar with bacterial concentration. On average, irrespective of genotype and explants, $OD_{600\ nm}$ = 0.2 was found optimum for further tissue-culture based transformation experiments.

Assessment of Infection Durations on Transformation Efficiency

Effect of various infection durations (the time for which explants were submerged in the co-cultivation liquid media containing the bacterial suspension) (2, 3, 5, 8 and 10 min) was investigated on TE. The bacterial $OD_{600\ nm}$ = 0.2 was maintained in all of the experiments. No transformant was obtained at 1 min infection duration in both types of explants in all the genotypes, indicating that bacterial culture did not properly associate with explants. Among

Table 6: Assessment of various pre-culture periods on transformation efficiency in tomato

Pre-culture period (h)	Transformation efficiency culturing hypocotyls (%)			Transformation efficiency culturing leaf discs (%)		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
0	1.39^{o-r}	1.83^{nop}	1.27^{o-s}	0.91^{qrs}	0.74^{rs}	0.57^{s}
24	4.38^{hi}	5.12^{gh}	4.05^{ij}	3.02^{kl}	3.20^{k}	3.42^{jk}
48	10.69^{a}	8.25^{c}	9.95^{ab}	9.18^{b}	6.40^{ef}	9.88^{b}
72	7.44^{d}	6.36^{ef}	7.00^{de}	5.83^{fg}	4.80^{hi}	5.81^{fg}
96	2.32^{lmn}	2.04^{mno}	2.82^{klm}	1.42^{o-r}	1.60^{n-q}	1.14^{p-s}

The values are means and percentages of three separate experiments. Mean values following by the different letters show significant differences ($p \leq 0.05$). LSD value is 0.79 at $p \leq 0.05$

Table 7: Assessment of various *Agrobacterium* (EHA105) cell densities on transformation efficiency in tomato

Cell densities of *Agrobacterium* ($OD_{600\ nm}$)	Transformation efficiency culturing hypocotyls (%)			Transformation efficiency culturing leaf discs (%)		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
0.1	5.66^{c}	4.00^{ef}	4.00^{ef}	3.87^{ef}	2.77^{ghi}	2.21^{hij}
0.2	8.83^{a}	7.33^{b}	6.66^{b}	5.55^{c}	5.00^{cd}	4.44^{de}
0.3	6.58^{b}	5.33^{c}	4.00^{ef}	3.06^{g}	3.88^{ef}	3.33^{fg}
0.4	2.89^{gh}	2.66^{ghi}	2.00^{ij}	1.10^{k}	1.66^{jk}	1.10^{k}
0.5	0.00^{l}	0.00^{l}	0.00^{l}	0.00^{l}	0.00^{l}	0.00^{l}

The values are means and percentages of three separate experiments. Mean values following by the different letters show significant differences ($p \leq 0.05$). LSD value is 0.79 at $p \leq 0.05$

Table 8: Assessment of different time periods of transfection on transformation efficiency in tomato

Infection time (min)	Transformation efficiency culturing hypocotyls (%)			Transformation efficiency culturing leaf discs (%)		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
2	6.31^{e}	5.00^{f}	6.22^{e}	4.22^{gh}	2.79^{j}	3.68^{hi}
3	9.21^{b}	8.50^{c}	10.66^{a}	7.44^{d}	7.64^{d}	8.94^{bc}
5	3.67^{hi}	4.50^{fg}	4.85^{fg}	2.66^{jk}	3.05^{ij}	2.10^{kl}
8	2.57^{jk}	1.85^{lm}	1.35^{mno}	1.33^{m-p}	1.46^{lmn}	1.04^{n-q}
10	0.52^{q}	1.00^{n-q}	1.47^{lmn}	0.66^{pq}	0.58^{q}	0.70^{opq}

The values are means and percentages of three separate experiments. Mean values following by the different letters show significant differences ($p \leq 0.05$). LSD value is 0.68 at $p \leq 0.05$

Table 9: Assessment of various concentrations of acetosyringone on transformation efficiency in tomato

Acetosyringone concentration (µM)	Transformation efficiency culturing hypocotyls (%)			Transformation efficiency culturing leaf discs (%)		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
0	2.41^{qr}	1.87^{rs}	3.05^{pq}	1.13^{st}	0.57^{t}	0.92^{st}
20	5.55^{lm}	4.28^{no}	4.90^{mn}	4.07^{nop}	3.63^{op}	3.72^{op}
40	9.00^{hi}	10.35^{g}	10.18^{g}	7.13^{j}	6.36^{jkl}	6.86^{jk}
60	18.32^{a}	16.13^{b}	15.73^{bc}	15.00^{c}	13.77^{d}	12.39^{e}
80	12.22^{e}	11.42^{ef}	11.69^{ef}	10.71^{fg}	8.63^{i}	9.76^{gh}
100	10.00^{gh}	6.78^{jk}	7.16^{j}	6.42^{jkl}	5.45^{lm}	6.04^{kl}

The values are means and percentages of three separate experiments. Mean values following by the different letters show significant differences ($p \leq 0.05$). LSD value is 1.02 at $p \leq 0.05$

various infection durations examined, 3 min at $OD_{600\ nm}$ = 0.2 was found optimum for explants survival and efficient transformation for all the cultivars. When the infection duration was increased beyond 3 min, TE gradually decreased and necrosis occurred in the same manner due to excessive amount of bacteria (Table 8). To prevent the explants from necrosis, they were sub-cultured on fresh MS media supplemented with 300 mg/L claforan® (cefotaxime sodium). The highest TE (10.66%) was achieved in Roma followed by Rio Grande (9.21%) and Moneymaker (8.5%) at 3 min infection period culturing hypocotyls explants (Table 8; Fig. 2d). Similarly from leaf discs, the best TE (8.94%) was recorded in Roma followed by Moneymaker (7.64%) and Rio Grande (7.44%) at 3 min infection period (Table 8; Fig. 2e). From the present investigation, we concluded that infection period more than three minutes caused necrosis in explant tissues and reduced TE (Table 8).

