Overexpression of \textit{AtDREB1} and \textit{BcZAT12} genes confers drought tolerance by reducing oxidative stress in double transgenic tomato \textit{(Solanum lycopersicum L.)}

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Abstract

\textbf{Key message} Double transgenic tomato developed by \textit{AtDREB1A} and \textit{BcZAT12} genes pyramiding showed significant drought tolerance by reducing oxidative stress with enhanced yield.

\textbf{Abstract} Although a large number of efforts have been made by different researchers to develop abiotic stress tolerance tomato for improving yield using single gene, however, no reports are available which targets \textit{AtDREB1} and \textit{BcZAT12} genes together. Hence, in the present study, double transgenic plants were developed using \textit{AtDREB1} and \textit{BcZAT12} genes to improve yield potential with better drought tolerance. Double transgenic (DZ1–DZ5) tomato lines showed enhanced drought tolerance than their counterpart non-transgenic and single transgenic plants at 0, 07, 14, and 21 days of water deficit, respectively. Double transgenic plants showed increased activity of antioxidant enzymes, like catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and guaiacol peroxidase (POD), and accumulation of non-enzymatic antioxidants like ascorbic acid, glutathione as compared to non-transgenic and single transgenic. Additionally, the transcript analysis of antioxidant enzymes revealed the increased level of gene expression in double transgenic tomato lines. Developed double-transgenic tomato plants co-over-expressing both genes exhibited more enzymatic and non-enzymatic anti-oxidative activities as compared to the non-transgenic and single transgenic control, respectively. This is the preliminary report in tomato, which forms the basis for a multigene transgenic approach to cope with drought stress.

\textbf{Keywords} \textit{AtDREB1A} · \textit{BcZAT12} · Co-overexpression · Drought · Gene pyramiding · Double transgenics · \textit{Solanum lycopersicum}

Introduction

In agriculture, a plant generally faces water scarcity due to abiotic stresses like drought, cold, and salinity. The inadequate water availability adversely affects plant growth, development, yield and crops quality which depends on the duration, degree of water deficit, and crop stage (Krishna et al. 2019, 2021; Karkute et al. 2019). Throughout the world, about 20\% of agricultural land is under irrigation, of which 14.51\% is supported by good irrigation (FAO 2012) while the remaining is rainfed, hence, frequently prone to drought/ water-deficit stress. The drought stress is considered as the most destructive stress among abiotic stresses which alters plants physiological activity primarily through disturbing plant–water relationship and by the generation of reactive oxygen species (ROS) Sharma et al. (2012),
consequently restricts plants' overall genetically predetermined performance (Rai et al. 2013a). Thus, plants' ability to resist drought stress is of great economic significance in changing the agro-ecosystem for agricultural sustainability. Abiotic stresses in general interrupt cellular homeostasis and produce an elevated level of ROS (Asada 2008; Gill and Tuteja 2010); which acts as a signal to trigger responses related to stress (Miller et al. 2010; Liu et al. 2018). The antioxidative defense machinery of plants relieves oxidative stress by ROS detoxification; thereby plays an important role in tolerance against drought (Sánchez Rodríguez et al. 2010; Sánchez-Rodríguez et al. 2016). These anti-oxidative defense systems composed of low-molecular-mass non-enzymatic antioxidants like carotenoids, ascorbic acid, phenolics, flavonoids, and enzymes like catalase (CAT), superoxide dismutase (SOD) and ascorbate glutathione of Halliwell Asada cycle (Gill and Tuteja 2010). The enhancement in the activity of total foliar antioxidants under abiotic stresses is well documented (Rai et al. 2012a, b; Karkute et al. 2019). When subjected to drought stress, a real-time increment in several other antioxidative defense system components also occurs in tolerant cultivars (Rai et al. 2013a, b; Baghour et al. 2019).

