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RESEARCH PAPER

Morphological analysis of cold-tolerant tomato (*Solanum lycopersicum* Mill.) plants expressing *CBF3* gene

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Key Message: The study evaluated morphological traits in three tomato transgenic lines (Rio Grande, Moneymaker, and Roma) expressing the *AtCBF3* gene under normal growth conditions. The findings revealed no significant differences in various parameters compared to non-transgenic plants that demonstrate the cold-inducible nature of Lip9 promoter.

Abstract

This research endeavor sought to assess the morphological attributes of transgenic tomato plants expressing the *AtCBF3* gene in comparison to their non-transgenic (NT) counterparts under standard growth conditions. The study focused on three distinct tomato lines (Rio Grande, Moneymaker, and Roma) evaluating a range of characteristics. The findings revealed that transgenic plants carrying the *AtCBF3* gene exhibited no statistically significant variations in parameters such as plant height, leaf count, fresh weight, dry weight, root length, days to flowering, and flower count when compared to their non-transgenic counterparts. However, remarkable differences were evident among the various genotypes concerning these morphological traits. Transgenic plants generally exhibited comparable or slightly reduced performance in terms of plant height, fresh weight, and number of flowers compared to NT plants. Notably, transgenic Rio Grande showed the highest values for plant height, root length, and number of flowers. Additionally, transgenic plants exhibited non-significant differences in first fruit set, number of fruit per plant, fruit diameter, fruit mean weight, and number of seeds per fruit compared to NT plants. The study concludes that under normal conditions, the *AtCBF3* gene driven by the lip9 promoter did not significantly influence various morpho-agronomical and yield parameters in tomato plants. The study provides insights into the morphological characteristics of transgenic tomato plants expressing the *AtCBF3* gene emphasizing the importance of considering genotypic variations in transgenic crops. The implications of the cold-inducible nature of the promoter and the need for further investigations into specific gene-environment interactions are highlighted.

Keywords: *AtCBF3* gene, Cold-tolerant tomato, Lip9 promoter, Morphological analysis, Normal growth conditions, *Solanum lycopersicum* Mill

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Introduction

The cultivated tomatoes are susceptible to chilling stress (0 to 12 ºC) at every developmental stage, ranging from germination to fruit setting (Liu et al., 2012). To counteract the adverse effects of chilling stress, it is essential to develop tomato cultivars with increased tolerance to low temperatures. Chilling tolerance is a complex phenomenon that relies on various physiological and biochemical changes occurring during cold acclimation (Heidarvand & Maali Amiri, 2010; Shah et al., 2016; Shah et al., 2017). The well-established CBFs pathway regulates multiple cold-inducible genes, contributing to cold tolerance in tomatoes (Chinnusamy et al., 2007; Zhou et al., 2011). For the tomato cultivar Punjab Upma, an efficient transformation procedure was established using the Agrobacterium tumefaciens strain GV3101. Kanamycin, under the control of the 35 S promoter, served as the selection agent during the transformation process. The optimal conditions for achieving the highest transformation efficiency included an Agrobacterium density of OD600 nm = 1.0, an acetosyringone concentration of 25 µM, and a kanamycin concentration of 30 mg/l (Rizwan & Bal, 2012). Guo et al. (2012) introduced a rapid transformation protocol for Micro-Tom tomatoes, investigating factors such as Agrobacterium optical density, infection time, co-cultivation duration, and the appropriate dose of carbancilin antibiotic for selection. The results indicated that a bacterial cell density of OD600 nm = 0.5, an infection time of 5 minutes, and the addition of 500 mg/l carbancilin in the selection media enhanced the production of transgenic plants. The overall transformation efficiency achieved was 5.1%. Islam et al. (2010) introduced an innovative method to transform tomatoes using cotyledonary explants. Their study highlighted that achieving a bacterial optical density (OD600 nm) of 0.8 coupled with a 3-day co-cultivation period significantly improved the efficiency of the transformation process. Transformants were precisely selected through exposure to a

kanamycin concentration of 200 mg/l, and confirmation of the presence of the GUS gene was conducted using PCR. In a related study, Siddig et al. (2009) outlined a transformation protocol specifically designed for the tomato cultivar CastleRock. The investigation revealed that infecting cotyledonary explants with a bacterial culture characterized by an optical density of OD600 nm = 0.8 for a duration of 30 minutes in combination with hygromycin at 25 mg/l led to a remarkable increase in transformation efficiency. In post-selection, hygromycin-resistant cotyledonary explants were transplanted to MS basal media supplemented with 5 mg/l BA and 1.0 mg/l IAA for regeneration.