Assessment of Acetosyringone on Transformation Efficiency

Different concentrations of acetosyringone (0, 20, 40, 60, 80 and 100 µM) were added in two experiments for the improvement of TE. In first experiment, 0–100 µM acetosyringone was supplemented with inoculation medium (Table 1; IM), while in second experiment, the hypocotyls

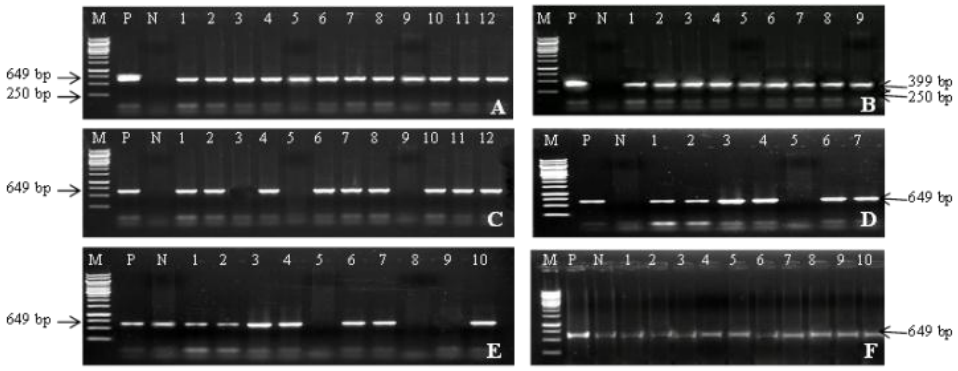

Fig. 3: Confirmation and expression analyses of T_0 – T_1 tomato transgenic lines by PCR, segregation analysis and semi-quantitative RT-PCR **(a)** PCR analysis showing 649 bp fragment of *AtDREB1A*; Lane M shows DNA ladder (1 kb) (Fermentas), Lane P shows positive control, Lane N shows negative control, Lanes 1–5 show transgenic Roma, Lanes 6-9 show transgenic Moneymaker, Lanes 10–12 show transgenic Rio Grande **(b)** PCR analysis showing 399 bp fragment of *hpt*; Lane M, P and N show DNA ladder, positive control and negative control, respectively, Lanes 1–3 show transgenic Roma, Lanes 4–6 show transgenic Moneymaker, Lanes 7–9 show transgenic Rio Grande **(c)** T_1 transgenic Roma showing Mendelian ratio (3:1) by segregation analysis **(d)** T_1 transgenic Moneymaker showing Mendelian ratio (3:1) by segregation analysis **(e)** T_1 transgenic Rio Grande showing Mendelian ratio (3:1) by segregation analysis **(f)** Expression analysis of *AtDREB1A* by semi-quantitative RT-PCR which depicts various expression pattern of desired gene in three tomato transgenic lines; Lanes 1-4 show transgenic Roma, Lanes 5–7 show transgenic Moneymaker, Lanes 8–10 show transgenic Rio Grande

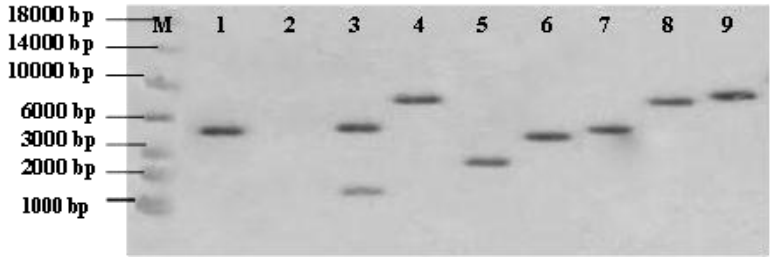

Fig. 4: Southern blot study showing stable integration of *AtDREB1A* gene in T_1 generation. Lane M shows molecular weight marker. Lane 1 shows pBIH-*AtDREB1A* plasmid, Lane 2 shows NT plants, Lane 1–3 shows transgenic Roma, Lane 4–6 shows transgenic Moneymaker, Lane 7–9 shows transgenic Rio Grande

and leaf discs were co-cultivated for two days on co-cultivation media (Table 1; CCM) supplemented with the same concentrations of acetosyringone. The TE was compared after four weeks of culture on selection medium. The minimum TE (0.57%) was recorded in an experiment with no acetosyringone (Table 9). It was found that TE increased up to 60 µM acetosyringone concentration, while further increase of acetosyringone gradually decreased TE. The TE increased considerably in explants cultured on co-cultivation media supplemented with 60 µM acetosyringone. The highest TE (18.32%) was noticed in cv. Rio Grande followed by Moneymaker (16.13%) and Roma (15.73%) culturing hypocotyls using 60 µM acetosyringone (Table 9; Fig. 2f). Similarly, leaf discs derived maximum TE was 15 and 13.77% in Rio Grande and Moneymaker followed by Roma (12.39%) at 60 µM acetosyringone (Table 9; Fig. 2g).