Tomato (Solanum lycopersicum L.) belongs to Solanaceae family is considered as the most important vegetable fruit crop and consumed worldwide. It was also extensively used as a model plant system for different genetic, molecular, and biological studies. Despite the fact that tomatoes can be cultivated profitably globally but its growth and development relatively sensitive to cold, drought, extreme temperatures, and salinity stresses (Foolad 2007; Rai et al. 2013a; Karkute et al. 2019). Drought potentially impacts tomato production globally (Pék et al. 2014; Krishna et al. 2021), wherein plants are unable to give higher yields (Foolad 2007; Krishna et al. 2019). The drought adaptation in plants is regulated at the transcription level and genes involve offer novel possibilities to improve drought-stress tolerance and also to understand signaling pathways (Rai et al. 2013a, b).

The Arabidopsis thaliana transcription factor AtDREB1A/CFB3 gene regulates drought tolerance, having significant importance for plants genetic engineering to attain improved drought tolerance (Rai et al. 2012b; Li et al. 2011; Krishna et al. 2021). Proteins encoded by AtDREB1A/CFB3 binds to C-repeat (CRT) cis-acting core DRE sequence CCGAC, present in A. thaliana various downstream drought-inducible genes promoter region of (Gilmour et al. 2000); and in transgenic plant systems, such as Lolium perenne (Li et al. 2011), peanut and chrysanthemum (Hong et al. 2006; Bhatnagar et al. 2009). Furthermore, ZFPs (Zinc finger proteins) are comparatively a large group of plants transcriptional regulators (Rai et al. 2012a, 2013b). The C_{2}H_{2} is a type of ZFPs widely different in structure and function with respect to their interaction with DNA or RNA or protein (Huang et al. 2007; Kielbowicz-Matuk 2012; Persikov et al. 2014; Persikov et al. 2015) and provides a defense to various stresses, abiotic and biotic (Sun et al. 2010; Rai et al. 2012a; Zhang et al. 2014; Saxena et al. 2016). The ZAT12 is one such stress-responsive C_{2}H_{2} type ZFP, which can manage numerous stress-induced gene expressions in plants arbitrated by ROS signaling (Shah et al. 2013; Zhang et al. 2014; Miller et al. 2018; Krishna et al. 2021). Stress-inducible transcription factor-encoding genes additionally regulate the downstream expression of the genes, consequently a similar enhancement as in stress tolerance (Kielbowicz-Matuk 2012; Ravikumar et al. 2014).

In the present study, we analyzed antioxidative mechanisms, which alleviate oxidative stress generated due to drought stress in AtDREB1A and BcZAT12 pyramided double transgenic (DT) lines of tomato, by exploring the impacts of rd29A::AtDREB1A and BcZAT12 over-expression on key non-enzymatic antioxidant, such as ascorbic acid (AsA), glutathione and antioxidant enzymes, under an increase in the days of drought stress.

**Methods and materials**

**Plant material and drought stress**

In the present study, tomato variety Kashi Vishesh (H86) developed at Indian Institute of Vegetable Research, Varanasi, India using Solanum hirsutum f. glabratum B ‘6013’ as donor parent following backcross pedigree selection method, used for the experiments as performs very well in the Indian sub-continent and show moderate field resistance to tomato leaf curl virus. The research materials consisted of a total eight (H86-cv. Kashi Vishesh, non-transgenic (NT); D86-AtDREB1A::CBF3 (Accession no. AF07460) and ZT1-BcZAT12 (Accession no. DQ166621.1) single transgenic (ST) lines and five (DZ1, DZ2, DZ3, DZ4, and DZ5) double transgenic (DT) lines expressing, AtDREB1A::BcZAT12 genes developed by crossing D86 and ZT1 transgenic lines were taken for the drought stress study. The 25-day-old seedlings were transplanted in 7 kg earthen pots having 5:1 ratios of soil and manure which were kept in the transgenic glass house in a completely randomized design (CRD) at ICAR-Indian Institute of Vegetable Research, located at longitude and latitude of 82.052/E and 25.010/N and altitude of 128.93 m above mean sea level at Varanasi, UP. Transgenic glass houses were equipped with an air cooling system to maintain the standard growth condition of day/night temperature of 25 °C/15 °C and 50% relative humidity; 16 h/8 h of photoperiod with a light intensity of 350 mmol m^{-2} s^{-1} flux. For drought-stress treatments, different days of water deficit (DWD) were imposed on 50 days (d) old tomato plants (from seed germination), during late vegetative stage.
by withholding 7, 14, or 21 d irrigation. The regularly irrigated plants were kept as control (0 d), which contains up to 80% moisture of total field capacity while 7, 14, 21 DWD exposed plants pot have nearly 40, 25 and 15% soil moisture contents of field capacity. After 0, 7, 14 and 21 DWD treatment, from the top, fully expanded fourth leaf was collected and stored at −80 °C till the completion of different analysis. The experiment was replicated thrice with three tomato plants in each replication. For various biochemical analyses, leaf samples were collected and pooled from three replications of each tomato lines/events/variety of each replicate.

