

Expression analysis and introgression of transcriptional factor (DREB1A) in tomato for cold tolerance

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Key Message: This is a novel study and it demonstrates that the progenies of T5 transgenic lines of tomato over-expressing *DREB1A* were used as pollinators in introgression into two elite cultivars of tomato BSX-935 and 95017. Successful F₁ hybrids were confirmed through molecular analysis.

ABSTRACT: Crop productivity is impaired by a wide range of abiotic stresses. Cold stress is the main factor that causes the decrease in productivity of tomato. Through genetic engineering, a single gene can be easily transferred with desired characteristics into local best performing varieties. *CBF/DREB1* is the most effectively used gene family to engineer cold stress tolerance in tomato. The aim of this study was to focus on the transgene expression and inheritance pattern in segregating generation of tomato lines and impact of transgene in different genetic backgrounds. In this study, transfer of transgene through conventional breeding into two elite cultivars (BSX-935 and 95017) was carried out. The lines used for detection and confirmation of transgene were previously developed by genetic transformation with a construct containing the *AtDREB1A* gene driven by the stress-inducible promoter *lip-9*. Transformants of T4 generation were identified by PCR and were self-pollinated to generate T5 progeny. The confirmed transgenics were used to evaluate different morphological traits such as plant height, fruit size, number of fruit per plant and number of seeds per fruit compared with non-transgenic counterparts. By using Completely Randomized Design, non-significant differences were found between transgenics and their non-transgenics plants. To check the expression pattern in transgenic plants, reverse transcription polymerase chain reaction was done. Progenies of T5 transgenic lines were used as pollinators for introgression into two elite cultivars BSX-935 and 95017. Successful F₁ hybrids were confirmed through molecular analysis. These F₁ hybrids may be used as a source for development of cold tolerant nearly isogenic lines through backcross breeding program in future.

Keywords: *AtDREB1A*, Cold stress, F₁ hybrids, Transgenic plants, T5 progeny

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INTRODUCTION

Tomato (*Solanum lycopersicum* Mill.) is one of the major vegetables, grown everywhere in the world (Rick, 1995). It requires less time period for cultivation and gives maximum produce due to which it has high economic value, and area for its cultivation is gradually increasing (Naika et al., 2005). Out of its production, 38% is contributed by each of Khyber Pakhtunkhwa (KPK) and Baluchistan provinces, 15% and 9% by Punjab and Sindh, respectively. It is susceptible to chilling temperature (0 – 12 °C). Because of sensitivity to low temperature, it includes lots of anatomical disturbances under subtropical temperate zones (Kalloo, 1991). When plants exposed to low temperature, it disturbs the cell stability and reactive oxygen species (ROS) in plants. These are the major cellular changes in plants; occur due to stress conditions (Suzuki & Mittler, 2006). ROS such as singlet oxygen (O₂[•]), hydrogen peroxide (H₂O₂) and HO affect bio-macromolecules like proteins, lipids, carbohydrates and DNA that lead to cell death in plants (Ruelland et al., 2009; Gill & Tuteja, 2010).

Many cultivated varieties of tomato are sensitive to temperature less than 15 °C that hinders its growth, fruit development, flower opening and fruit maturation (Foolad & Lin, 2001; Ploeg & Heuvelink, 2005). General

symptoms that occur due to chilling injury in tomato are surface lesions, loss of chlorophyll, plant death and programmed cell death (Saltveit & Morris, 1990). When exposed to low temperature, plants respond with changes in their pattern of gene expression and protein products (Wray, 1991). Thus, acclimatization plays an important role on the survival and distribution of the plant and on crop yields. New varieties of tomato can be developed through transferring gene of interest by traditional breeding or gene transfer technology from a great range of germplasm available for tomato crop improvement (Finkers et al., 2007).

Use of transgenic or traditional crop varieties for the improvement of wild species of crops, is the part of different research studies (Baack et al., 2008). The main objective to introgress a gene in wild relatives from crops is to study the effect of transgene transfer on host genetic background (Chapman & Burke, 2006). Back crossing is one of the traditional breeding approaches used for the introduction of one or few genes in best performing current cultivars. At present, molecular breeding is the most widely used technique for crop improvement. Backcross breeding is mostly preferred when the aim is to develop the varieties with desired characters that may be monogenic or polygenic in nature. Through backcrossing, it becomes easy to transfer these multigenic and monogenic traits in crops (Moose & Mumm, 2008). Crop improvement through crossing between two different species or within the species has met with limited success in plant breeding practices. Various studies relating to sequencing, mutational analysis and transgenic studies have played a profound understanding of the complex transcriptional mechanism that functions in cold stress condition (Sanghera et al., 2011).

