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Research Article

Molecular characterization of transgenic tomato *(lycopersicum esculentum)* for cold tolerance

Mazhar Ali Mastoi[1,*], Noor Nabi Mastoi[1], Amesh Kumar Oad[1], Tanveer Ali Soomro[1], Kashif Ali Shar[2] and Raina Ijaz[3]

[1] Department of Plant Breeding and Genetics, Sindh Agriculture University, Tandojam-Pakistan.
[2] Department of Zoology, Shah Abdul Latif University, Khairpur, Sindh Pakistan
[3] Department of Horticulture, University of Poonch Rawalakot, AJK, Pakistan
* Correspondence: mazharalimastoi@gmail.com

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Abstract | Cold stress is the main factor underlying the reduction in productivity of tomato. This study was carried out at National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agriculture Research Center (NARC), Islamabad during the year 2018-2019. The research was aimed to focus on the confirmation of presence of cold tolerance gene DREB1A and transgene expression in T5 generation of and impact of transgene in different genetic backgrounds of tomato lines. The five transgenic cold tolerant tomato lines and three non- transgenic lines were chosen for recognition and corroboration of transgene, previously developed by genetic transformation with a construct containing DREB1A gene energetic by the stress inducible promoter lip-9. Transformation of T4 generation were identified by PCR and were self- pollinated to generate T5 progeny. The confirmed transgenes were used to estimate different morphological traits such as plant height, fruit size, no. of fruit per plant and no. of seeds per fruit, shelf life and compared with non-transgenic plants. The results indicated that T5 generations lines carrying DREB1A gene and it express successfully in promising T5 generations lines. By using CRD design non-significant differences were found between transgenes and non-transgenic plants for all the character except shelf life demonstrating that shelf life was improved in transgenic lines providing resistance against cold tolerance. The results suggested that these transgenic lines can be used as a source for emerging cold tolerant nearly isogenic lines through backcross breeding program in future.

Keywords | Cold stress; DREB1A; T5 generation; genetic transformation; transgenic tomato

Key findings | Transformation of T4 generation were identified by PCR and were self- pollinated to generate T5 progeny. Transgene DREB1A was successfully expressed in T5 generation

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1. Introduction

Tomato (*Lycopersicon esculentum Mill.*) is the second most important vegetable crop of the world and has been used as a model plant in genetic evolution and molecular biology research. Its growth and development can be easily affected by cold stress. . It is rich in nutrition with zero cholesterol, but highly sensitive to abiotic stresses, especially salt, drought, and high temperature (Zhang *et al.*, 2020). Tomato has become a model organism due to its smaller genome, short life span, absence of gene duplication, ability to grow in a wide variety of climatic conditions, and ease of controlled pollination and hybridization (Schwarz 2014). In most plants, proteins involving response to cold stress are located in cytoplasm or nucleus. However, studies have shown that some membrane-localized proteins are involved in plant response to cold stress (Ding *et al.*, 2019). Severe climatic conditions further pose a significant challenge to the sustainable production of agriculture. Roughly, the biotic factors cause 20–40% direct loss of global agricultural productivity (Bom Marco *et al.*, 2013). As we know that the more demand of tomato (Lycopersicon esculentum) for its nutritional value, there is needed to improve the tomato varieties through modern methods of breeding and sometimes with the help of conventional breeding. Conventional breeding plays very important role to increase yield and quality (Iqbal *et al.*, 2019). The characters like biotic and abiotic are produced in different crops mainly by modern methods of biotechnology for example transformation, soma clonal variation, QTL mapping. Through different breeding methods, many goals are achieved and many varieties have been developed of different better traits or combine traits in same variety. Agriculture and biotechnology is trying to improve the tomatoes as per demand Hutton and Scott (2015). Abiotic stresses also lead to osmotic stress which can further cause loss of turgor pressure and ultimately restrict growth and development. Plants maintain their osmotic homeostasis partially via accumulation of osmoregulatory compounds such as free proline (Gujjar *et al.*, 2018)

2. Materials and Methods

The research was conducted in Kharif season 2019 at glasshouse of National Institute of Genomics and Advanced biotechnology (NIGAB), National Agricultural Research Center (NARC) Islamabad, during the year 2018-19 for molecular characterization of transgenic tomato for cold tolerance.