Molecular Analyses for Confirmation and Expression of Desired Gene

Identification and segregation analysis of desired gene: To confirm the integration of *AtDREB1A* and *hpt* genes, PCR was done. For that purpose, genomic DNA from transgenic and NT plants was extracted. The plasmid DNA of *Agrobacterium* was used as positive control, while DNA from NT plants was used as negative control. The gene fragments (649 and 399 bp) of *DREB1A* and *hpt*, respectively were amplified from T_0 putative transgenic plants only by using specific primer pairs (Fig. 3a and b). The seeds of T_0 putative transgenic lines were harvested individually. Then, healthy seeds were self-fertilized under controlled conditions to give rise T_1 progeny that was scrutinized by PCR analysis. T_1 generation of all the

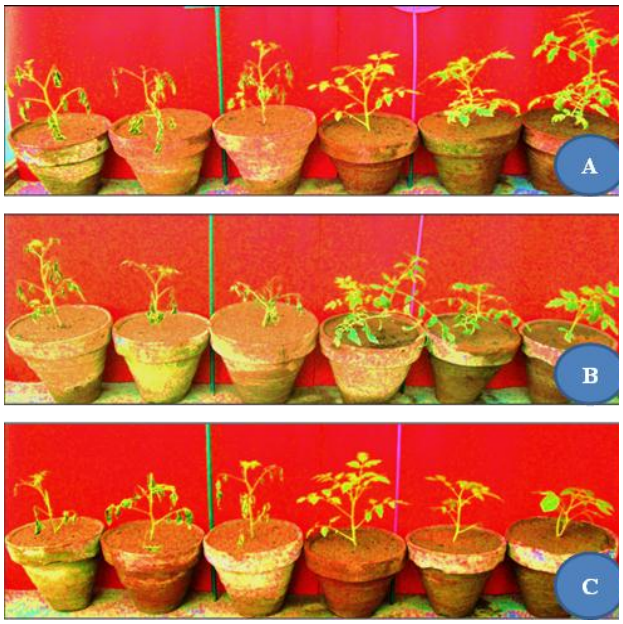

Fig. 5: Evaluation of cold tolerance in 3 tomato transgenic lines (T_2) at 4°C under controlled environment in glasshouse **(a)** Phenotypic responses of first three transgenic plants from right and NT plants of Rio Grande, where NT plants showed severe wilting symptoms as compared to transgenic plants after 4°C cold stress **(b)** Phenotypic changes of first three NT plants from left and transgenic plants of Moneymaker by exposing to low temperature (4°C) for seven days followed by maintenance at 25°C for one day **(c)** Phenotypes of first three NT plants of Roma from left showing clearly the wilting and dried leaves symptoms compared to their counterparts showing almost normal growth at 4°C temperature stress

transgenic lines showed normal Mendelian ratio (3:1) by segregation analysis done by gel electrophoresis (Fig. 3c–e).

Expression Analysis

To assess the expression of mRNA transcript, we performed semi-quantitative RT-PCR in three tomato transgenic lines. The T_1 transgenic and NT plants were exposed to 4°C for 3 days plus one day recovery period and then RT-PCR analysis was done. This assay depicted that relative transcript was expressed only under chilling stress and it produced differential pattern of mRNA transcript in leaf tissues of transgenic lines (Fig. 3f).

Southern Blot Analysis

To investigate the stable integration of *AtDREB1A* in T_1 generation, we performed Southern blot experiment. The putative transgenic lines (T_0) were self-pollinated to generate T_1 transgenic lines. Afterwards, segregation analysis was done via PCR that confirmed 3:1 of transgenic lines. The plants of each line were exposed to Southern blotting. The DNA of 8 transgenic plants was incubated with BamH1 for digestion. The results showed the presence of one to two copies of *AtDREB1A* in transgenic lines, while no signal was found in NT lines (Fig. 4).

Chilling Trials

To investigate the cold tolerance potential of *AtDREB1A* gene, transgenic as well as NT plants of three transgenic lines (T_2) were subjected to 4°C for seven days under controlled conditions in transgenic containment. Both transgenic and NT plants of same age were given cold stress. The phenotypic differences were evident among transgenic and NT plants after stress application (Fig. 5). NT plants of all the genotypes showed severe wilting symptoms after 3–4 days of cold stress. After 8–9 days of cold stress, the maximum leaves of NT plants became brown, dried up and ultimately died. The converse was true in case of all transgenic plants having *AtDREB1A* gene (Fig. 5). These plants exhibited healthy appearance and survived under severe cold stress period. After two weeks of cold spell, the transgenic plants were shifted to normal phenotype and yielded new leaves and branches, while NT plants failed to regain normal growth (Fig. 5).