Through genetic engineering plant breeders can easily transfer a single gene with desired characteristics into local best performing varieties. This is the most reliable and time saving technique to transfer gene of interest in different genetic backgrounds (Sharma et al., 2002). *CBF/DREB1* is the most effectively used gene family to engineer cold stress tolerance in tomato (Thomashow, 2010). Plants that are able to cope with cold stress conditions like *Arabidopsis thaliana* (L.) detect low temperature conditions and turn on expression of TFs, members of the *CBF/DREB1* gene family, which includes *CBF1*, 2 and 3. The resultant protein products of these genes control the expression of cold-regulated genes (Thomashow, 2010). In this study, a combination of modern biotechnology and conventional hybridization has been used for transfer of transgene from transgenic plants generated by *Agrobacterium tumefaciens*-mediated genetic transformation technique. This will lead ultimately to the development of elite transgenic cultivars with cold tolerance in addition to other desirable agronomic traits. The resultant cultivars will also be able to grow under open field conditions during the winter season, thereby eliminating the use of plastic tunnels. This study was aimed to detect transgenes (*DREB1A*) in segregating populations (T5) of tomato lines and to check the expression analysis of transgenic lines through semi quantitative RT-PCR. During this study, comparison was made between transgenic and non-transgenic plants on the basis of morphological parameters. Introgression of transgene (*DREB1A*) was also done into two pure lines followed by expression analyses.

MATERIALS AND METHODS

Plant material

Fifteen T5 advance transgenic tomato lines of Moneymaker transformed with *DREB1A* gene and one non-transgenic genotype was selected and grown in the glass house where temperature was maintained at 25 – 30 °C. The seeds of these transgenic and non-transgenic lines (Table 1) were provided by NIGAB and Horticultural Research Institute (HRI), NARC, Islamabad, Pakistan.

DNA extraction and quantification

Genomic DNA was isolated from fresh tomato leaves of both transgenic and non-transgenic plants using CTAB method described by Doyle and Doyle (1990). Hundred (100) mg fresh leaf sample was ground in liquid nitrogen using mortar and pestle to a fine powder and added 1.0 ml of pre-heated (60 °C) 2X CTAB extraction buffer. The mixture of plant extract was transferred to an eppendorf tube for incubation temperature 65 °C in water bath for 30-40 min. Then centrifugation was performed at 12000 rpm for 10 minutes and their supernatant was transferred to fresh tube. Then chloroform: isoamyl alcohol (24: 1) was added and mixed it gently. Again centrifuge it at 12000 rpm for ten minutes. Then supernatant was transferred into new eppendorf tube and equal volume of ice chilled isopropanol was added. Mix it gently and then incubate for 30 min to precipitate DNA. Again centrifuge for 10 minutes at 12000 rpm and supernatant was removed. Then pellet was washed with 70% ethanol and simultaneously tube was air-dried by putting on autoclaved filter paper. Then pellet was re-suspended in 50-100 µl of nuclease free water and treated with RNaseA (1 µl/100 ml TE buffer). The DNA concentration was quantified by Bio Spec-nano Micro-volume UV-Vis spectrophotometer (IMPLEN, Germany) at 280 nm wavelength, whereas the extracted DNA was run on agarose gel.

Confirmation of transgene presence

For the purpose to confirm the presence of *DREB1A* and associated *hpt* genes in advance tomato lines (advanced to T5 progeny), molecular analysis using PCR was performed. *DREB1A* gene primer pair was used to amplify 632 bp fragment. The sequence of forward primer is; *DREB1A*-F 5'TGAATCATTCTGCTTT-3' and reverse primer: *DREB1A*-R 5'-TAATAACTCCATAACGATA-3'. Similarly both primers for selectable marker gene hygromycin phosphotransferase (*hpt*) was also used for confirmation. The PCR conditions were optimized for amplification of transgenes.

Gel electrophoresis and documentation

The Amplified products were resolved on 0.6 µg/ml ethidium bromide containing 1.5% agarose gel in 1X TBE (10 mM Tris-Borate, 1 mM EDTA) buffer at 100V for 35 min. After electrophoresis, amplified products were visualized at the wavelength of 280nm on transilluminator and photographed by a gel documentation system.

RNA extraction

Total RNA from transgenic and non-transgenic tomato plants was extracted by using TRIzol® Reagents. Leaf sample was ground in liquid nitrogen using mortar and pestles. The crushed tissue was taken into eppendorf tube and mixed with 1.0 ml of Trizol and 200 µl chloroform. The homogenized sample incubated on ice for 2-3 min. After incubation, the sample was centrifuged at 12000 rpm for 12 min at 4 °C. The supernatant was transferred into new eppendorf tube and chilled isopropanol was added in equal amount. After an incubation period of 10 min on ice, samples were again centrifuged at 12000 rpm for 10 min. The pellet obtained after centrifugation was washed with 75% ethanol and centrifuged for 5 min at 7500 rpm. Ethanol was removed and pellet was dried for 5 min at open air. After drying the pellet, RNase free water (50-60 µl) was added and pellet was dissolved. Finally, RNA samples were stored at -80 °C for future use.

Transgene expression analyses

Two steps reverse-transcriptase PCR was performed to detect the expression of desired gene (*DREB1A*). In first step, complementary DNA (cDNA) was prepared from good quality pure RNA following the instructions of manufacturer (Revert Aid Reverse Transcriptase, Fermentas) (Verma, 1981; Gerard & D'Alessio, 1993; Sambrook & Russell, 2001). In the second step, 2.0 µl of cDNA from reverse transcription was used as a template and *DREB1A* gene specific primers were employed to amplify the desired fragment through conventional PCR with optimized conditions as shown in the Fig.1. The product of reverse transcription-PCR was resolved on 1.5% agarose gel by the process of electrophoresis and photographed through gel documentation system.