Plant Material

Transformed five transgenic tomato lines with *DREB1A* gene were selected and three non-transgenic lines of tomato were grown in glasshouse, where temperature controlled at 25- 30^{o}C. The seeds of transgenic and non-transgenic tomato lines (Table 1) was provided by (NIGAB), NARC Islamabad correspondingly.

Table 1: Transgenic and non-transgenic tomato lines details

S. No.	Transgenic tomato lines
1	**MM5-1**
2	**MM-7**
3	**MM1320**
4	**MM14-9**
5	**MM14-10**
	Non-transgenic Lines
1	**B-16**
2	**95017**
3	**BSX935**

Molecular characterization of transgenic tomato for cold tolerance

Twenty four wells of a tray were used for sowing single line of tomato. The tray was placed at glasshouse where temperature was maintained at 25-30ºC. 120 wells occupied by transgenic lines and remaining non-transgenic lines of tomato occupied with 72 wells. The leaves of fully germinated plants of tomato were grown for DNA extraction and PCR analysis. DNA extracted from the leaves of transgenic and non-transgenic tomato plants by CTAB method and the thermos cycler machine was used to determine the presence of cold tolerant gene (*DREB1A*) in tomato plant. The pair of primer *(DREB1A)* gene were used to amplify 632 bp fragments. The sequence of forward primer was DREB1A–F 5'TGAACTCATTTTCTGCTTT-3' and the sequence of reverse primer was DREB1A–R-5'-TAATAACTCCATAACGATA-3'. The banding pattern of this gene was visualized in Gel Documentation monitor.

DNA extraction

DNA was extracted from both transgenic and non-transgenic tomato leaves, the leaves were fully mature and healthy in appearance, and Genomic DNA was isolated by CTAB method. Initially explants of both transgenic and non-transgenic lines of tomato collected to isolate the DNA by CTAB method. 500ml chilled CTAB was added in leaves of tomato lines for initiating the process of grinding by mortar and pestle. After grinding leaves of explant, the mixture of plant extract was transferred to Eppendorf tubes and incubated at 65oC in water bath for 30-40 minutes. During incubation, the samples was inverted and shaken 2-3 times to mix all material in Eppendorf tube. 650ml Chloroform Isoethyl alcohol was added in samples and extracted mixture was centrifuged at 12000 rpm for 10 minutes to lower down the cell debris. The supernatant was transferred to a new Eppendorf tube, afterward 480ml chilled isopropanol added in extracted layer of DNA and Centrifuge again for 10 minutes, the liquid layer was discarded made inside the tubes remaining minute material was DNA in Eppendorf tubes. The tubes were placed at clean area to dry DNA and then Centrifuge process started for five minutes after adding 300µl of ethanol to wash pallet, the pallet was air-dried by inverting the tubes on tissue paper. The pallet was re-suspended in 50-100µl of nuclease free water and treated with RNase. The DNA concentration was confirmed in agarose Gel-electrophoresis.

Confirmation presence of transgene by PCR

To confirm transgene gene presence by *DREB1A*in advance tomato lines, molecular breakdown using PCR process was performed. The sequence of primer used to amplify 632bp fragment of *DREB1A*gene were F: 5'TGAACTCATTTTCTGCTTT-3 and R: 5'- TAATAACTCCATAACGATA-3'. The PCR conditions were optimized for amplification of transgenes in a thermocycler (Applied Bio system). The total volume of reaction was 20µl containing 10X PCR buffer (10 mMTris-HCl; pH 8.3; 50mMKCl), 10 mM of deoynucleotide triphosphate (DNTPs) mix, 25 mM $MgCl_2$, forward and reverse primers (10 pM) and one unit Taq DNA polymerase. The PCR was programed for an initial denaturation step of 4 minutes at 94ºC followed by 35 cycles of denaturation for 40s at 95ºC, 45s at 52ºC for primer annealing and 45s at 72ºC for primer extension and an extension cycle of 10 minutes at 72ºC and a hold temperature of 4ºC at the end.

Gel electrophoresis and documentation

The improved products were resolved on 0.6µg/ml ethidium bromide containing 1.5% agarose gel in 1X TE buffer at 100v incubator for 35 minutes. After electrophoresis, mixture of amplified products was visualized at the wavelength of 280nm on transilluminator and photographed by a gel documentation system.