Zhang et al. (2012) illustrated significant chilling tolerance in cotton by introducing the *betA* gene leading to increased glycinebetaine synthesis. The transgenic plants characterized by *betA* gene overexpression, displayed elevated transpiration rates, stomatal conductance, and net photosynthesis in comparison to their wild-type counterparts. In a similar study, Singh et al. (2011) enhanced cold tolerance in tomatoes by engineering plants that overexpressed the *At-CBF1* gene. T1 generation transformants were marker-free and exhibited inducible expression solely under cold stress (4 ºC) for three days. These transgenic lines displayed a significantly higher survival rate (50%) compared to non-transgenic plants (10%). Additionally, the transgenic plants maintained higher relative water contents after exposure to chilling stress, indicating superior adaptive capabilities under such conditions. Khare et al. (2010) conducted a study on tomatoes, introducing the *mtlD* gene to enhance cold tolerance. The resulting transgenic plants survived for two days under chilling stress (4 ºC), whereas control plants died gradually. The accumulation of mannitol in transgenic plants increased leaf osmotic potential, thereby retaining water potential in the surrounding environment and safeguarding against dehydration stress induced by cold. Liu et al. (2013) improved the chilling tolerance of tomatoes through the incorporation of the *Lefad7* gene. Transgenic tomato plants demonstrated increased resistance to cold temperatures, effectively modulating trienoic fatty acids and accumulating higher chlorophyll contents in comparison to their wild-type counterparts when subjected to low-temperature stress (4 °C). The authors concluded that overexpressing *Lefad7* led to chilling tolerance in tomatoes, attributed to alterations in membrane lipid composition.

Materials and Methods

Plant material and genetic transformation via *Agrobacterium tumefaciens*

Seeds of tomato (*Solanum lycopersicum* Mill. cvs. Rio Grande, Moneymaker, and Roma) were obtained from Horticultural Research Institute (HRI), NARC Islamabad, Pakistan. Seeds were treated for dormancy break and surface sterilized before cultivation. Hypocotyls, epicotyls, internodes, leaf discs, and cotyledons from 15-day-old *in vitro* seedlings were evaluated for shoot production on MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l IAA. The transformation protocol involved optimizing bacterial culture preparation, Agrobacterium cell densities, and acetosyringone (AS) concentrations. Hypocotyls and leaf discs were used as explants, and their age and pre-culturing duration were varied. Co-cultivation duration and AS concentrations were optimized, and co-infected explants were cultured on media with varying AS concentrations and pH. Bacterial overgrowth was controlled using filter papers, and incubation was in the dark at 28 ºC. Co-cultivated explants were washed with different concentrations of claforan® and transferred to pre-selection medium for varying durations to optimize pre-selection. Various concentrations of hygromycin B were tested on shoot regeneration frequency, determining the lethal dose for maximum survival of transformants. Pre-selected explants were moved to selection media fortified with hygromycin and cefotaxime to select transformants. Resistant calli were initiated and transferred to shoot induction medium for regeneration. Shoots were then moved to root induction medium, and the transformation efficiency was calculated. Transformed plantlets with efficient roots were acclimatized and shifted to mini plastic bags for further growth until maturity. Plantlet survival rates were recorded periodically.

Techniques employed in analyzing morphological characteristics

The study was conducted at the Post-Harvest Laboratory, Department of Horticulture at Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan. Three distinct transgenic lines of tomatoes (*Solanum lycopersicum* Mill.) namely Rio Grande, Moneymaker, and Roma (T1 generation) were cultivated in greenhouse conditions and allowed to undergo self-pollination for fruit production. The seeds from each line were harvested individually and subsequently planted in nine-centimeter Petri plates within a growth room. The growth room maintained a light period of 16 hours at a temperature of 22 degrees Celsius followed by a dark period of 8 hours at 18 degrees Celsius, all with a relative humidity of 75%. Germination occurred within 5-6 days resulting in the T2 generation. Six days after germination, the healthy seedlings were transferred to small plastic bags containing a 1: 1 mixture of vermiculite and soil. Regular watering every three days continued for about three weeks, after which the plantlets were transplanted into pots and placed under standard growth conditions in a glasshouse. Total plant DNA was extracted using the CTAB method, and PCR analysis was conducted to confirm the presence of *CBF3* and *HPT* genes. Transgenic plants that exhibited Mendelian segregation ratio (3: 1) at thirty days old were selected for morphological analysis and