Evaluation of morphological parameters

Morphological data on plant height (cm), number of fruit per plant, fruit length, fruit width and number of seeds per fruit of T5 transgenic and non-transgenic plants were also recorded and were compared with non-transgenic plants. Seed of fifteen transgenic tomato lines were separately grown in trays. The plantlets were watered regularly and after four weeks, they were transferred to pots and were kept under normal growth conditions in glasshouse. Confirmed transgenic plants were chosen for morphological analysis. To measure the plant height, three transgenic plants from each line were randomly selected; data were collected in centimeters from ground level to upper level by using a meter rod. The diameter of three fruits per plant was measured in centimeters using a measuring tape and then average was used for statistical analysis. Data for number of fruits/plant was measured by randomly selecting three plants per line. To count number of seeds/fruit, three fruits/plant were selected from each line and then averaged for statistical analysis.

Data analysis

The data of given morphological traits were subjected to analysis of variance at 5% confidence level.

Introgression of transgene

The progeny of transgenic lines (T5 generation) were used for hybridization. Progeny of transgenic lines and recipient elite tomato cultivars 95017 and BSX-935 (provided by Horticultural Research Institute, NARC, Islamabad) were grown in transgenic containment for crossing. Ten plants from each of two recipients were

used as a maternal parent for hybridization. Florets were emasculated from mother plant. Pollens were collected from PCR confirmed T5 plants of transgenic lines and used for pollination.

Molecular analysis for the detection of transgene in F₁ hybrids

F₁ hybrid seed was grown; sample from every single plant was collected to confirm the successful transfer of transgene into non-transgenic tomato lines through PCR analysis. For the confirmation of successful transfer of transgene in cross combinations molecular analysis was done by using PCR. To amplify 632 bp fragment of *DREB1A* gene, the primers sequences used were: forward primer: *DREB1A*-F 5'TGAACTCATTTTCTGCTTT-3'and reverse primer: *DREB1A*-R 5'-TAATAACTCCATAACGATA-3'. The PCR was optimized for amplification of transgenes in a thermo cycler (Applied Biosystem) for 20µlreaction volume containing 10X PCR buffer (10 mM Tris-HCl; pH 8.3; 50 mM KCl), 10 mM of deoxynucleotide triphosphate (dNTPs) mix, 25 mM MgCl₂, Each forward and reverse primers (10 pM) and 1 unit Taq DNA polymerase.

RESULTS

Molecular analysis for transgene in segregating population

PCR analysis was carried out to confirm the transgene in segregating population in T5 generation of transgenic tomato lines. Total 100 plants were grown for T5 population which was screened through PCR analysis (Figure 6). Out of 100 samples, 59 transgenic plants were identified positive for the presence of *DREB1A* as shown in Fig. 2. A band of 632 bp was successfully amplified in transgenic plants and positive control (plasmid pBIH). On the other hand, no amplification was detected in non-transgenic plants.

Inheritance pattern of transgene

Fifty-nine (59) plants out of 100 were found to be transgenic. It showed that PCR data obtained for *DREB1A* was well fit into 3:1 ratio which confirmed the normal inheritance pattern for single gene according to First Mendelian Law of Segregation.

Transgene expression analyses

To assess the expression of transgene at cytoplasmic level, we performed semi-quantitative RT-PCR in transgenic tomato lines. The cDNA was synthesized from 30 confirmed transgenic plants (Fig. 3). After that reverse transcriptase PCR was performed by using cDNA as template to confirm the expression behavior of *DREB1A* with gene specific forward and reverse primers by standardized PCR conditions as shown in Fig. 4. A band of 632 bp was successfully amplified in all of the transgenic plants, thereby confirming the expression consistency in T5 generation.

Table 1 Details of transgenic and non-transgenic tomato lines

S. No.	Lines	S. No.	Lines	S. No.	Lines	S. No.	Control lines
1	MM-5(1)	6	MM-7(10)	11	MM-36(16)	16	Moneymaker
2	MM-5(8)	7	MM-7(12)	12	B-5(15)		Moneymaker
3	MM-5(18)	8	MM-7(18)	13	B-6(6)		Moneymaker
4	MM-22(2)	9	MM-2(5)	14	B-6(9)		Moneymaker
5	MM-22(6)	10	MM-2(15)	15	B-16(3)		Moneymaker