RNA extraction

The total RNA collection from the transgenic and non-transgenic tomato plant was extracted by using TRIzol Reagents. Tomato Leaves were collected to grind with mortar and pestles. The grinded tissue was ground by liquid nitrogen by using mortar and pestles. The crushed tissue was taken into Eppendorf tube and mixed with 1ml of Trizol and 200µl chloroform. The homogenize sample incubated on ice for 2-3 min. After incubation, the sample was centrifuged at 12,000 rpm for 12 min at 4ºC. 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 12,000rpm for 10 min. The pellet obtained after centrifugation was washed with 75% ethanol and centrifuged for 5 min at 7500rpm.Ethanol was removed, and pellet was dried for 5min at open air. After drying the pellet, RNase free water (50-60µl) was added, and pellet was dissolved. Finally, RNA sample were stored at -80ºC until used.

Transgene analysis expression

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). Briefly, 4.0µl of RNA template was mixed with 1.0µl of Oligo (dT) 18 primer and 9.5µl DEPC-treated H2O. This material was mixed gently and incubated at 65 ºC for five minutes with cover temperature of 70 ºC and then chilled on ice for 2 – 3 min. Subsequently, µl of RT buffer, 0.5µl of reverse transcriptase (Revert Aid) and 1.0µl of dNTP mix were added in the above mixture making a total volume of 20µl. The mixture was centrifuged gently and incubated at 42 ºC for 60 min. Then the reaction was terminated by incubating at 70ºC for ten minutes. In second step, 2.0µl 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. The product of RT-PCR was resolved on 1.5% agarose gel by the process of electrophoresis and photographed through gel documentation system in the lab.

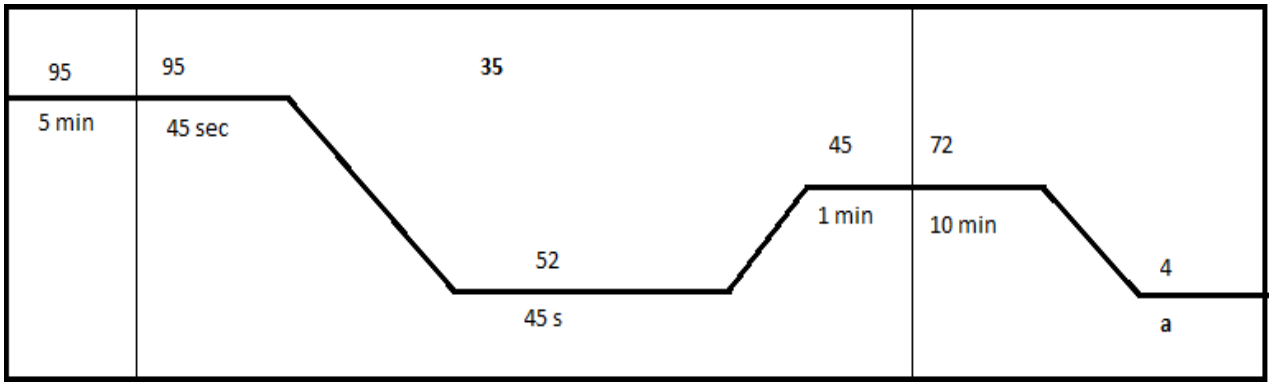

Figure 1. PCR profile for the expression of *DREB1A* gene in transgenic tomato genotypes. Evolution of morphological parameters

Morphological data on Fruit size, no. of fruit plant^{-1}, No. of seed fruit^{-1}, Plant height, Shelf life of T5 transgenic and non-transgenic plants were also recorded and comparison was observed. Seed of fifteen transgenic tomato lines were separately grown in trays according to completely randomized design. The plantlets were watered regularly and after four weeks they were transferred for plots and were kept under normal growth conditions in glasshouse.

The transgenic plants carrying cold tolerance genes were chosen for recording morphological characters. To measure the plant height, three transgenic plants 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^{-1} was measured by randomly selecting three plants per line. To count no. of seeds fruit^{-1}, three fruits per plant were selected from each line and then average for statistical analysis. For the estimation of shelf life, fruit of each variety was kept into plastic bags and then placed into refrigerator at controlled temperature (4.5 ℃).

Data analysis

The data collected of given morphological traits, were subjected to analysis of variance by using Completely Randomized Design. However, the difference among the mean were compared through least significant difference (LSD) at 5% probability level.