**Phyto-pigments (chlorophyll and carotenoid)**

The leaf samples (200 mg) were homogenized in 80% acetone to extract phyto-pigments like chlorophyll and carotenoids as described by Porra et al. (1989). From the extract, the supernatant was collected and subsequently measured the absorbance at 663, 645 and 480, 510 nm for chlorophyll and carotenoid, respectively. Arnon (1949) equation was used to compute the concentration of chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids. Results were expressed in milligrams per gram of fresh weight of the sample.

**Superoxide radical (O−2) measurement**

The superoxide anion (O−2) generation rate was quantified by following the procedure of Shah et al. (2001). The assay reaction was set with 3 ml final volume of 100 mM sodium phosphate buffer (pH 7.2) containing sodium diethyl dithiocarbamate (1 mM) and 0.25 mM nitro blue tetrazolium (NBT). Approximately 200 mg of fresh leaf sample was powdered by following the procedure of Shah et al. (2001). The assay was initiated by adding NADPH to the mixture at room temperature, the reaction was initiated and the reduction in the absorbance of NADPH for 5 min was monitored at 290 nm with an extinction coefficient of 2.8 mM−1 cm−1. The oxidation rate of ascorbate was measured and the specific activity of ascorbate peroxidase was expressed as µmol ascorbate oxidized mg−1 (protein) min−1.

**Glutathione reductase (GR), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) activity assay**

The glutathione reductase (GR; EC 1.6.4.2), dehydroascorbate reductase (EC 1.8.5.1), and monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) activity were assayed described by Sánchez-Rodríguez et al. (2010) from the fresh leaf (200 mg) samples. The leaf samples were homogenized in 5 ml of 100 mM Tris–HCl buffer (pH 7.8), 25 mM sodium phosphate buffer (pH 7.0), and 100 mM HEPES–HCl buffer (pH 7.6), respectively, for GR, DHAR, and MDHAR at 4 °C in a pre-chilled mortar and pestle. The enzymatic extract was centrifuged at 22,000×g for 20 min on 4 °C and the supernatant was collected subsequently. In the presence of O−2, NBT gets oxidized to form a dark blue insoluble precipitate. Absorbance was recorded at 540 nm and O−2 generation was expressed as ΔA540 min−1 mg−1 protein.

**Ascorbate peroxidase (APX) activity assay**

According to the procedure of Nakano and Asada (1981) ascorbate peroxidase (APX; EC 1.11.1.11), activity was estimated from 200 mg of fresh leaf sample homogenized in 5 ml of 50 mM potassium phosphate buffer (pH 7.8) having 1 mM EDTA, 1 mM ascorbic acid, 1% PVP; and 1 mM phenylmethylsulfonyl fluoride in a pre-chilled mortar and pestle at 4 °C. The extract was centrifuged at 22,000×g for 15 min at 4 °C. The resultant supernatant was used for the enzymatic assay. A 3 ml assay reaction mixture prepared, comprising 50 mM potassium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM AsA, 200 µl enzymatic extract and at last 0.2 mM H2O2 was mixed at last at 25 °C. The absorbance of the reaction mixture was monitored at 290 nm for 5 min with an extinction coefficient of 2.8 mM−1 cm−1. The oxidation rate of ascorbate was measured and the specific activity of ascorbate peroxidase was expressed as µmol ascorbate oxidized mg−1 (protein) min−1.