Table 2 Outcomes of introgression experiment

Pure line	Transgenic (T5)	PCR response of crosses										Total PCR positive
		1	2	3	4	5	6	7	8	9	10	
BSX-935	MM-14(10)	-	+	+	+	-	-	-	-	+	-	4
	MM-14 (19)	+	-	-	+	-	+	+	+	-	-	5
	MM-14(14)	-	+	-	+	-	+	-	-	-	-	3
	MM-13 (20)	-	+	-	-	-	+	+	-	-	+	4
	MM-49(1)	+	-	-	-	-	+	+	-	-	-	3
	MM-49 (2)	+	-	-	-	-	+	+	-	+	-	4
	MM-15 (6)	-	+	-	+	+	-	-	-	+	-	4
Pure line	Transgenic (T5)	PCR response of crosses										Total PCR positive
		1	2	3	4	5	6	7	8	9	10	
95017	MM-14(10)	+	+	+	+	-	-	-	-	-	-	4
	MM-14 (19)	+	+	-	-	-	-	-	-	-	-	2
	MM-14(14)	+	+	+	-	-	-	-	-	-	-	3
	MM-13 (20)	+	-	+	+	-	-	-	+	-	-	4
	MM-49(1)	+	+	-	-	-	+	-	-	-	-	3
	MM-49 (2)	-	-	-	+	+	-	+	+	-	-	4
	MM-15 (6)	+	-	-	-	-	-	+	+	-	-	3
	MM-15(11)	+	+	+	+	-	+	-	-	-	-	5

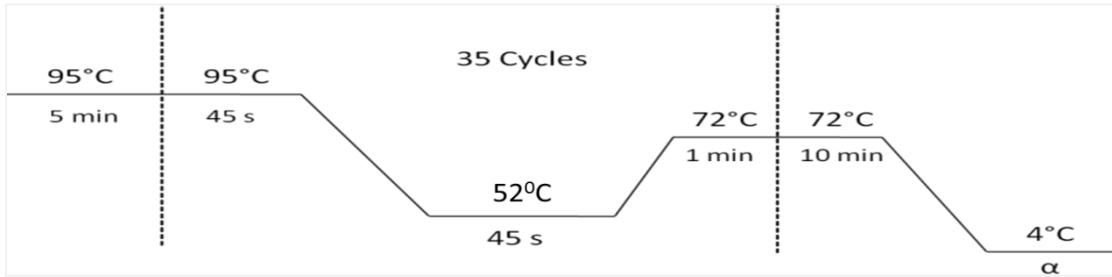


Fig. 1 PCR profile for the expression of *DREB1A* gene in transgenic tomato genotypes

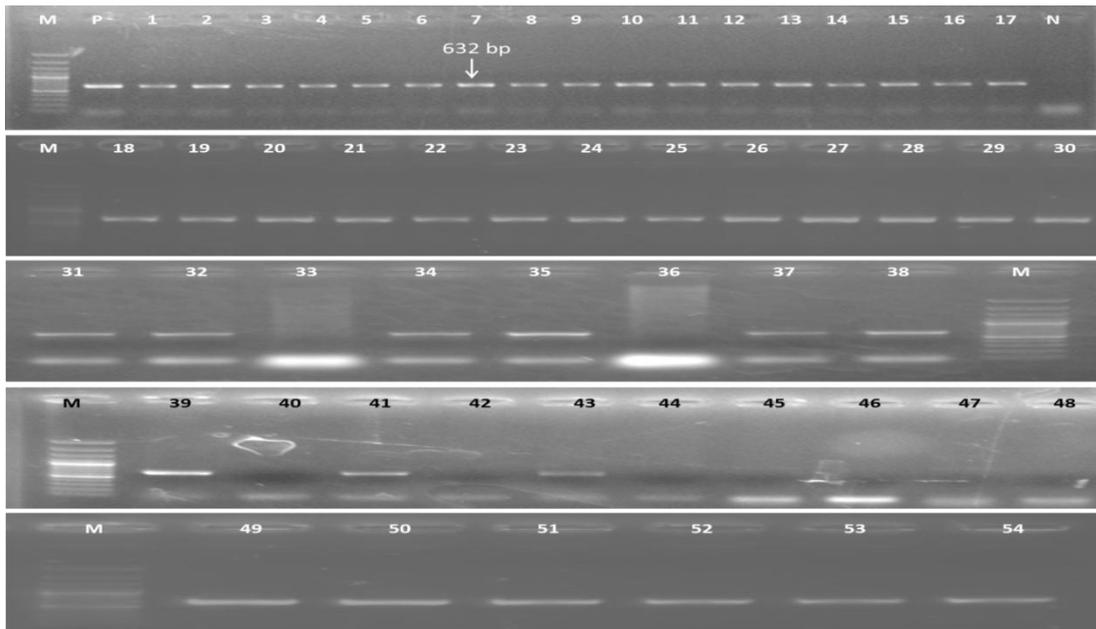


Fig. 2 PCR analysis for presence of *DREB1A* gene fragment (632 bp) in transgenic tomato lines: Lanes M 1 kb DNA Ladder (Fermentas), Lane P positive control (plasmid pBIH), Lane N negative control (NT tomato), Lanes 1–54 transgenic plants of Moneymaker.