Discussion

The comprehensive investigation of this study demonstrated that gene transfer efficiency decreased by increasing more than fifteen days old seedlings. TE was approximately two times higher in 15 days-old seedlings than that of 25 days old seedlings. Our results demonstrated that younger seedlings (15 days old) were more effective for gene transfer than that of older ones. It might be due to the reason that younger seedlings were more susceptible to *Agrobacterium* and might also be due to increase in active surface area i.e. more number of dividing plant cells facilitating efficient T-DNA transfer in younger seedlings than that of older ones, consistent with the other recent report (Ribas *et al.*, 2011). Sharma *et al.* (2009) optimized an efficient *Agrobacterium*-mediated transformation protocol culturing cotyledons from ten days old seedlings and reported the highest TE (41.4%) in tomato cv. Pusa Ruby. Li *et al.* (2009) used FAST technique for transient expression analysis of desired gene in *Arabidopsis.* The cotyledons from 3–5 days old seedlings were employed in transformation experiments and more than 50% TE was obtained. They also found that older seedlings (more than seven days old) had a sharp decline in TE. Dan *et al.* (2006) reported that twenty days old seedling proved to be the best age of explants for TE that ranged between 1.00 to 4.08%. Our findings were not consistent with the earlier report by Arcos-Ortega *et al.* (2010) who evaluated the influence of seedling age over TE in *Capsicum chinense* Jacq. The maximum TE (55%) secured culturing four weeks old leaf

explants, while it decreased and serious tissue necrosis occurred when eight week old explants were cultured. Hence, it is clear from this study that age of *in vitro* seedlings has a significant effect on TE in tomato.

Several studies have revealed the animation of "window of competence"; a time during which the plant cells are more vulnerable to *A. tumefaciens* transformation. It has been suggested that during the pre-culturing stage, the plant cells become more competent for transformation by physiological adaptation (Gao *et al.,* 2009). The pre-culture period increases the regeneration percentage by eliminating the hypersensitive response of explants induced by *Agrobacterium* in co-cultivation stage (Arcos-Ortega *et al.,* 2010). The pre-culture period is an important factor that significantly affects the TE in tomato (Rai *et al.,* 2012). TE is closely linked with cell division or dedifferentiation (Arias *et al.,* 2006). The major limiting factor in regeneration and TE is the phase of plant cell cycle. It has been reported that TE of cells with nuclei at the S phase of the cycle was higher (Pena *et al.,* 2004). The delivery of foreign DNA through *A. tumefaciens* occurs through S phase of cell cycle; therefore the actively growing cells particularly in S phase are the most responsive for plant genetic transformation via *Agrobacterium.* Our findings were supported by the earlier reports in which the highest TE recorded at 48 h pre-culture period (Sharma *et al.,* 2009). In contrast, Park *et al.* (2003) established an efficient *Agrobacterium*-mediated transformation system in tomato culturing cotyledons and hypocotyls explants from 10 days-old seedlings and ascertained that pre-culturing of these explants for 24 h gave the highest TE (20%). These differences might be due to difference in *Agrobacterium* strain, type and age of explants and genotypes. Rai *et al.* (2012) optimized an efficient *Agrobacterium*-mediated transformation protocol for tomato cultivars using cotyledons explants and reported that six days pre-culturing period significantly improved the TE upto 44.3% in tomato. Similarly, Duan *et al.* (2010) conducted a study on tomato cv. MicroTom and reported that pre-culturing of cotyledon on pre-cultured medium for 24 h decreased the TE and promoted browning of some tissues. While freshly isolated cotyledon explants improved the TE. These diverse observations might be due to disparity of tomato genotypes and explants used.

Our findings coincided with the earlier report by Qiu *et al.* (2007) who engineered tomato cv. Micro-Tom with *CsZCD* gene utilizing an improved transformation protocol via *Agrobacterium* and reported that among various *Agrobacterium* concentrations, $OD_{600\ nm}$ = 0.2 was found to be the best level for securing the highest TE (20.87%) in tomato. Duan *et al.* (2010) generated tomato (cv. Micro-Tom) transgenic plants through *Agrobacterium*-mediated method based on bacterial titers and argued that at OD reading of 1.0, the explants death occurred due to bacterial overgrowth, while the intermediate *Agrobacterium* titer ($OD_{600\ nm}$ = 0.1 – 0.5) was found optimum for efficient genetic transformation in tomato and no significant differences were noticed between TE at $OD_{600\ nm}$ = 0.1 and at $OD_{600\ nm}$ = 0.5. They also claimed that $OD_{600\ nm}$ = 0.1 was used in further transformation experiments. Contrary to our findings, high concentration of OD increased the TE in some species as supported by Guo *et al.* (2012) who optimized various factors for efficient gene transfer in tomato cv. Micro-Tom and concluded that *Agrobacterium* suspension ($OD_{600\ nm}$ = 0.5) along with other optimized parameters yielded the maximum TE (5.1%) culturing cotyledonary explants. Similarly, Cruz-Mendivil *et al.* (2011) conducted transformation study and inferred that leaf explants co-infected with bacterial density ($OD_{600\ nm}$ = 0.5) produced the maximum TE (19.1%) in tomato cv. Micro-Tom.