3. Results

Molecular analysis for transgene in T5 population

PCR analysis was carried out to confirm the transgene in T5 generation of transgenic tomato lines. Total 40 plants were grown for T5 population which was screened through PCR analysis. Out of 40 samples, 24 were transgenic plants showing positive result for the presence of *DREB1A*gene. A band of 620 bp was successfully amplified in transgenic plants. On other hand, no amplification was detected in non-transgenic plants (Figure 2).

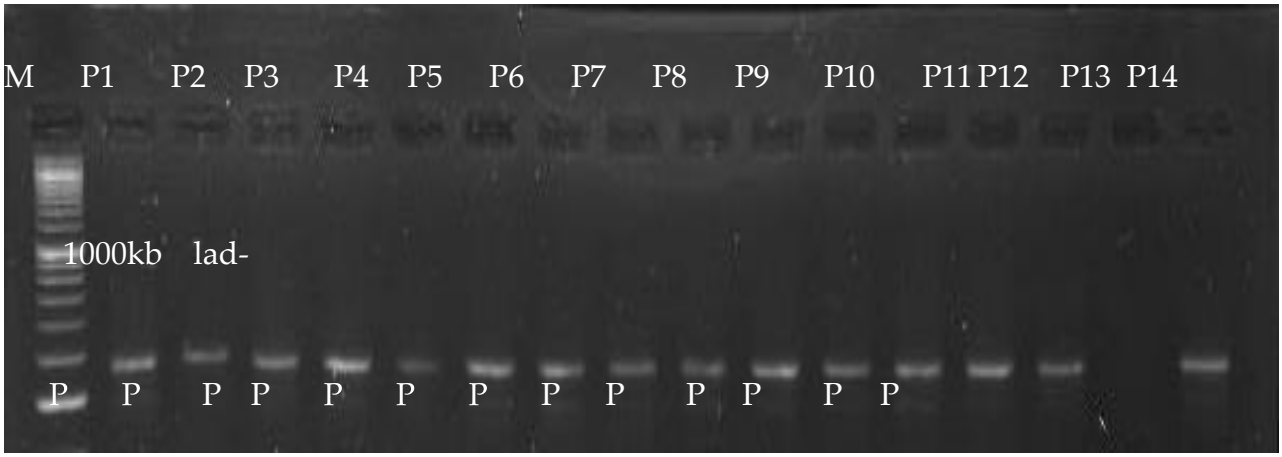

Figure 2: PCR analysis for presence of *DREB1A* gene fragment (632 bp) in transgenic and non- transgenic tomato: M; Marker, 1000 bp DNA Ladder P; Plant No. P; Present A; Absent

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 generated from mRNA for total RNA was extracted from 30 confirmed transgenic plants (Figure 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 Figure 4. A band of 632 bp was successfully amplified in all of the transgenic plants, thereby, confirming the expression consistency in T5 generation.

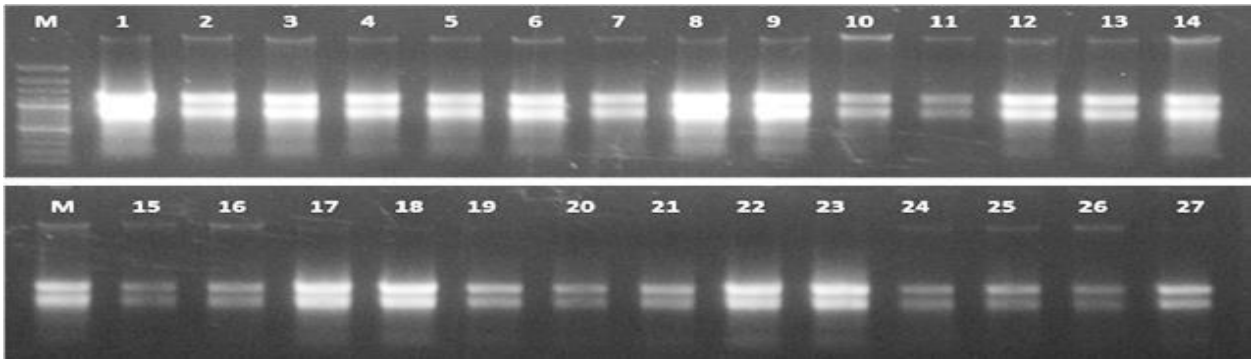

Figure 3: Total RNA extraction from transgenic plants of Moneymaker.