compared with their isogenic non-transgenic (NT) counterparts. The height of 12 randomly chosen transgenic and NT plants was measured from ground level to the upper surface of the main photosynthetic tissues using a meter rod, and the values were recorded in centimeters. Subsequently, the plants were carefully uprooted and root length was measured using the meter rod. Fresh weight was determined immediately after harvesting transgenic and NT plants followed by incubation at 80 ºC for two days to record dry weight. The mean fresh weight (g) of mature fruits was calculated by harvesting 12 mature fruits from each plant line and the average was recorded. The diameter of twelve fully developed fruits from various plants, was gauged in centimeters utilizing a measuring tape, and the mean value was documented for subsequent statistical analysis. Additionally, morphological data encompassing parameters such as the number of leaves per plant, days to flowering, number of flowers per plant, time to the first fruit set, number of fruits per plant, and the count of seeds per fruit were systematically recorded.

Results

Comparison of four-week-old transgenic and non-transgenic (NT) plants from three tomato lines was conducted based on various morphological characteristics:

Plant height (cm)

An analysis of variance (ANOVA) conducted for plant height in both transgenic and non-transgenic (NT) plants indicated no significant differences between the two groups and their interaction with genotypes (plants × genotypes). However, significant variations were observed among genotypes. The plant height of transgenic plants, though insignificantly lower than that of NT plants, was evident (Fig. 1 A & B). The highest plant height was recorded in transgenic Rio Grande (71.35 cm), followed by Roma (65.95 cm) and Moneymaker (57 cm). In comparison, their non-transgenic counterparts exhibited heights of 72.55, 67.26, and 59.63 cm, respectively (Table 1).

Number of leaves per plant

Data on the number of leaves per plant (Table 2) indicated no significant variations between transgenic and NT plants. From a statistical perspective, no significant distinctions were identified in the number of leaves among transgenic and non-transgenic (NT) plants and their interaction (plants × genotypes). However, substantial differences were evident among genotypes. The highest number of leaves per plant was documented in transgenic Rio Grande (46.75), followed by Roma (33.57) and Moneymaker (24.69), in contrast to their non-transgenic counterparts Rio Grande (45.26), Roma (30.93) and Moneymaker (22.92) (Table 2).

Fresh weight (g/plant)

Data on plant fresh weight (Table 3) exhibited minor differences between transgenic and NT plants. The fresh weight per plant for transgenic and NT plants showed no significant variations between the two groups and their interaction with genotypes. However, highly significant differences were noted among genotypes. The highest fresh weight per plant was recorded in transgenic Rio Grande, Roma, and Moneymaker (64.45, 56.5 and 45.77 g, respectively) compared to their NT counterparts with weights of 63.17, 53.82, and 43.71 g, respectively (Table 3).

Dry weight (g/plant)

The dry weight per plant exhibited non-significant variations among both transgenic and non-transgenic (NT) plants and their interaction with genotypes, although significance was observed for genotypes. The highest recorded dry weight per plant (6.95 g) was noted in transgenic Rio Grande followed by Roma (6.09 g) and Moneymaker (5.26 g) compared to their isogenic NT counterparts with weights of 6.21, 5.49, and 4.52 g (Table 4).

Root length (cm)

For the measurement of root length at 30 days after germination, twelve uniform plants from each line were randomly selected. The growth rate of root length displayed non-significant differences among transgenic and NT plants including their interaction (plants × genotypes) at 25 °C, whereas significant differences were observed among genotypes. Data presented in Table 5 indicated that the highest root length (26.65 cm) was achieved in transgenic Rio Grande followed by Roma (22.15 cm) and Moneymaker (19.23 cm). Conversely, NT plants of Rio Grande exhibited a root length of 24.99 cm followed by NT Roma (20.38 cm) and NT Moneymaker (16.95 cm) (Table 5).