A peculiar infection period plays an important role for T-DNA transfer into plant genome as low infection duration ensures very less TE due to improper association of *Agrobacterium* with host tissues. While high infection time generates hypertonic conditions due to excessive growth of bacteria that ultimately burst the plant cells (Thiruvengadam *et al.,* 2013). Optimization of transformation protocols is necessary to combat all these problems faced in the development of transgenic tomato (Gao *et al.,* 2009). Random integration of T-DNA in multiple copies can be found in the plant genome. Gene silencing can result very poor or no expression at all (Chalfun *et al.,* 2003). As a result, the rate of expression of the desired characters might be very low. For this purpose, an efficient transformation protocol can raise the amount of transgenic plants (Van *et al.,* 2010). Our findings indicated a specific range of *Agrobacterium* density and infection period for obtaining maximum TE. Guo *et al.* (2012) optimized various transformation factors in tomato cv. Micro-Tom culturing cotyledon explants and proclaimed that bacterial concentration and infection time had significant effect on contamination, necrosis and ultimately TE. They recommended 5 min infection period for maximum TE (5.1%) in Micro-Tom. Similarly, Rai *et al.* (2012) developed an efficient procedure for genetic transformation of tomato culturing cotyledonary explants from six days old *in vitro* seedlings and suggested 5 min infection duration for maximum TE (44.3%). This is contradictory to our findings, as in our experiment, necrosis started in explants at five min infection duration. Duan *et al.* (2012) optimized various transformation factors in tomato (cv. Lichun) and pointed out that inoculation period of ten minutes was optimum for obtaining maximum TE (27%) in tomato. Higher infection period of 30 min has been reported in the previous report by Sharma *et al.* (2009) who developed *Agrobacterium*-mediated system for tomato transformation culturing cotyledonary explants. This inconsistency of infection duration might be due to different genotypes and bacterial strain used.

Pitzschke and Hirt (2010) identified several steps involved for studying the interaction between

Agrobacterium and plant cells such as recognition and expression of Vir genes, targeting of Vir factors, transfer and integration of T-DNA into host cells. The Vir region (30–40 kb) of Ti plasmid was responsible for T-DNA transfer into host plant cells. This region made up of six necessary operons (Vir A, Vir B, Vir C, Vir D, Vir E and Vir G) and two unnecessary operons (Vir F and Vir H). The only two operons (Vir A and Vir G) constituted two component systems (Vir A/Vir G) that activated the transcription of other Vir genes (Riva *et al.,* 1998). This Vir A/Vir G system recognized a diversity of phenolic compounds such as AS that behaves as the inducers of Vir gene expression for stable genetic transformation. *Agrobacterium* strains showed different sensitivity responses to various concentrations of AS (Subramanyam *et al.,* 2011). Similarly, TE varied with different concentration of AS and their interaction with various genotypes. Although, AS like compounds were secreted by dicotyledonous plants themselves but the addition of AS in inoculation and co-cultivation media improved tomato TE (Mahmoudian *et al.,* 2002). Therefore, for the improvement of TE, different concentrations of AS (0–100 μM) were added in inoculation and co-cultivation media and were scrutinized in three tomato genotypes. In our study, 60 μM AS concentration enhanced TE in all the genotypes, while further increase of AS gradually decreased the transformation efficiencies. Our findings assisted the findings of Duan *et al.* (2012) who recorded the highest TE (73.2%) with the application of 50 μM AS using *A. tumefaciens* strain LBA4404 culturing hypocotyls and cotyledons of ten days old seedlings. Guo *et al.* (2012) used 100 μM acetosyringone in culture medium for *Agrobacterium* strain, EHA105 inoculating cotyledonary explants of tomato cv. Micro-Tom and obtained 5.1% TE. AS (100 μM) has also been reported in other previous studies (Costa *et al.,* 2002). Our results clearly demonstrated the effect of low levels of AS on regeneration and TE along with high levels which adversely affected the regeneration and subsequent TE in tomato due to overgrowth of *Agrobacterium.*

Zhang *et al.* (2004) conducted a similar type of study and reported that like *Arabidopsis CBF1-3* genes, tomato possesses 3 CBF homologs namely *Lycopersicum esculentum CBF1-3* (*LeCBF1-3*). Among them, only *LeCBF1* was functional in cold stress. They transformed *LeCBF1* in *Arabidopsis* under the control of 35S constitutive promoter and found aberrant growth and phenotypes of *Arabidopsis* transgenic plants. But the overexpression of this gene induced the expression of cold regulated (COR) genes and hence increased freezing tolerance in *Arabidopsis* without any chilling stimulus. From this experiment they inferred that *LeCBF1* was an active homolog of *AtCBF* genes. To prove these logic consequences, they performed another experiment in which they transformed *LeCBF1* or *AtCBF3* in tomato under constitutive 35S promoter and reported inhibitory effects of *LeCBF1* or *AtCBF3* on growth as well as yield characteristics including stunted growth, delay in flowering and few numbers of fruit per plants in transgenic plants as compared to their isogenic control plants. From this experiment, tomato transgenic plants didn't exhibit any freezing tolerance. But our research study is contrasting from that of Zhang *et al.* (2004) and signifies the importance of lip9 inducible promoter which triggers *AtDREB1A* gene to regulate the expression of multiple cold stress responsive genes to create chilling tolerance in tomato with no abnormal phenotypic changes. From present study, we also conclude that under normal growth conditions no distinguishable differences were noticed in transgenic and NT tomato plants. But under various levels of chilling stress, improved chilling tolerance was noticed in all the transgenic lines with no inhibitory effects of *AtDREB1A* gene on growth as well as yield characteristics of all the transgenic lines.