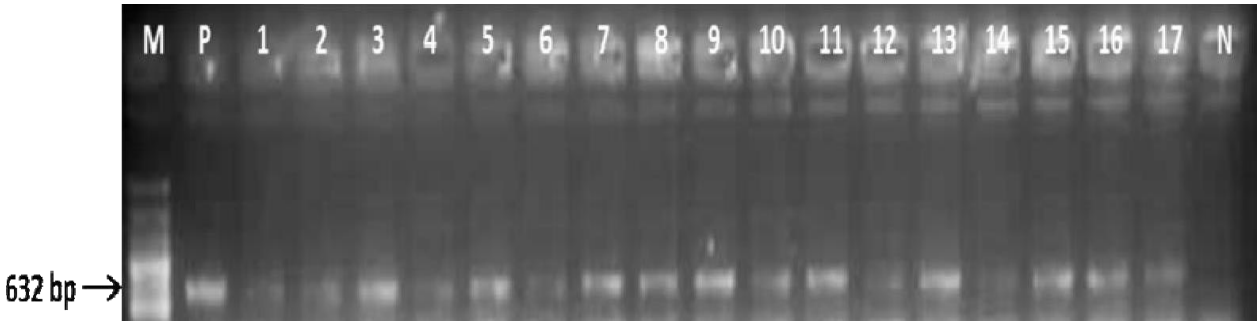

Figure 4: RT-PCR analysis of transgenic tomato plants. Lanes M 100 bp DNA Ladder (Ferments), Lane P positive control (pBIH-CBF3 as template), Lane N negative control (non-transgenic tomato), Lanes 1–28 transgenic plants of Moneymaker.

Comparison of morphological parameters between transgenic and non-transgenic plants Analysis of variance

The significance of mean squares from analysis of variance (Table 1) showed that fruit size, no. of fruits plant^{-1}, no. of seed fruit^{-1}, plant height were non-significant. Thus, indicating that transgenic lines were more or less similar in performance as compared to commercial money maker non-transgenic lines. Moreover, there was highly significant difference among the lines for shelf life of fruit expressing that shelf life of transgenic lines were improved as compared to the non-transgenic lines.

Table 2: Mean squares from analysis of variance for various morphological traits of transgenic and non-transgenic lines

Source of variation	DF	Characters				
		Fruit size	**No. of fruit plant^{-1}**	**No. of seed fruit^{-1}**	**Plant height**	**Shelf life**
Genotypes	7	0.080^{ns}	2.851^{ns}	6.803^{ns}	2.9463^{ns}	24.803**
Error	16	0.178	6.125	5.000	3.833	0.708
Total	23					

****,*=** significant at 1 and 5% of probability levels, respectively.

Mean performance for different morphological parameter Fruit size

This study demonstrated that the fruit size of transgenic plants was non- significantly less than their non-transgenic plants. The biggest fruit size (4.4 cm) was observed in transgenic line MM19 while minimum fruit size was observed as 3.986 cm in Non-transgenic line BSX935 (Figure 5; Table 2).