**Catalase (CAT) activity assay**

As per the method suggested by Rai et al. (2012a), catalase (CAT, EC 1.11.1.6) activity was determined. Approximately 200 mg of fresh leaf tissue was homogenized in 50 mM Tris–NaOH buffer (pH 8.0) containing 0.5 mM EDTA, 0.5% (v/v) Triton X-100, and 2% (w/v) polyvinyl pyrrolidone (PVP), using pre-chilled mortar and pestle. Further, the extract was centrifuged at 20,000×g for 15 min at 4 °C; the dialyzed supernatant was utilized for the assay. A 3 ml assay reaction mixture prepared consisting of 50 mM H2O2, 200 µl enzyme, and 100 mM KH2PO4 buffer (pH 7.0). Utilizing 0.036 mM−1 cm−1 of extinction coefficient and the consecutive decline in absorbance for 5 min due to H2O2 decomposition was recorded at 240 nm. Enzyme specific activity was expressed in µmol of H2O2 oxidized mg−1 (protein) min−1.
DHA at ambient temperature. The increase in absorbance due to ascorbate was monitored at 265 nm for 3 min with an extinction coefficient of 14 mM⁻¹ cm⁻¹ and as µmol of NADPH oxidized mg⁻¹ (protein) min⁻¹, the enzyme-specific activity was expressed. For non-enzymatic reduction in DHA by GSH, the reaction rate was corrected. The MDHAR activity measured by a reaction mixture of 2 ml was prepared from 200 µl of enzymatic supernatant with 0.15 mM NADP, 2.5 mM AsA and 100 mM HEPES–HCl buffer (pH 7.6). The NADPH oxidation rate detected as an increase in absorbance up to 3 min at 340 nm (extinction coefficient of 6.2 mM⁻¹ cm⁻¹) and was expressed as µmol of ascorbate oxidized mg⁻¹ (protein) min⁻¹.

**Superoxide dismutase (SOD) activity assay**

According to the procedure as suggested by Nahakpam and Shah (2011) superoxide dismutase (SOD, EC 1.15.1.1), activity was determined from 200 mg of fresh leaf tissue samples crushed in a pre-chilled mortar and pestle with 5 ml potassium phosphate buffer (100 mM; pH 7.8), containing EDTA (0.1 mM), Triton X-100 (0.1% v/v), and polyvinyl pyrrolidone (PVP) (2% w/v). The enzymatic extract was then centrifuged in 4 °C for 15 min at 22,000 × g; and the resultant supernatant was dialyzed with the help of cellophane membrane tube against cold extraction buffer, sodium carbonate–bicarbonate buffer (50 mM; pH 9.8) for 4 h. For the enzyme assay, a 3 ml reaction mixture was prepared using 100 µl of enzyme extract and EDTA (0.1 mM), to which epinephrine was added finally. The formation of adrenochrome during next 5 min was monitored at 470 nm. The amount of enzyme required to stimulate 50 percent epinephrine oxidation under experimental conditions is described as one unit of SOD activity.

**Guaiacol peroxidase (POD) activity assay**

Guaiacol peroxidase (EC 1.11.1.7) activity was estimated according to the method suggested by Shah et al. (2001). The fresh leaf sample (200 mg) was crushed with 5 ml sodium phosphate buffer (60 mM; pH 7.0) at 4 °C using a pre-chilled mortar and pestle. The supernatant was collected after centrifuging the homogenates at 22,000×g for 15 min, and used for enzymatic preparation. A reaction mixture of 2 ml was set up consisting of 40 mM sodium phosphate buffer (pH 6.0), 9 mM guaiacol, 2 mM H₂O₂, and 50 µl enzyme extract. The absorbance of the reaction mixture was recorded at 470 nm with 26.6 mM⁻¹ cm⁻¹ extinction coefficient for 5 min and expressed as µmol of H₂O₂ reduced mg⁻¹ (protein) min⁻¹.