Conclusion

An efficient and quick transformation system has been established for three local tomato cultivars namely Rio Grande, Moneymaker and Roma assessing various critical factors that affect transformation efficiency in tomato. The resultant transgenic plants at T_0 stage were identified by PCR using gene specific *AtDREB1A* primers. These putative transgenic plants of all the lines were self-fertilized that generated T_1 progeny. For expression analysis, two steps RT-PCR was done that confirmed differential expression profile of our transgene in transgenic lines, while stable integration of the transgene was confirmed by southern blot analysis. The chilling tolerance of these transgenic lines was assessed at T_2 stage by exposing the transgenic lines as well as NT counterparts to 4°C, which showed that only transgenic lines exhibited chilling tolerance, while their NT counterparts showed symptoms of wilting, necrosis and ultimately died. The transgenic lines developed in this study could be further used in breeding programs to develop tomato cold tolerant cultivars.

Acknowledgments

This study was financially supported by Indigenous PhD Fellowship Program Higher Education Commission, Pakistan and National Institute for Genomics and Advanced Biotechnology (NIGAB), NARC is gratefully acknowledged. We are highly thankful to acknowledge the cooperation of Department of Horticulture, PMAS Arid Agriculture University, Rawalpindi in providing the facilities for evaluation of cold tolerance in tomato transgenic lines.

References

Analytical Software, 2005. *Statistix Version 8.1. User's Manual*. Analytical Software, Tallahassee, Florida, USA

Arcos-Ortega, G.F., R.A. Chan-Kuuk, W.A. Gonzalez-Kantun, R. Souza-Perera, Y.E. Nakazawa-Ueji, E. Aviles-Berzunza, G. Godoy-Hernandez, M.A. Lawton and J.J.J.Z. Aguilar, 2010. *Agrobacterium tumefaciens*-transient genetic transformation of Habanero pepper (*Capsicum chinense* Jacq.) leaf explants. *Electron. J. Biotechnol.*, 13: 1–9

Arias, R.S., S.A. Filichkin and S.H. Strauss, 2006. Divide and conquer: development and cell cycle genes in plant transformation. *Trends Biotechnol.*, 24: 267–273

Arokiaraj, P., F. Rueker, E. Obermayr, A.R.S. Bahri, J. Hafsah, D.C. Carer and H.Y. Yeang, 2002. Expression of human serum albumin in transgenic *Hevea brasiliensis*. *J. Rubber Res.*, 5: 157–166

Birnboim, H.C. and J. Doly, 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.*, 7: 1513–1523

Carvalho, R.F., M.L. Campos, L.E. Pino, S.L. Crestana, A. Zsogon, J.E. Lima, V.A. Benedito and L.E.P. Peres, 2011. Convergence of developmental mutants into a single tomato model system: 'Micro-Tom' as an effective toolkit for plant development research. *Plant Methods*, 7:18.

Chalfun, J.A., J.J. Mes, L. Mlynarova, M.G.M. Aarts and G.C. Angenent, 2003. Low frequency of T-DNA based activation tagging in *Arabidopsis* is correlated with methylation of CaMV 35S enhancer sequences. *FEBS Lett.*, 555: 459–463

Costa, M.G.C., W.C. Otoni and G.A. Moore, 2002. An evaluation of factors affecting the efficiency of *Agrobacterium*-mediated transformation of *Citrus paradise* (Macf.) and production of transgenic plants containing carotenoid biosynthetic genes. *Plant Cell Rep.*, 21: 365–373

Cruz-Mendivil, A., J.R. Lopez, L.J. German-Baez, M. Lopez-Meyer, S. Hernandez-Verdugo, J.A. Lopez-Valenzuela, C. Reyes-Moreno and A. Valdez-Ortiz, 2011. A simple and efficient protocol for plant regeneration and genetic transformation of tomato cv. Micro-Tom from leaf explants. *HortScience*, 46: 1655–1660

Dan, Y., H. Yan, T. Munyikwa, J. Dong, Y. Zhang and C.L. Armstrong, 2006. Micro-Tom – a high-throughput model transformation system for functional genomics. *Plant Cell Rep.*, 25: 432–441

Duan, Y., C. Zhai, H. Li, J. Li, W. Mei, H. Gui, D. Ni, F. Song, L. Li, W. Zhang and J. Yang, 2012. An efficient and high-throughput protocol for *Agrobacterium*-mediated transformation based on phosphomannose isomerase positive selection in Japonica rice (*Oryza sativa* L.). *Plant Cell Rep.*, 31: 1611–1624

Duan, Y.X., J. Fan and W.W. Guo, 2010. Regeneration and characterization of transgenic kumquat plants containing the *Arabidopsis APETALA1* gene. *Plant Cell Tiss. Org. Cult.*, 100: 273–281

Gao, N., W. Shen, Y. Cao, Y. Su and W. Shi, 2009. Influence of bacterial density during preculture on *Agrobacterium*-mediated transformation of tomato. *Plant Cell Tiss. Org. Cult.*, 98: 321–330