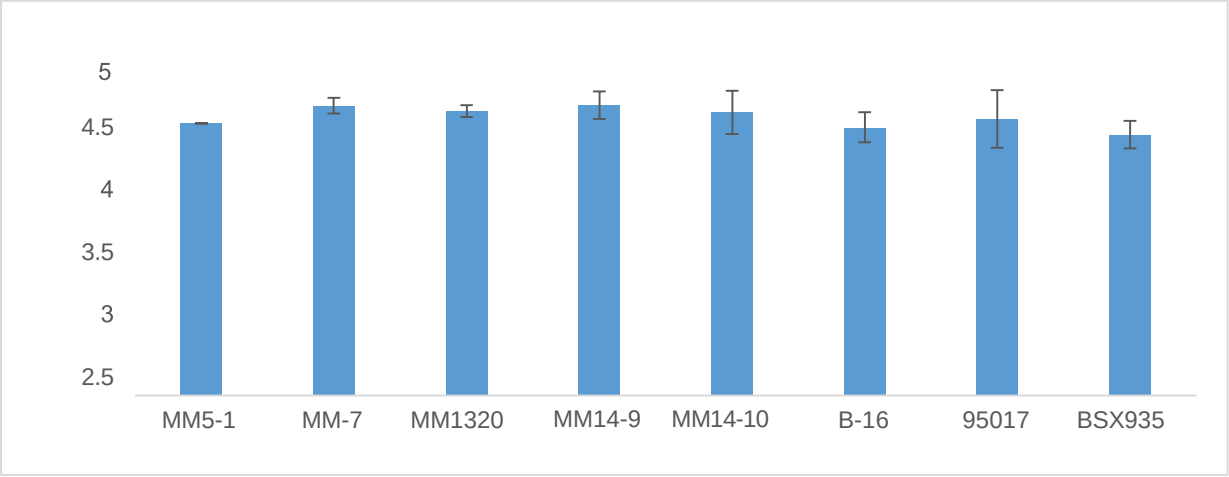

Figure 5. Mean performance for fruit size among different transgenic and non-transgenic varieties of tomato

Number of fruit plant^{-1}

During this study, it has been noticed that there is no significant effect of cold tolerant gene *AtDREB1A* on the no. of fruit per plant in transgenic lines without any stress as compared to their non-transgenic lines. The highest number of fruits plant^{-1} was counted as 23.33 fruits in BSX935 while minimum number of fruit was counted as 20.66 fruits in MM14-9 (Figure 6; Table 1).

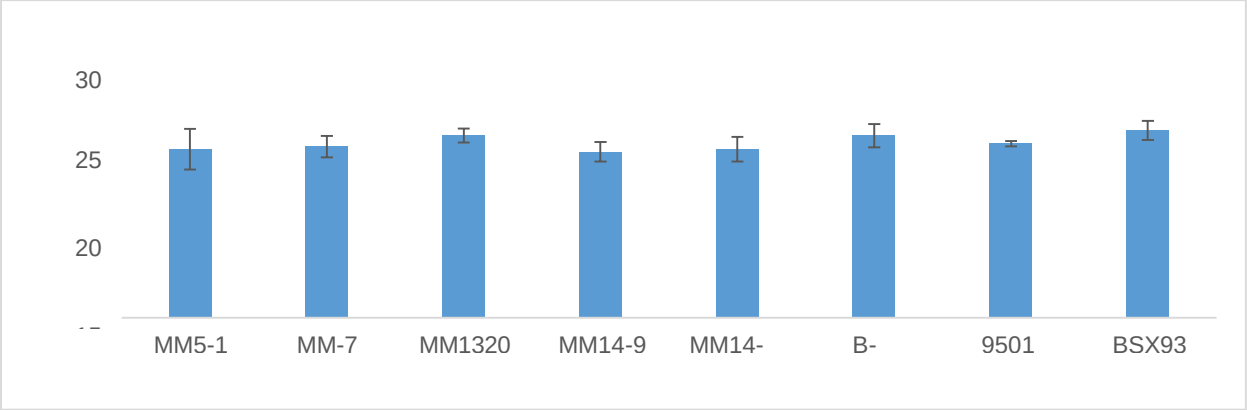

Figure 6. Mean performance for fruits per plant between different transgenic and non-transgenic varieties of tomato

Number of seed fruit^{-1}

The maximum number of seeds fruit^{-1} (44.33) was observed in MM14-9 while, minimum no. of seed fruit^{-1} (39.33) was noticed in 95017 (Figure 7; Table 2)