**Glutathione assay**

Owens and Belcher (1965) described procedure was used for glutathione assay; in which a fresh leaf (200 mg) sample was powdered in mortar and pestle with liquid nitrogen and mixed with 5 ml of 5% (w/v) meta-phosphoric acid at 4 °C. The extract was centrifuged at 22,000×g for 20 min at 4 °C and the supernatant collected for assaying both ascorbate and glutathione. Total glutathione and reduced glutathione (GSH) were assayed following the procedure. The reduced glutathione was oxidized by 5,5-dithio-bisnitrobenzoic acid (DTNB), and the reaction between GR and NADPH, the oxidized glutathione (GSSG) was reduced to GSH. A reaction mixture of 2 ml was prepared using 100 mM potassium phosphate buffer (pH 8.0), consisting of 50 µl of the extract, 30 µl of 4% (w/v) DTNB, and 1 mM EDTA. The reaction mixtures were incubated at room temperature for 4 min then the absorbance was recorded at 412 nm. Total glutathione was also assayed by adding 0.25 mM NADPH and 0.5 units of glutathione reductase to the same reaction mixture. The concentration of GSH and total glutathione was measured with a standard reference curve of 1–5 µg.

**Ascorbate (AsA) assay**

Ascorbate (AsA) DHA, total AsA, and reduced AsA were estimated according to Law et al. (1983) procedure. Total AsA was estimated using Dithiothreitol (DTT) by reducing DHA to AsA. From fresh leaf tissue (200 mg), the sample was crushed in 5% of metaphosphoric acid consisting of the total of 10 mM DTT and 500 µl of 150 mM sodium phosphate buffer (pH 7.5). The reaction mixture was incubated for 10 min at room temperature in dark, after incubation, 100 µl of 0.5% (w/v) N-ethylmaleimide was added along with 200 µl FeCl₃ (3% w/v), 400 µl 2, 20-bipyridyl (4% w/v, in 70% v/v ethanol) and 400 µl orthophosphoric acid (44% v/v) followed by incubation for 30 min at 40 °C in dark. The absorbance was recorded at 525 nm, the total AsA content was analyzed using a standard AsA reference curve. With the substitution of 100 µl of DTT with 100 µl of distilled water, the reduced AsA was measured in the similar method as described in the earlier procedure. The DHA was analyzed as a difference between the reduced AsA and total AsA and expressed as mg g⁻¹ FW. [Note: The optical density for all the biochemical parameters were recorded by SmartSpec 3000 UV/Visible Spectrophotometer (Bio Rad, Hercules, California, USA) spectrophotometer.]

**Total fruit number and fruit weight**

The total number of fruit per plant was counted and fruit yield per plant was recorded by weighing total fruit and expressed in gram per plant.
Gene expression analysis

Antioxidative enzymes gene, namely APX, CAT, DHAR, GR, MHDAR, POD, and SOD, were selected and their sequences were retrieved from the NCBI database (www.ncbi.nlm.nih.gov) to study their expression analysis. qRT-PCR primer designing Primer3 v. 0.9 software (Rozen and Skaletsky 1998) was used (Table 1). RNA was isolated from top young leaves utilizing TRI reagent (Ambion, California, USA) according to manufacturer’s protocol. cDNA strand was synthesized using 1 µg RNA in a 20 µl of reaction volume using Bio-Rad cDNA synthesis kit (Bio-Rad, Hercules, California, USA), and for RT-qPCR, IQ SYBR Green Supermix (Bio-Rad, Hercules, California, USA) was utilized, the reaction was set in thermal cycler (iQ5, Bio-Rad, Hercules, California, USA), actin gene was used as an internal control. The sequences of actin and other gene-specific primer are tabulated in Table 1. For PCR reaction, 5 µl of cDNA was taken and PCR was programmed as initial denaturation at 95 °C for 1 min, then at 95 °C for 45 s denaturation, at 56–60 °C for 45 s annealing, 72 °C for 45 s extension, repeated for 36 cycles and a final extension of 72 °C for 5 min. The relative gene expression was calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Statistical analysis

All the analyses were done by SPSS statistical package (SPSS Inc., Version 16.0). Three samples were taken for each replication and the mean value was used for statistical analysis. One-way analysis of variance (ANOVA) was performed to estimate the significant value difference and Duncan’s multiple-range analysis was also computed. The level of significance for post hoc test was set at 5% and $P < 0.05$ significance.