Giovannoni, J.J., 2004. Genetic regulation of fruit development and ripening. *Plant Cell*, 16: S170–S180

Guo, M., Y.L. Zhang, Z.J. Meng and J. Jiang, 2012. Optimization of factors affecting *Agrobacterium*-mediated transformation of Micro-Tom tomatoes. *Genet. Mol. Res.*, 11: 661–671

Jan, N., M. Hussain and K.I. Andrabi, 2009. Cold resistance in plants: A mystery unresolved. *Electron. J. Biotechnol.*, 12: 1–15

Kasuga, M., Q. Liu, S. Miura, K. Yamaguchi-Shinozaki and K. Shinozaki, 1999. Improving plant drought, salt and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotechnol.*, 17: 287–291

Khan, M.S., 2011. The role of DREB transcription factors in abiotic stress tolerance of plants. *Biotechnol. Biotec. Eq.*, 25: 2433–2442

Lee, H., Y. Guo, M. Ohta, L. Xiong, B. Stevenson and J.K. Zhu, 2002. LOS2, a genetic locus required for cold-responsive gene transcription encodes a bi-functional enolase. *EMBO J.*, 21: 2692–2702

Li, J.F., E. Park, A.G. Arnim and A. Nebenfuhr, 2009. The FAST technique: a simplified *Agrobacterium*-based transformation method for transient gene expression analysis in seedlings of *Arabidopsis* and other plant species. *Plant Methods*, 5: 1–15

Li, W., M. Li, W. Zhang, R. Welti and X. Wang, 2004. The plasma membrane–bound phospholipase Dδ enhances freezing tolerance in *Arabidopsis thaliana*. *Nat. Biotechnol.*, 22: 427–433

Liu, Q., M. Kasuga, Y. Sakuma, H. Abe, S. Miura, K.Y. Shinozaki and K. Shinozaki, 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell*, 10: 1391–406

Mahmoudian, M., M. Yucel and H.A. Oktem, 2002. Transformation of Lentil (*Lens culinaris* M.) cotyledonary nodes by vacuum infiltration of *Agrobacterium tumefaciens*. *Plant Mol. Biol. Rep.*, 20: 251–257

Murray, M.G. and W.F. Thomson, 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.*, 8: 4321–4325

Paramesh, H., B. Fakrudin and M.S. Kuruvinashetti, 2010. Genetic transformation of a local variety of tomato using gus gene: an efficient genetic transformation protocol for tomato. *J. Agric. Technol.*, 6: 87–97

Park, S.H., J.L. Morris, J.E. Park, K.D. Hirschi and R.H. Smith, 2003. Efficient and genotype-independent *Agrobacterium*-mediated tomato transformation. *J. Plant Physiol.*, 160: 1253–1257

Pedley, K.F. and G.B. Martin, 2003. Molecular basis of Pto-mediated resistance to bacterial speck disease in tomato. *Annu. Rev. Phytopathol.*, 41: 215–243

Pena, L., R.M. Perez, M. Cervera, J.A. Juarez and L. Navarro, 2004. Early events in *Agrobacterium*-mediated genetic transformation of citrus explants. *Ann. Bot.*, 94: 67–74

Pitzschke, A. and H. Hirt, 2010. New insights into an old story: *Agrobacterium*-induced tumour formation in plants by plant transformation. *EMBO J.*, 29: 1021–1032

Porta, N.L., G. Sablok, G. Emilliani, A.M. Hietala, A. Giovannelli, P. Fontana, E. Potenza and P. Baldi, 2014. Identification of low temperature stress regulated transcript sequences and gene families in Italian cypress. *Mol. Biotechnol.*, 57: 407–418

Qiu, D., G. Diretto, R. Tavarza and G. Giuliano, 2007. Improved protocol for *Agrobacterium*-mediated transformation of tomato and production of transgenic plants containing carotenoid biosynthetic gene *CsZCD*. *Sci. Hortic.*, 112: 172–175

Rai, G.K., N.P. Rai, S. Kumar, A. Yadav, S. Rathaur and M. Singh, 2012. Effects of explants age, germination medium, pre-culture parameters, inoculation medium, pH, washing medium, and selection regime on *Agrobacterium*-mediated transformation of tomato. *In Vitro Cell. Dev-Plant*, 48: 565–578

Rein, D., E. Schijlen, T. Kooistra, K. Herbers, L. Verschuren, R. Hall, U. Sonnewald, A. Bovy and R. Kleemann, 2006. Transgenic flavonoid tomato intake reduces C- reactive protein in human C-reactive protein transgenic mice more than wild-type tomato. *J. Nutr.*, 136: 2331–2337

Ribas, A.F., E. Dechamp, A. Champion, B. Bertrand, M.C. Combes, J.L. Verdeil, F. Lapeyre, P. Lashermes and H. Etienne, 2011. *Agrobacterium*-mediated genetic transformation of *Coffea arabica* (L.) is greatly enhanced by using established embryogenic callus cultures. *BMC Plant Biol.*, 11: 92

Riva, G.A., J.G. Cabrera, R.V. Padron and C.A. Pardo, 1998. *Agrobacterium tumefaciens*: a natural tool for plant transformation. *Electron. J. Biotechnol.*, 1: 118–133