Days to flowering

The evaluation of a crucial growth parameter, the time required for flowering, was conducted across transgenic and non-transgenic (NT) tomato genotypes. The results indicated no significant differences among transgenic and NT plants and their interaction (plants × genotypes), while a significant difference was observed for genotypes at normal temperature. Transgenic plants exhibited a non-significantly prolonged duration to flower compared to NT plants across all lines (Table 6). The most extended time to flowering (67 days) was observed in transgenic Moneymaker, followed by Roma (62 days) and Rio Grande (55 days), in contrast to their non-

transgenic counterparts, Moneymaker (65 days), Roma (61 days), and Rio Grande (52 days).

Table 1 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of plant height (cm) in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	71.35 ± 3.87	57.00 ± 2.01	65.95 ± 3.81	64.76
NT plants	72.55 ± 3.15	59.63 ± 2.91	67.26 ± 2.74	66.48
Mean	71.95	58.00	66.60	

Each dataset represents the mean of three independent replicates. The values following the ± sign indicate standard deviation (n = 3). The LSD (Least Significant Difference) value was 4.75 at $p \leq 0.05$.

Table 2 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of number of leaves per plant in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	46.75 ± 4.53	24.69 ± 2.36	33.57 ± 3.36	35.00
NT plants	45.26 ± 3.28	22.92 ± 3.16	30.93 ± 3.71	33.03
Mean	46.00	23.80	32.25	

Each dataset represents the mean of three independent replicates. The values following the ± sign indicate standard deviation (n = 3). The LSD (Least Significant Difference) value was 4.59 at $p \leq 0.05$.

Table 3 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of fresh weight (g/plant) in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	64.45 ± 5.46	45.77 ± 4.07	56.50 ± 3.89	55.57
NT plants	63.17 ± 7.56	43.71 ± 4.78	53.82 ± 6.11	53.56
Mean	63.81	44.74	55.16	

Each dataset represents the mean of three independent replicates. The values following the ± sign indicate standard deviation (n = 3). The LSD (Least Significant Difference) value was 5.1 at $p \leq 0.05$.

Table 4 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of dry weight (g/plant) in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	6.95 ± 1.47	5.26 ± 1.15	6.09 ± 1.44	6.1
NT plants	6.21 ± 1.44	4.52 ± 1.32	5.49 ± 0.76	5.4
Mean	6.58	4.89	5.79	

Each dataset represents the mean of three independent replicates. The values following the ± sign indicate standard deviation (n = 3). The LSD (Least Significant Difference) value was 1.42 at $p \leq 0.05$.

Table 5 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of root length (cm) in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	26.65 ± 3.62	19.23 ± 3.24	22.15 ± 3.92	22.67
NT plants	24.99 ± 1.71	16.95 ± 3.27	20.38 ± 4.54	20.77
Mean	25.82	18.09	21.26	

Each dataset represents the mean of three independent replicates. The values following the ± sign indicate standard deviation (n = 3). The LSD (Least Significant Difference) value was 6.35 at $p \leq 0.05$.

Table 6 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of days to flowering in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	55.00 ± 5.57	67.00 ± 7.94	62.00 ± 5.29	61.33
NT plants	52.00 ± 5.29	65.00 ± 4.58	61.00 ± 4.58	59.33
Mean	53.5	66.00	61.5	

Each dataset represents the mean of three independent replicates. The values following the ± sign indicate standard deviation (n = 3). The LSD (Least Significant Difference) value was 10.02 at $p \leq 0.05$.

Number of flowers per plant

The count of flowers per plant within all transgenic lines exhibited no significant variation from non-transgenic (NT) plants at 25 °C (Table 7). ANOVA analysis for the number of flowers per plant revealed no significant variations for transgenic and NT plants, as well as the interaction between genotypes and plants. However, considerable differences were identified among genotypes concerning the number of leaves per plant. The data in Table 7 showed a non-significant decrease in the number of flowers per transgenic plant compared to NT plants across all tested genotypes (Fig. 1 C & D). The highest number of flowers (46.62) was observed in transgenic Rio Grande, followed by Roma (40.78) and Moneymaker (32.59), in contrast to their isogenic NT plants, which produced 37.29, 32.91, and 24.93 flowers per plant, respectively (Table 7).