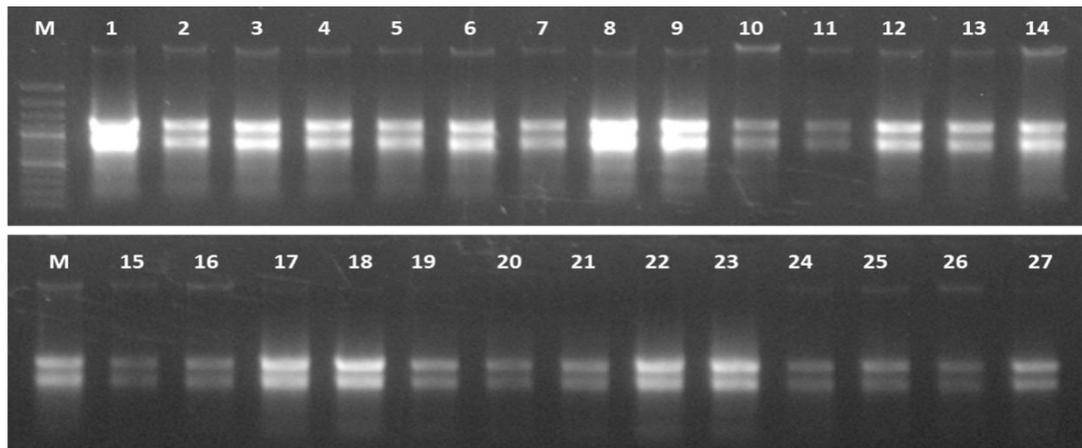


Fig. 3 Total RNA extraction from transgenic tomato plants of Moneymaker

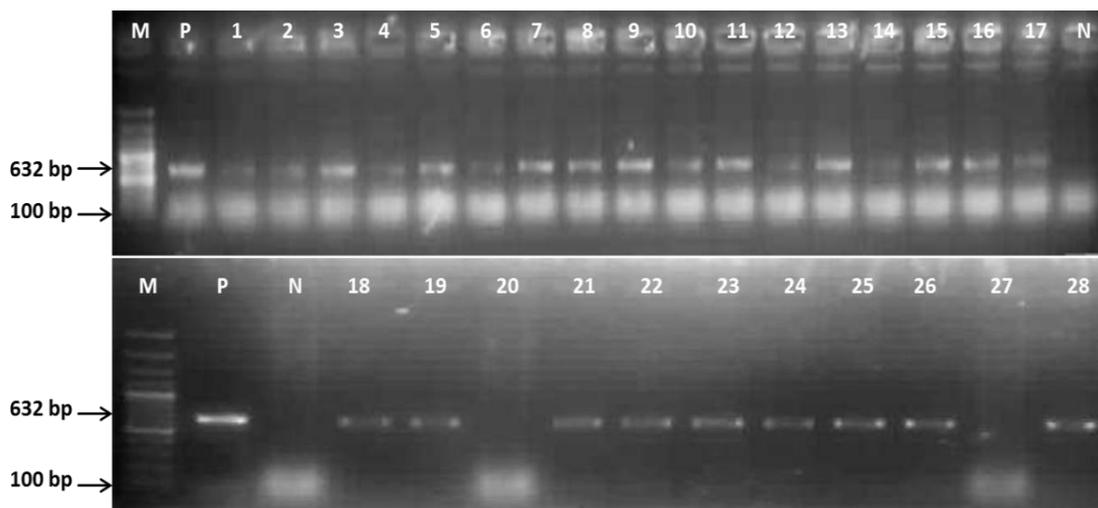


Fig. 4 RT PCR analysis of transgenic tomato plants. Lanes M 1 kb DNA Ladder (Fermentas), Lane P positive control (pBIH-CBF3 as template), Lane N negative control (non-transgenic tomato), Lanes 1–28 transgenic plants of Moneymaker.

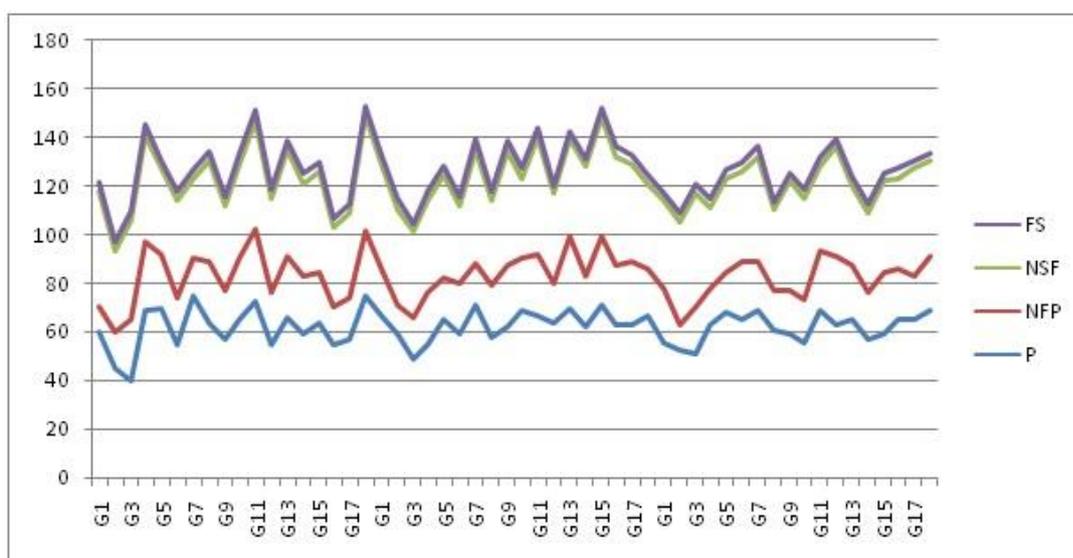


Fig. 5 Comparison of morphological parameters, FS (Fruit size), NSF (no. of seeds per fruit), NFP (no. of fruits per plant), P (Plant height)

Comparison among transgenic and non-transgenic plants on morphological basis

The T5 transgenic lines were compared with their non-transgenic plants on the basis of following morphological characteristics:

Plant height

From this study, it has been confirmed that transgenic lines didn't show any significant difference for plant height as compared to non-transgenic lines under normal growth condition as shown in Fig. 5. The average plant height in transgenic plants was observed as 71 cm in comparison with non-transgenic plants (G-16) in which average plant height was observed is 70 cm.