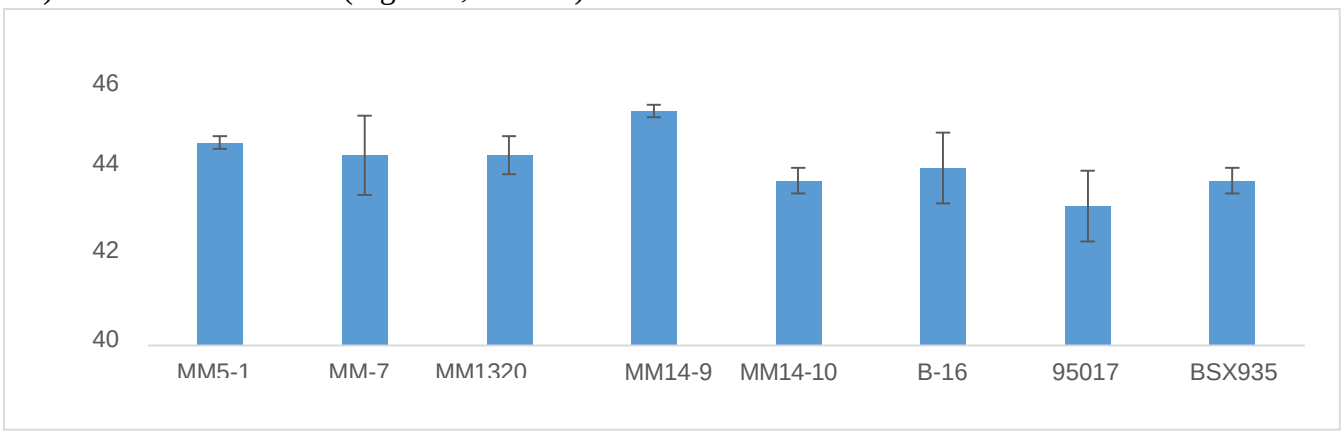

Figure 7. Mean performance for seeds per fruit between different transgenic and non-transgenic varieties of tomato

Plant height

From this study, it has been confirmed that transgenic lines did not show any significant difference for plant height as compared to non-transgenic lines under normal growth condition. The maximum plant height (73.66) was observed in BSX935, and minimum plant height (71.00) was found in B-16.

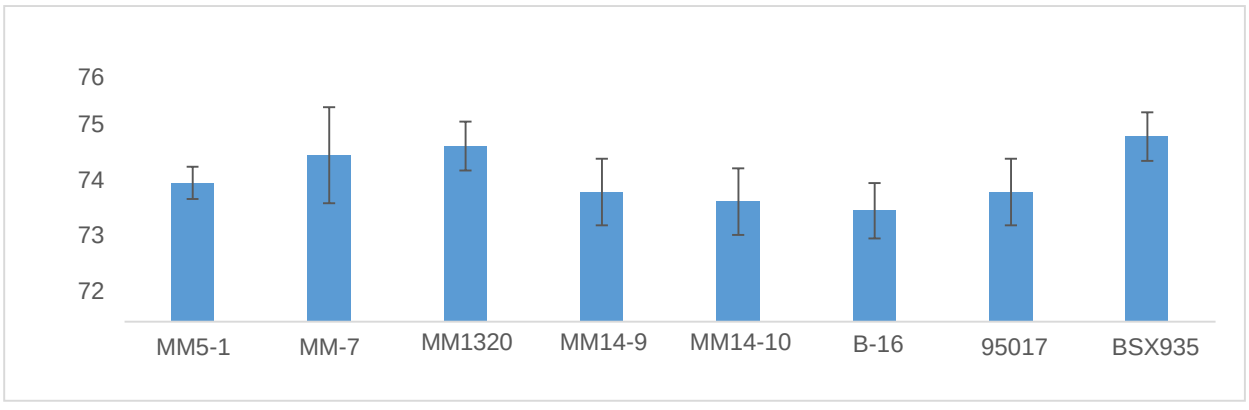

Figure 8. Mean performance for Plant height among different transgenic and non- transgenic varieties of tomato

Shelf life

From this study, it has been confirmed that there was significant difference between transgenic and non-transgenic lines of tomato for shelf life. The maximum shelf life (43.00) was observed in MM5-1 and minimum shelf life (33.33) was noticed in B-16 (Figure 9; Table 1).