First fruit set (days)

The agronomic performance of lip9-CBF3 and NT plants was assessed concerning the first fruit set at normal temperature. The results in Table 8 indicated that the time required for the initiation of fruiting in transgenic lines was nearly identical to that of NT plants under optimal growth conditions. Statistically non-significant differences were observed among transgenic and NT plants, as well as in their interaction (genotypes × plants), while significant differences were found among genotypes. The longest days to fruiting (76 days) were recorded in transgenic Moneymaker followed by Roma (70) and Rio Grande (65) compared to their isogenic NT plants, where days to fruiting (74, 69, and 62) were recorded in Moneymaker, Roma, and Rio Grande (Table 8).

Number of fruit per plant

ANOVA analysis showed non-significant differences among transgenic and NT plants and in the interaction of genotypes × plants, while it was significant for genotypes. Results from the investigation of transgenic lines (T2 generation) revealed that the average maximum number of fruit per plant (21.66) was obtained in the transgenic line of Rio Grande followed by Roma (15.72) and Moneymaker (10.66) compared to their NT plants of Rio Grande (24.61), Roma (18.49) and Moneymaker (13.86) (Table 9; Fig. 2 A & B).

Fruit diameter (cm)

Analysis of variance (ANOVA) for fruit diameter revealed non-significant variations among transgenic and non-transgenic (NT) plants and also in the interaction of genotypes × plants, while significant differences were observed among genotypes. The fruit diameter was non-significantly lower in transgenic plants compared to NT plants (Fig. 2 C & D). The maximum fruit diameter (5.46 cm) was documented in the transgenic Rio Grande, followed by Moneymaker (4.18 cm) and Roma (3.34 cm) in contrast to their isogenic NT plants that produced 6.15, 4.86, and 4.46 cm fruit diameter for Rio Grande, Moneymaker and Roma, respectively (Table 10).

Fruit mean weight (g)

The average fruit weight of lip9:CBF3 expressing lines and NT tomato plants was examined at 25 °C. Data in Table 11 indicated non-significantly lower fruit weight in transgenic lines compared to their counterparts. The maximum fruit weight (39.59 g) was observed in the transgenic Rio Grande followed by Roma (35.65 g) and Moneymaker (30.84 g), as opposed to NT plants that produced 42.72, 38.31 and 32.45 g fruit mean weight for Rio Grande, Roma, and Moneymaker, respectively. ANOVA for fruit weight indicated significant variations among genotypes, while it exhibited non-significant differences among transgenic and NT plants.

Number of seeds per fruit

ANOVA for the number of seeds per fruit showed no significant variations for transgenic and NT plants and also in their interaction with genotypes. Data in Table 12 indicated that the maximum number of seeds per fruit (120.78) was obtained in the Rio Grande transgenic line followed by Roma (116.67) and Moneymaker (110.37), in comparison to their NT counterparts that produced 123.87, 121.59 and 115.23 seeds per fruit for Rio Grande, Roma, and Moneymaker, respectively (Table 12).

Table 7 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of number of flowers per plant in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	35.65 ± 4.82	21.98 ± 2.99	30.84 ± 3.71	29.49
NT plants	37.29 ± 5.22	24.93 ± 4.84	32.91 ± 5.21	31.71
Mean	36.47	23.45	31.87	

Each dataset represents the mean of three independent replicates. The values following the ± sign indicate standard deviation (n = 3). The LSD (Least Significant Difference) value was 8.87 at $p \leq 0.05$.

Table 8 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of first fruit set (days) in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	65.00 ± 5.20	76.00 ± 6.08	70.00 ± 5.00	70.33
NT plants	62.00 ± 4.36	74.00 ± 6.24	69.00 ± 5.29	68.33
Mean	63.5	75.00	69.5	

Each dataset represents the mean of three independent replicates. The values following the ± sign indicate standard deviation (n = 3). The LSD (Least Significant Difference) value was 10.44 at $p \leq 0.05$.

Table 9 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of number of fruit per plant in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	21.66 ± 3.94	10.66 ± 2.67	15.72 ± 4.44	16.01
NT plants	24.61 ± 5.09	13.86 ± 2.72	18.49 ± 3.83	18.98
Mean	23.13	12.26	17.1	

Each dataset represents the mean of three independent replicates. The values following the ± sign indicate standard deviation (n = 3). The LSD (Least Significant Difference) value was 6.74 at $p \leq 0.05$.