Number of seeds per fruit

ANOVA for number of seeds/fruit showed no significant variations for transgenic and non-transgenic plants. The average number of seeds in transgenic plants was observed as 43 seeds as compared to non-transgenics in G-16 in which average number of seeds per plant were observed as 40 seeds.

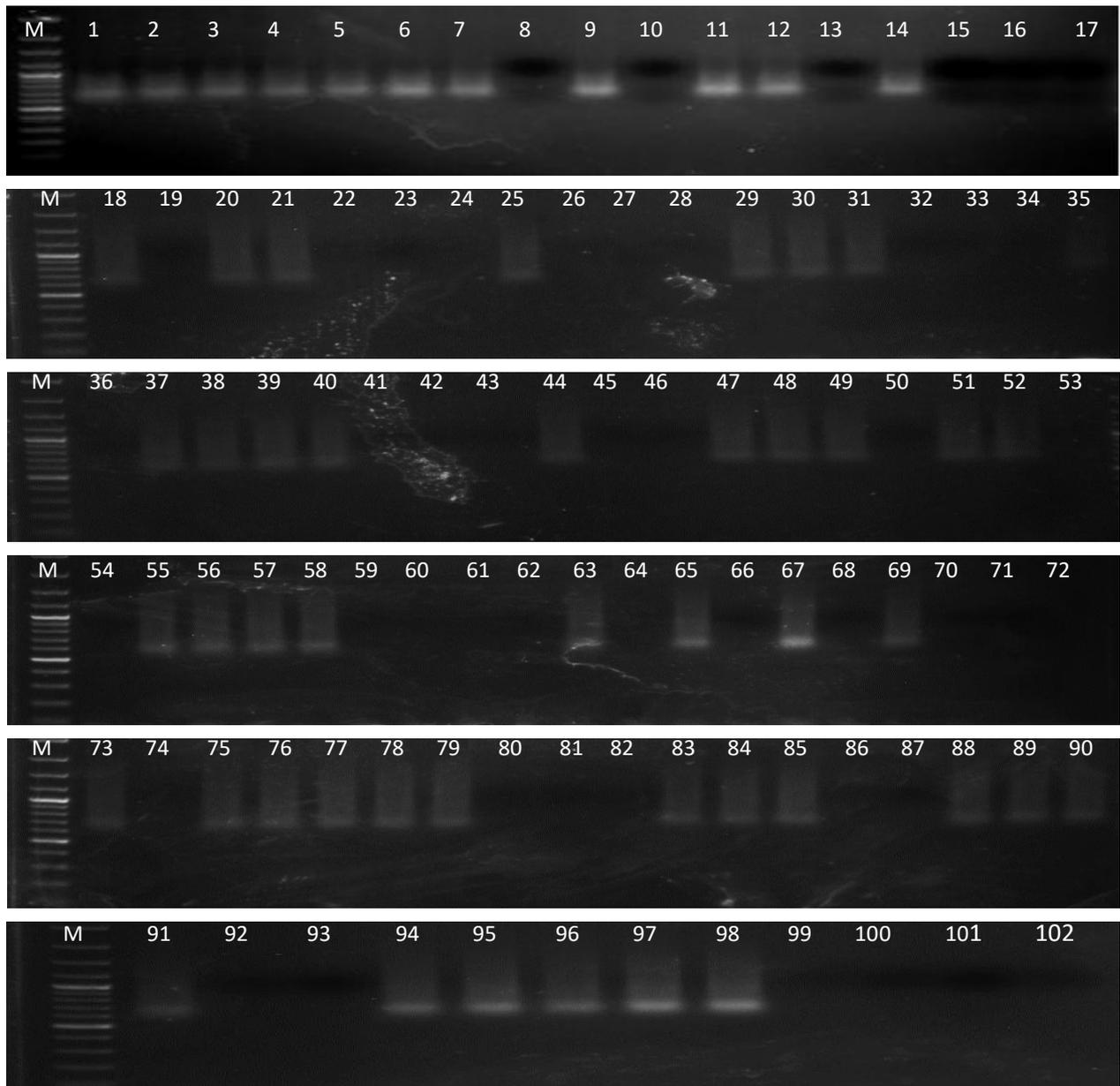


Fig. 6. PCR analysis for presence of *DREB1A* (632 bp) in F_1 hybrids: M represents marker/ladder (Fermentas), Lanes 1–102 plants from introgression.