Romero, J.P., G. Houlne, L. Canas, R. Schantz and J. Chamarro, 2001. Enhanced regeneration of tomato and pepper seedling explants for *Agrobacterium*-mediated transformation. *Plant Cell Tiss. Org. Cult.*, 67: 173–180

Roy, R., R.S. Purty, V. Agrawal and S.C. Gupta, 2006. Transformation of tomato cultivar 'Pusa Ruby' with *bspA* gene from *Populus tremula* for drought tolerance. *Plant Cell Tiss. Org. Cult.*, 84: 55–67

Sambrook, J. and D.W. Russel, 2001. *Molecular Cloning: A Laboratory Manual*, 3rd edition. Cold Spring Habour, NY

Shah, S.H., S. Ali and G.M. Ali, 2013. A novel approach for rapid *in vitro* morphogenesis in tomato (*Solanum lycopersicum* Mill.) with the application of cobalt chloride. *Eur. Acad. Res.*, 1: 2702–2721

Shah, S.H., S. Ali, S.A. Jan and G.M. Ali, 2014a. Assessment of carbon sources on *in vitro* shoot regeneration in tomato. *Pak. J. Agri. Sci.*, 51: 197–207

Shah, S.H., S. Ali, S.A. Jan, J. Din and G.M. Ali, 2014b. Assessment of silver nitrate on callus induction and *in vitro* shoot regeneration in tomato (*Solanum lycopersicum* Mill.). *Pak. J. Bot.*, 46: 2163–2172

Shah, S.H., S. Ali, S.A. Jan, J. Din and G.M. Ali, 2015a. Piercing and incubation method of *in planta* transformation producing stable transgenic plants by overexpressing *DREB1A* gene in tomato (*Solanum lycopersicum* Mill.). *Plant Cell Tiss. Org. Cult.*, 120: 1139–1157

Shah, S.H., S. Ali, S.A. Jan, J. Din and G.M. Ali, 2015b. Callus induction, *in vitro* shoot regeneration and hairy root formation by the assessment of various plant growth regulators in tomato (*Solanum lycopersicum* Mill.). *J. Anim. Plant Sci.*, 25: 528–538

Sharma, M.K., A.U. Solanke, D. Jani, Y. Singh and A.K. Sharma, 2009. A simple and efficient *Agrobacterium*-mediated procedure for transformation of tomato. *J. Biosci.*, 34: 423–433

Singh, S., M. Rathore, D. Goyary, R.K. Singh, S. Anandhan, D.K. Sharma and Z. Ahmed, 2011. Induced ectopic expression of *At-CBF1* in marker-free transgenic tomatoes confers enhanced chilling tolerance. *Plant Cell Rep.*, 30: 1019–1028

Southern, E., 2006. Protocol southern blotting. *Nat. Protoc.*, 1: 518–525

Steel, R.G.D., J.H. Torrie and D.A. Dicky, 1997. *Principles and Procedures of Statistics: A Biological Approach*, 3rd edition, pp: 352–258. McGraw Hill Book Co. Inc., New York, USA

Subramanyam, K., K. Subramanyam, K.V. Sailaja, M. Srinivasulu and K. Lakshmidevi, 2011. Highly efficient *Agrobacterium*-mediated transformation of banana cv. Rasthali (AAB) via sonication and vacuum infiltration. *Plant Cell Rep.*, 30: 425–436

Thiruvengadam, M., J. Jeyakumar, M. Kamaraj, I.M. Chung and J.J. Kim, 2013. Optimization of *Agrobacterium*-mediated genetic transformation in gherkin (*Cucumis anguria* L.). *Plant Omics*, 6: 231–239

Thomashow, M.F., 1998. Role of cold-responsive genes in plant freezing tolerance. *Plant Physiol.*, 118: 1–7

Van, D.T., N. Ferro and H.J. Jacobsen, 2010. Development of a simple and effective protocol for *Agrobacterium tumefaciens* mediated leaf disc transformation of commercial tomato cultivars. *GM Crops*, 1: 312–321

Wang, Y., X. Tang, Z. Cheng, L. Mueller, J. Giovannoni and S.D. Tanksley, 2006. Euchromatin and pericentromeric heterochromatin: comparative composition in the tomato genome. *Genetics*, 172: 2529–2540

Yarra, R., S.J. He, S. Abbagani, B. Ma, M. Bulle and W.K. Zhang, 2012. Overexpression of a wheat Na^+/H^+ antiporter gene (*TaNHX2*) enhances tolerance to salt stress in transgenic tomato plants (*Solanum lycopersicum* L.). *Plant Cell Tiss. Org. Cult.*, 111: 49–57

Zhang, X., S.G. Fowler, H. Cheng, Y. Lou, S.Y. Rhee, E.J. Stockinger and M.F. Thomashow, 2004. Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon that differs from that of freezing-tolerant *Arabidopsis*. *Plant J.*, 39: 905–919

Zhang, Y., H. Liu, B. Li, J.T. Zhang, Y. Li and H. Zhang, 2009. Generation of selectable marker-free transgenic tomato resistant to drought, cold and oxidative stress using the Cre/loxP DNA excision system. *Transgenic Res.*, 18: 607–619

(Received 28 May 2015; Accepted 05 October 2015)