Table 10 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of fruit diameter (cm) at ripening in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	5.46 ± 0.84	4.18 ± 0.96	3.34 ± 0.63	4.32
NT plants	6.15 ± 1.14	4.86 ± 0.70	4.46 ± 1.07	5.15
Mean	5.8	4.52	3.9	

Each dataset represents the mean of three independent replicates. The values following the ± sign indicate standard deviation (n = 3). The LSD (Least Significant Difference) value was 1.67 at $p \leq 0.05$.

Table 11 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of fruit mean weight (g) in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	39.59 ± 4.17	30.84 ± 3.71	35.65 ± 4.82	35.36
NT plants	42.72 ± 4.91	32.45 ± 4.35	38.31 ± 5.96	37.82
Mean	41.15	31.64	36.98	

Each dataset represents the mean of three independent replicates. The values following the ± sign indicate standard deviation (n = 3). The LSD (Least Significant Difference) value was 8.19 at $p \leq 0.05$.

Table 12 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of number of seeds per fruit in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	120.78 ± 9.91	110.37 ± 6.91	116.67 ± 7.76	115.94
NT plants	123.87 ± 5.18	115.23 ± 5.87	121.59 ± 4.26	120.23
Mean	122.32	112.8	119.13	

Each dataset represents the mean of three independent replicates. The values following the ± sign indicate standard deviation (n = 3). The LSD (Least Significant Difference) value was 12.79 at $p \leq 0.05$.

Fig. 1 Morphological comparison of transgenic and NT plants of three tomato genotypes under normal growth environment in glasshouse (A) Plant height of NT plants (B) Plant height of transgenic plants (C) Transgenic plants at flowering stage (D) NT plants at flowering stage

Fig. 2 Comparison of transgenic and NT plants of three tomato genotypes on the basis of morphological parameters under normal growth conditions in glasshouse (A) Transgenic plants at fruiting stage (B) NT plants at fruiting stage (C) Fruit size of transgenic plants (D) Fruit size of NT plants

Discussion

During this investigation, *AtCBF3* transgenic plants exhibited no distinct phenotypic alterations in plant height under normal growth conditions compared to their isogenic NT counterparts. This observation contrasts with the findings of Jin et al. (2012) who modified tomatoes with the *PL1* gene and asserted that transgenic tomato plants displayed reduced plant height compared to control plants. Bo et al. (2006) contradicted our results, reporting conflicting outcomes and reported that chrysanthemum plants transformed with rd29A: AtDREB1A exhibited greater plant height than NT plants. In alignment with our results, Singh et al. (2011) engineered tomato cv. Shalimar with the *At-CBF1* gene driven by an rd29A inducible promoter to withstand chilling stress. They reported that transgenic tomato plants exhibited no significant differences in plant height compared to NT plants under normal conditions without chilling stress. In our investigation, tomato transgenic plants harboring the *AtCBF3* gene produced a non-significantly higher number of leaves per plant than NT plants. This finding parallels the earlier study on tomato plants by Garcia-Hurtado et al. (2012) wherein transgenic tomatoes overexpressing *CcGA20ox1* demonstrated a higher leaf count than WT plants. However, our findings diverged from those of Safdar et al. (2013) who developed transgenic tomato cv. Rio Grande overexpressing yeast halotolerance genes (*HALI* and *HALII*). They concluded that six transgenic lines exhibited a lower number of leaves compared to the control line suggesting a potential influence of genotype differences on the observed discrepancy.

The present study did not provide support for the involvement of the *AtDREB1A* gene in enhancing fresh weight in three tomato transgenic lines under normal growth conditions. This lack of support may be attributed to the fact that the lip9 promoter did not induce the expression of *AtDREB1A* possibly due to its cold-inducible nature. Our findings align with the earlier proposition by Garcia-Hurtado et al. (2012), who observed that transgenic tomato plants overexpressing the *CcGA20ox1* gene exhibited similar fresh weight to wild-type plants under normal growth conditions. Similarly, Bo et al. (2006) reported that tomato transgenic plants overexpressing *AtDREB1A* under the influence of the 35S promoter displayed undesirable growth phenotypes including reduced fresh weight compared to control plants. This undesired phenotype could be mitigated by replacing the 35S CaMV promoter with an inducible promoter (rd29A) in transgenic Arabidopsis plants overexpressing *DREB1A* as demonstrated by Kasuga et al. (1999). In contrast, another research group investigated the impact of *HALI* and *HALII* on fresh weight in transgenic tomatoes, suggesting that transgenic lines showed improvements in fresh weight and water content compared to non-transgenic plants (Safdar et al., 2013).