Number of fruit per plant

During this study, it has been noticed that there is no significant effect of cold tolerant gene *AtDREB1A* on the number of fruit per plant in transgenic lines without any stress as compared to their NT lines (Fig. 5). In transgenics the highest number of fruits per plant was counted as 26 fruits, while in non-transgenic (G-16), the maximum number of fruits was counted as 23 fruits.

Fruit size

This study demonstrated that the fruit size of transgenic plants was non-significantly less than their non-transgenic plants (Fig. 5). The highest fruit size (4.39 cm) was observed in transgenics in comparison with non-transgenic G-16 in which maximum fruit size was observed as 4.20 cm.

Introgression in pure lines

After confirmation of transgenics in T5 generation regarding transgene presence, expression and inheritance we used those lines to introgress transgene in high yielding pure lines (95017 and BSX-935). Ten (10) crosses were developed from each transgenic line. Matured seeds from 102 successful crosses were harvested out of total 140. Samples from these F₁ hybrids were taken and PCR analysis was performed for confirmation of 632bp of *DREB1A*. Fifty-five plant samples were found to be successfully introgressed as shown in Fig. 6.

DISCUSSION

Tomato (*Solanum lycopersicum*) is one of the major vegetables grown everywhere in the world (Rick, 1995). But its growth and development are severely affected by low, non-freezing temperatures. Low temperature disturbs the cell stability and reactive oxygen species (ROS) in plants. These are the major cellular changes in plants; occur due to stress conditions (Suzuki & Mittler, 2006). Under cold stress different genes in downstream activate and cause low temperature tolerance in plants (Artus et al., 1996). Cold acclimate tempts the expression of the CBF as a result it activates other downstream genes that cause cold tolerance to plants (Artus et al., 1996). Interspecific hybridization is a common natural phenomenon observed both in animals and plants that lead to introgression (Mallet, 2008). The main objective to introgressing a gene in wild relatives of crops is to study the effect of transgene transfer on host genetic background (Chapman & Burke, 2006). Introduction of one or few genes in best performing current cultivars is one the traditional plant breeding approaches. At present, molecular breeding is most widely used technique for crop improvement. Backcross breeding is mostly preferred when the aim is to develop the varieties with desired characters that may be monogenic or polygenic in nature (Moose & Mumm, 2008). The transcriptional factor (*DREB1A*) is involved in enhancing cold tolerance under stress conditions in T2 generation, which has already been proved in previous research studies (Shah et al., 2015; Shah et al., 2017a; Shah et al., 2017b). The present study is the extension of these previous studies and we have confirmed successful expression of the transcriptional factor in T4 generation. The key objective of our study is the introgression of desired gene into two pure tomato lines for the induction of cold tolerance through conventional breeding.

Similar to our study, Zong et al. (2016) cloned and characterized *AaDREB1* gene from the cold-tolerant plant *Adonis amurensis*, encoding the *DREB1* transcription factor. Results from qRT PCR showed that *AaDREB1* expressed itself under chilling, salinity and water deficit conditions. From these results, it was concluded that by transferring *AaDREB1* through genetic engineering could play an important role in crop improvement against different stresses. Sarkar et al. (2016) developed eight transgenic lines in peanut by transferring *AtDREB1A* gene through *Agrobacterium*. Transgene confirmation was done by PCR and Dot-blot analysis in T0, while southern blot analysis was done to confirm the copy numbers. The results indicated that the expression of transgene was significantly correlated with physiological and biochemical characteristics. Similar results were secured by Aakash et al. (2013) who studied the cold stress response in Jumli Marshi and found 4636 genes that were expressing significantly in 24 h of low temperature.

Our findings were in line with Patade et al. (2013) who studied osmotin and other stress responsive genes in transgenics and wild type tomato plants. After exposure to cold stress, it has been observed that changes occurred that demonstrated degree of transcriptional regulation in transgenic plants. From these results, it has been concluded that chilling stress tolerance in transgenic plants was mainly due to over expression of Osmotin modulate transcript and functional products of other stress responsive genes. In an earlier study, Singh et al. (2011) worked against to enhance such type (cold) of tolerance in tomato by over-expressing *At-CBF1* gene. The transgenic plants had higher relative water contents than that of NT plants after contact to chilling stress and indicated better adaptive capability under chilling stress.

The proposed study was envisaged to screen the transcription factor in T5 generation. Our findings were in confirmatory with the earlier findings by Elizondo and Oyanedel (2010) who evaluated two Nearly Isogenic Lines of cold-resistant wild tomato *Solanum habrochaites* (S. Knapp & D. M. Spooner) with introgression in chromosome 2 and 3. Four plantings were used as experimental material and exposed to different environmental stresses. Different morphological parameters like yield, plant growth and fruit setting were calculated after two successive 10 days period. No significant differences were found for these morphological traits and it was recommended that near isogenic lines should not be used to get cold tolerant varieties because of linkage drag and inability to perform under different environmental stresses. Our results agreed with the earlier proposition by James et al. (2008) who isolated *DREB1/C*-repeat binding factor from wild barley. Sequence comparison between *Hordeum spontaneum* L. and *Hordeum vulgare* L. was done. Lots of conservative sequences were found between them. It was reported that *HsDREB1A* gene with stress-inducible promoters *HVA1s* and *Dhn8* can activate different reporter genes. In this study, *HsDREB1A* was sub cloned with *HVA1s* promoter and transferred into bahiagrass (*Paspalum notatum* Flugge) cultivar 'Argentine'. It was observed that under severe salinity and drought stress conditions plants with *HsDREB1A* survived under stress conditions