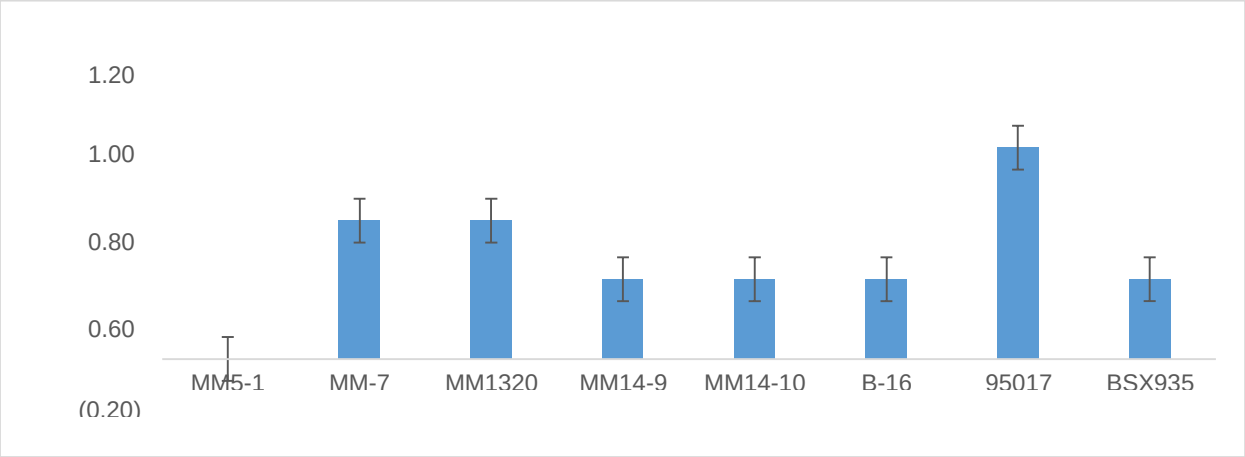

Figure 9. Mean performance for shelf life between different transgenic and non-transgenic varieties of tomato

Table 3: over all mean performance of tomato genotypes for fruit size, Number of fruit plant^{-1}, Number of seed plant^{-1}, Plant height and shelf life.

Genotype	Fruit Size	NFPP	NSPF	P. Height	Shelf life
MM5-1	4.156	21.00	42.66	72.00	**43.00**
MM-7	4.433	21.33	42.00	73.00	**40.00**
MM1320	4.346	22.66	42.00	73.33	**39.00**
MM14-9	4.440	20.66	44.33	71.66	**41.33**
MM14-10	4.233	21.00	40.66	71.33	**39.33**
B-16	4.100	22.66	41.33	71.00	**33.33**
95017	4.233	21.66	39.33	71.66	**37.33**
BSX935	3.986	23.33	40.66	73.66	**38.33**
LSD (5%)	**9.92**	**11.36**	**5.37**	**2.71**	**1.45**

=NFPP; No. of fruits plant^{-1}NSPF; No. of seed fruit^{-1}

Confirmation of DREB1A gene into transgenic lines

Sample from each transgenic line were taken and PCR was performed for confirmation of DREB1A gene into transgenic lines. The maximum positive PCR response was found in MM5-1 confirming the presence of cold tolerance gene. However, minimum PCR positive response was noticed in MM1320. Thus, MM5-1 can be utilized as donor parent in hybridization program against cold tolerance in tomato.

Table 3: Confirmation of DREB1A gene into transgenic lines

Trans-genic	PCR Response										Total +ve PCR results
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	
MM5-1	+	+	+	+	+	+	-	+	+	-	8
MM-7	-	-	+	+	+	-	+	-	+	+	6
MM1320	-	+	-	-	+	-	-	+	+	+	5
MM14-9	+	-	+	+	-	+	+	+	-	+	6
MM14-10	-	-	+	+	+	+	+	+	-	-	6
B-16	-	-	-	-	-	-	-	-	-	-	0
95017	-	-	-	-	-	-	-	-	-	-	0
BSX935	-	-	-	-	-	-	-	-	-	-	0

4. Summary

Tomato (*Lycopersicum esculentum* L.) is an important vegetable crop that has a higher economic value as compared to other vegetables. It is mostly affected by low temperature below 120C. The present study was conducted to confirm the expression of transgene in T5 generation of transgenic tomato lines transformed with DREB1A. PCR results confirmed that transgene has successfully been transferred in T5 population and this population can further be utilized for confirmation in T6 generation of tomato lines. Moreover, DREB1A gene was successfully expressed in T5 generation. Thus, it has been confirmed from the above experiment that DREB1A gene successfully introgressed and expressed in T5 generation.

The T5 transgenic lines were compared with non-transgenic lines on the basis of morphological traits under normal growth conditions. It has been observed that no significant differences were found between transgenic and non-transgenic lines except for shelf life. Shelf life was increased in transgenic lines confirming the presence of cold tolerance gene and providing resistance against cold tolerance. Moreover, for other characters it is considered that there is no direct linkage of DREB1A gene with agronomic as well as yield parameters.

5. Conclusions

From the current study it was concluded that transgene DREB1A was successfully expressed in T5 generation. Transgene was successfully introgressed into transgenic line through agro-bacterium tumefaciens 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 for all the characters except shelf life and transgenic lines were stable in morphological parameters still in T5 generation.

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