Table 1 List of primers used for PCR and real-time PCR

| Sr. No | Gene Name | Forward Primer (5’–3’) | Reverse Primer (5’–3’) | Tm (°C) |
|--------|-----------|------------------------|------------------------|--------|
| 1      | AtDREB1   | AGGGCGGCTGATGTAATTTCCGAGGC | TCACCGGTTTGAATATGGCCAGGC | 55     |
| 2      | BcZAT12   | TGTCCGTATAGCCGGAGTGGATTT | TTTCTTCAACGTACATCAACCGGC | 56     |
| 3      | APX       | GAGGGTGGACCTAATGGAAGGC | ACATCCATCCTCCATATTTTGTATTT | 52     |
| 4      | CAT       | TTTCCATCATGTCGAGCTTTGCTGTT | TTTGTTGGGCCAGCTTTGCTGTT | 54     |
| 5      | GR        | CGTGTCTTGATACCTTGGTTGAG | GCTGGATAAGGATGATGCTATGTCG | 53     |
| 6      | SOD       | CGGA TGGGGCCTGCGGTTACTG | AGCATGCGCTCCAA AGCGTCGA | 55     |
| 7      | DHAR      | CCAGAATACCAAGGGTGAAGGAAA | TGCTAATCCCGGAGCAGCAACAA | 54     |
| 8      | MDHAR     | GTGTTTCTGCTGTCGATGTCGATG | GGTATGCTTCTGTAATGTCG | 51     |
| 9      | POD       | GAATTGTGGCCACTTGGTAGAAGA | GAGCAACGAGAAACTCTGACAA | 52     |
| 10     | Actin     | TGGTCGGATATGCGGAGAAGAAGC | CTCAGTCAGGAGAAACAGGGT | 51     |

Results

Chlorophyll and carotenoid

Total chlorophyll content was measured under control and 7, 14, and 21 DWD, and it was observed that the total chlorophyll was maximum under control condition in non-transgenic (NT), transgenic (ST), and double transgenic (DT), however, it got reduced after 7, 14 and 21 DWD (Table 1). Total chlorophyll under control condition recorded highest in DZ4 and DZ5 with value 3.90 mg g⁻¹ FW for each, while it was lowest in H86 (3.11 mg g⁻¹ FW). After 7 DWD, the total chlorophyll content was higher in DZ5 (3.57 mg g⁻¹ FW), while it was lowest in H86 (2.06 mg g⁻¹ FW). Similarly, a continuous reduction in total chlorophyll content recorded after 21 DWD with a lowest value in H86 (0.823 mg g⁻¹ FW) and highest in DZ5 (2.59 mg g⁻¹ FW) (Table 1). Chlorophyll a-and-chlorophyll b ratio does not showed any fixed trend, under control condition the chl a/chl b ratio was highest in DZ4 and DZ5 (2.28 mg g⁻¹ FW each) whereas it was lowest in H86 (1.84 mg g⁻¹ FW). After 7 DWD, it was the highest in DZ5 (2.56 mg g⁻¹ FW) while lowest in H86 (1.84 mg g⁻¹ FW). After 14 and 21 DWD, it was maximum in DZ5 (2.06 mg g⁻¹ FW) and ZT1 (2.20 mg g⁻¹ FW), respectively. However, it was minimum in H86 (1.41 and 1.21 mg g⁻¹ FW), respectively (Table 2).