During the current study, T2 transgenic plants carrying *AtCBF3* were compared with non-transgenic plants based on dry weight (g/plant) in three tomato genotypes at 25 °C. The results confirmed that transgenic plants did not exhibit significant differences in dry weight compared to non-transgenic plants. These findings corroborate with the earlier studies by Zhao et al. (2007) who reported that transgenic *Festuca arundinacea* plants overexpressing rd29A:AtCBF3 did not show significant differences in dry weight per plant compared to control plants. In a study by Gupta and Rajam (2013), tomatoes were modified through the incorporation of the mtlD gene, resulting in a notable 40-71% increase in dry weight when compared to control plants. In contrast, Dai et al. (1999) utilized the *AtHXK1* gene to engineer tomato plants, and their findings indicated an inverse correlation between the overexpression of hexokinase activity in photosynthetic tissues and the growth as well as the dry weight of transgenic plants. This effect was potentially linked to the inhibition of *CAB1*, a sugar-responsive photosynthetic gene, as discussed by Jang et al. (1997). The disparities observed between our study and earlier reports could potentially be ascribed to variations in the choice of transgenes, promoters, and genotypes under examination.

Root growth plays an essential role in a plant's ability to adapt to challenging environments, serving as the primary mechanism to fulfill transpiration needs and supply water to the plant (Liu & Huang, 2000). In the context of normal growth conditions, our study found that transgenic lines exhibited root lengths comparable to their isogenic counterparts due to the absence of *AtCBF3* gene expression at standard temperatures. This observation differs from the greenhouse investigation conducted by Saad et al. (2010) on transgenic tobacco carrying the *AlSAP* gene, which reported a slight increase in root length compared to control plants under normal growth conditions. Additionally, Garcia-Hurtado et al. (2012) conducted a greenhouse study on transgenic tomatoes expressing gibberellin 20-oxidase (*CcGA20ox1*) from citrus. Their research revealed a significant enhancement in various characteristics of transgenic plants, including root length, under non-stressed conditions when compared to wild-type (WT) plants. Similarly, previous studies by Eriksson et al. (2000), Vidal et al. (2001); Phillips (2004) documented longer roots in various plant species overexpressing *GA20ox* compared to their isogenic counterparts. Discrepancies between our findings and previous reports may be attributed to differences in transgenes, promoters, genotypes, and growth conditions.

To evaluate the overexpression effects of the *CBF3* gene on both vegetative and reproductive growth in three tomato genotypes, we analyzed transgenic plants with a single copy of the gene. These plants showed a non-significant delay in the time to flower initiation compared to non-transgenic (NT) plants. Although a delay in flowering was observed in transgenic plants, the overall growth rate was similar to that of isogenic counterparts. In contrast, Ellul et al. (2004) reported that overexpression of *AtAPETALA1* in tomatoes reduced

flowering time without affecting average plant production. Similarly, Gilmour et al. (2000) observed significant differences in flowering time between transgenic Arabidopsis harboring the *CBF3* gene and NT plants. Our study demonstrated that transgenic lines produced a similar number of flowers per plant compared to NT plants under normal conditions. This contrasts with Hammond and Zhao (2009) proposition of increased flower number and height in tobacco cv. Xanthi overexpressing the *pkv* gene. Additionally, transgenic tobacco plants with the *rolD* gene exhibited enhanced flowering and a higher number of flowers compared to their isogenic counterparts. These discrepancies may be attributed to variations in transgenes, promoters, genotypes, and growth conditions.

In this study, we conducted a comparative analysis of three transgenic tomato lines and non-transgenic (NT) plants focusing on the time taken for the first fruit set under normal growth conditions. Our results revealed that the transgenic lines exhibited similar fruiting times compared to the NT plants. This aligns with the findings of Singh et al. (2011) who reported no significant differences in morpho-agronomical characteristics between transgenic tomato var. Shalimar overexpressing the *AtCBF1* gene and NT plants including growth habit, fruit shape, and days to fruiting. In contrast, our results deviated from those of Komakhin et al. (2010) who engineered tomato cv. Marglob with the *NLS-recA-licBM3* gene, showing that the transformants yielded fruits 10-15 days earlier than the control plants. Our morphological analysis contradicted an earlier report by Mauro et al. (1996) in tobacco where transgenic plants exhibited an earlier reproductive phase possibly attributed to higher proline accumulation or ornithine depletion. These disparities may be attributed to variations in transgenes, genotypes, and culture conditions. Our investigation also revealed no significant impact of the *AtCBF3* gene on the number of fruits per plant under normal conditions without chilling stress. This contradicts the findings of Jin et al. (2012) who observed increased fruit production in tomato plants overexpressing the *PL1* fusion gene. According to Jin et al. (2012), there were no adverse effects on the growth of sixteen transgenic lines due to *PL1* gene overexpression. Similarly, a study on tomato transgenic plants harboring the *rolD* gene indicated a significant increase in fruit number per plant compared to NT plants, potentially attributed to a higher number of flowers and a shortened plant life cycle.