Boyce et al. (2003) found that the *sfr6* mutant of *Arabidopsis* show inefficiency when plants acclimatized to low temperature. It has also been confirmed that *sfr6* mutant was incapable to join with stress inducible genes of cold, drought and abscisic acid when they had C- repeat binding elements in their promoter. It has also been confirmed that *sfr6* also affected levels of proteins and their expression when mutation occurred. Through microarray analysis, it has been observed that *sfr6* had ability to affect promoters with C-repeat binding elements. Our findings were harmonious with the earlier report by Hsieh et al. (2002) who studied effects of overexpression of *DREB* in tomato, an *Arabidopsis DREB* gene with β -glucuronidase (*GUS*) as marker gene were transferred into the tomato genetic background through *Agrobacterium* mediated transformation. Plants were tested on kanamycin-containing medium and confirmed that transgenics were recognized through *GUS* histochemical staining assays to confirm the expression of transgene. Using Southern-blot analysis it has been confirmed that 35S: *CBF1* transgene was successfully transferred.

Our results noticeably signify that engineering of tomato with *DREB1A* gene has improved ability to survive under low temperature conditions and improved its yield during antagonistic environment via cold stress. Our conclusions are consistent with the earlier report by Murata and Tasaka (1997) who demonstrated that the efforts have been made to improve cold tolerance in plant through genetic engineering. Desaturation of fatty acids and higher rate of unsaturated fatty acids in plants cell membrane can improve the plants ability to cope with cold temperature. Contrary to our findings, Challam et al. (2015) reported that *CBF1* and *CBF2* were two TFs that played key roles in improving plant's stress tolerance ability under cold conditions. In order to confirm whether these two TFs have ability to tolerate low temperature, these two transcription factors were transferred in different varieties of rice to check their performance under cold conditions. Through expression analysis gene was confirmed in shoots of both susceptible and tolerant varieties. Sequence analysis of 20 varieties revealed that the difference of eight nucleotides was found. To find the nucleotide diversity *In silico* analysis was done in 400 varieties of rice and very low difference was found between *DREB* loci and one other gene (*MYB2*) involved in *DREB* pathway. No significant differences were found for traits like no linkage between plant seedling stage and CBFs; they are only responsible for cold stress tolerance in plants.

Under normal growth conditions, no significant difference was found between transgenic and non-transgenic plants. Similar type of findings was reported by Singh et al. (2011) who engineered tomato with *AtDREB1* gene with cold inducible promoter rd29A for chilling stress and reported that transgenic plants had no significant difference for plant height when compared with NT plants under normal growth conditions. During our study, no significant differences were found for number of fruit per plant between transgenic and NT plants without any stress. Similar to our findings Jin et al. (2012) found that there was no effect of over-expression of *PLI* gene in tomato for higher number of fruits per plant in transgenic plants as compared to their wild type plants. In order to determine the role of transgenic element *AtDREB1A* gene on seed yield, a comparative analysis revealed that there was no significant difference between transgenic and non-transgenic plants. It was also noticed that transgenic plants had non-significantly less fruit size as compared to non-transgenic plants. Similar type of results was recorded by Singh et al. (2011) in comparative analysis of morpho-agronomical characteristic of transgenic tomatoes and reported non-significant differences between transgenic and their isogenic counterparts. The non-significant analysis of morphological traits between transgenic and non-transgenic lines depicts that transgenic lines did not exhibit somaclonal variation. These lines were at par with non-transgenic lines in all of the parameters studied. Somaclonal variation could arise from number of factors such as plant tissue culture conditions (growth hormones, prolonged culture conditions etc.) and genetic transformation.

CONCLUSION

From the current study it was concluded that transgene *DREB1A* was still successfully expressed in segregating population (T5 generation). Transgene was also successfully introgressed into two high yielding varieties by conventional breeding approach, which was confirmed by PCR analysis. From morphological study it has also been concluded that there was no significant difference among transgenic and non-transgenic plants and transgenic lines were stable in morphological parameters still in T5 generation. F₁ hybrids produced by introgression can be used in backcrossing for the development of Near Isogenic Lines (NILs). These NILs can be evaluated for cold stress tolerance under field conditions and released as commercial cold tolerant varieties following the National Biosafety Rules of Pakistan.

Author Contribution Statement: Shaukat Ali planned, designed and supervised the research study. Kiran Kareem conducted the research project and wrote the manuscript. Muhammad Amir Zia and Siffat Ullah Khan contributed in research experiments. Armghan Shahzad contributed in the introgression of transcriptional factor (*DREB1A*) in tomato for cold tolerance. Jalal-ud-Din contributed in data analysis, description and edited the manuscript. Ghulam Muhammad Ali is the program leader and provided the research facilities. All the authors read and approved the manuscript to be published in Journal of Pure and Applied Agriculture.

Conflict of Interest: The authors declare that they have no conflict of interest.

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