In the same way, carotenoid content also measured under control, 7, 14, and 21 DWD, and it gets reduces with increasing DWD (Table 2). It was significantly higher in DZ4 (0.678 mg g⁻¹ FW) under control condition while lowest in H86 (0.558 mg g⁻¹ FW) After 7 DWD, DZ4 showed a significantly increased value of 0.643 mg g⁻¹ FW while lowest in H86 (1.84 mg g⁻¹ FW). After 14 and 21 DWD, maximum carotenoid content was noted in DZ3 (0.595 mg/g⁻¹ FW) and DZ1 (0.517 mg/g⁻¹ FW), while it was minimum in H86 (0.425 and 0.247 mg g⁻¹ FW). Chlorophyll/
carotenoid ratio was highest in DZ2 (6.49 mg g\(^{-1}\) FW), DZ2 (6.27 mg g\(^{-1}\) FW), ZT1 (6.08 mg g\(^{-1}\) FW) and DZ2 (5.34 mg g\(^{-1}\) FW), under control, 7, 14, and 21 DWD, respectively. While it was lowest in H86, with respective values 5.64, 5.02, 4.12, and 3.31 mg g\(^{-1}\) FW, under control, 7, 14 and 21 DWD (Table 3).

### Superoxide radical (O\(^{-2}\))

Superoxide anion increases with increase in DWD in NT, ST and DT plants, after 7 DWD, the higher fold change was noted in H86 while it was lowest in DZ5. While after 14 and 21 DWD, maximum increase was recorded in H86, while it was minimum in the case of DZ2 (Table 4).

### Ascorbate peroxidase and catalase activity

APX increases up to 14 DWD in NT, ST, and DT plants, however, after 21 DWD, it reduces in H86 and D86 but a continuous increase was noted in ZT1, DZ1, DZ2, DZ3, DZ4 and DZ5. After 7 DWD highest, 2.23-fold increase compared to control was noted in DZ5, while it was least in case of H86 (1.23-folds). Highest 3.02-fold increase compared to control was recorded in DZ5 after 14 DWD, while it was lowest in H86 (2.08-fold). After a maximum 21 DWD, a higher increase compared to control was noted in DZ5 (3.93-fold), whereas it was lowest in H86 (1.91-fold) (Fig. 1a).

Under control condition, highest catalase activity was recorded in DZ5, while it was lowest in H86. After 7 DWD, increase in catalase activity was recorded in NT, T, and DT lines with a maximum increase of 2.01-fold in ZT1, while it was lowest in the case of H86 (1.49-fold). Similarly, after 14 and 21 DWD increase recorded, it was maximum for DZ1, whereas it was minimum for H86 (Fig. 1b).

### Glutathione reductase and super oxide dismutase activity

GR specific activity noted maximum in ZT1 under controlled condition, it increases compared to control in NT, ST, and DT plants after 7 DWD with highest fold increase compared to control was noted in DZ5, while it was least in case of H86 (1.23-folds). The results are mean±SE of triplicate measurements. Means followed by the same letter along same row are not significantly different (\(P \leq 0.05\), according to Duncan’s multiple-range test.

### Table 2 Total chlorophyll and chlorophyll a/b ratio in different tomato genotypes

| Events | Total Chlorophyll (mg/g\(^{-1}\) FW) | Chlorophyll A/B (mg/g\(^{-1}\) FW) |
|--------|------------------------------------|-----------------------------------|
|        | 0 Days | 07 Days | 14 Days | 21 Days | 0 Days | 07 Days | 14 Days | 21 Days |
| H86    | 3.109±0.017*b | 2.058±0.135*b | 1.732±0.118*a | 0.823±0.034*b | 1.840±0.077*c | 1.603±0.115*c | 1.417±0.092*c | 1.212±0.087*c |
| D86    | 3.847±0.023*a | 3.441±0.139*b | 3.200±0.135*b | 2.535±0.125*a | 2.044±0.134*b | 2.066±0.093*b | 1.770±0.112*b | 1.890±0.138*b |
| ZT1    | 3.807±0.019*a | 3.412±0.221*a | 3.282±0.192*a | 2.487±0.129*a | 1.963±0.119*a | 2.010±0.129*a | 1.806±0.135*c | 2.204±0.119*a |
| DZ1    | 3.851±0.028*a | 3.432±0.176*a | 3.247±0.138*a | 2.556±0.118*a | 2.240±0.172*a | 2.024±0.157*b | 1.818±0.145*b | 1.940±0.158*b |
| DZ2    | 3.811±0.015*a | 3.372±0.234*a | 3.169±0.223*a | 2.489±0.139*a | 2.176±0.138*a | 2.055±0.089*b | 1.649±0.093*c | 1.809±0.137*b |
| DZ3    | 3.852±0.023*a | 3.473±0.198*a | 3.255±0.167*a | 2.521±0.145*a | 2.118±0.145*a | 2.342±0.129*a | 1.898±0.137*a | 1.862±0.155*b |
| DZ4    | 3.905±0.013*a | 3.491±0.251*a | 3.268±0.159*a | 2.518±0.113*a | 2.282±0.129*a | 2.353±0.114*a | 1.900±0.118*a | 1.810±0.129*b |
| DZ5    | 3.904±0.019*a | 3.573±0.177*a | 3.345±0.128*a | 2.596±0.110*a | 2.770±0.112*a | 2.569±0.129*a | 2.059±0.074*a | 2.156±0.091*a |