Furthermore, our study noted a non-significant reduction in fruit size, specifically in terms of fruit diameter, in the transgenic lines compared to their isogenic counterparts. This observation aligns with the findings of Kevany et al. (2008) who reported that overexpression of the *LeETR4* gene in transgenic tomatoes did not adversely affect fruit size and yield. Singh et al. (2011) also reported non-significant variations in fruit diameter between transgenic plants carrying the *AtCBF1* gene and NT plants. However, Me et al. (2007) reported contradictory results indicating a significant enhancement of fruit size in transgenic lines with *LeETR2* expression compared to wild-type plants. Our study revealed that, under normal temperature conditions, the fruit weight of 4-week-old transgenic lines was not influenced by lip9:CBF3 activity. The fruit weight of transgenic lines was nearly identical to that of their non-transgenic (NT) counterparts. Our findings aligned with Bettini et al. (2003) who found no significant differences in mean fruit weight (g) in tomato transgenic cv. Tondino overexpressing the *rolD* gene compared to isogenic control plants. However, our results contradicted those of Ellul et al. (2004) who conducted a comparative analysis of transgenic tomato plants with the *AP1* gene and reported lower mean fruit weight (g) in the transgenic line compared to control plants. Similarly, Singh et al. (2011) observed comparatively lower average fruit weight in marker-free transgenic tomatoes with the *AtCBF1* gene, while Me et al. (2007) reported significantly heavier fruit in transgenic lines compared to wild-type plants.

To assess the impact of the *AtCBF3* gene on overall yield, we conducted a comparative analysis of three transgenic tomato lines and their respective NT plants. Our findings indicated a non-significantly lower number of seeds per fruit in transgenic lines, resulting in lower yields compared to NT plants. In contrast, a controlled-environment study by Gilmour et al. (2000) on Arabidopsis transgenic plants overexpressing the *CBF3* gene reported significantly fewer seeds per fruit than control plants. This difference was attributed to the overexpression of the *CBF3* gene in specific parts of the transgenic plants leading to reduced axillary shoots. The detailed morphological study of three tomato transgenic lines in our investigation clearly demonstrated that the expression of the *CBF3* gene did not show a clear association with agronomic and yield parameters in tomatoes. This lack of association might be attributed to the fact that the lip9 cold-inducible promoter could not induce the expression of the *CBF3* gene under normal growth conditions, resulting in no significant differences between transgenic and NT plants.

Conclusion

Transgenic Rio Grande consistently displayed higher values in plant height, number of leaves, fresh weight, and dry weight compared to its NT counterpart but these differences did not reach statistical significance. Similarly, root length in transgenic plants did not significantly differ from NT plants, with the most substantial length observed in transgenic Rio Grande. Days to flowering, a crucial growth determinant, demonstrated non-significant differences between transgenic and NT plants, despite a slight delay in flowering observed in transgenic plants. The number of flowers per plant showed a non-significant decrease in transgenic plants across all genotypes. The timing of the first fruit set in transgenic lines aligned closely with that of NT plants, with significant differences apparent among genotypes. While the number of fruits per plant did not exhibit significant differences between transgenic and NT plants, notable genotypic variations were

evident. Similarly, fruit diameter and mean fruit weight showed non-significant variations between transgenic and NT plants, with the highest fruit weight recorded in transgenic Rio Grande. Although some morphological variations recorded between transgenic and NT plants but the overall differences were generally non-significant. This research sets the foundation for future studies focused on improving the cold tolerance of tomato plants through genetic modification. It highlights the complex connections between different plant types and also the complex interaction of genes and environmental factors in genetically modified crops.

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