The results are mean±SE of triplicate measurements. Means followed by the same letter along same row are not significantly different (\(P \leq 0.05\), according to Duncan’s multiple-range test.

### Table 3 Carotenoids and chlorophyll/carotenoids ratio in different tomato genotypes

| Events | Carotenoids (mg/g\(^{-1}\) FW) | Chlorophyll/Carotenoids (mg/g\(^{-1}\) FW) |
|--------|--------------------------------|----------------------------------|
|        | 0 Days | 07 Days | 14 Days | 21 Days | 0 Days | 07 Days | 14 Days | 21 Days |
| H86    | 0.558±0.013*b | 0.410±0.031*d | 0.426±0.031*c | 0.248±0.011*c | 5.644±0.331b | 5.022±0.343d | 4.124±0.175d | 3.307±0.178d |
| D86    | 0.620±0.019*a | 0.586±0.029*b | 0.559±0.045*a | 0.508±0.035*a | 6.194±0.319*a | 6.026±0.291a | 5.802±0.331c | 4.946±0.295c |
| ZT1    | 0.613±0.011*a | 0.584±0.037*b | 0.539±0.029*b | 0.483±0.031*b | 6.192±0.452*b | 5.875±0.373b | 6.082±0.465*b | 5.298±0.382b |
| DZ1    | 0.653±0.024*a | 0.615±0.051*c | 0.587±0.047*a | 0.517±0.035*a | 5.889±0.371*b | 5.604±0.299*b | 5.833±0.378*b | 5.002±0.245b |
| DZ2    | 0.595±0.029*b | 0.564±0.031*c | 0.527±0.033*b | 0.472±0.041*b | 6.499±0.451*b | 6.271±0.475*b | 6.015±0.313*a | 5.341±0.177a |
| DZ3    | 0.658±0.024*a | 0.634±0.045*a | 0.595±0.051*a | 0.500±0.037*a | 5.836±0.393*b | 5.482±0.391*c | 5.518±0.475*b | 5.278±0.378*b |
| DZ4    | 0.678±0.037*a | 0.643±0.051*a | 0.587±0.019*a | 0.490±0.019*a | 5.765±0.413*b | 5.436±0.225*b | 5.692±0.199*c | 5.189±0.292*b |
| DZ5    | 0.670±0.032*a | 0.640±0.033*d | 0.572±0.027*a | 0.506±0.017*a | 5.789±0.470*b | 5.608±0.313*b | 5.890±0.377*b | 5.236±0.197*a |

The results are mean±SE of triplicate measurements. Means followed by the same letter along same row are not significantly different (\(P \leq 0.05\), according to Duncan’s multiple-range test.
in DZ1 (1.93), and lowest fold change in H86 (1.50). After 14 and 21 DWD, highest increase was noted in DZ2 with respective fold increase of 3.43 and 4.36, while it was lowest in H86 with respective fold change of 1.76 and 1.0 (Fig. 1c).

The activity of SOD enhanced with each water-deficit treatment viz; 7, 14 and 21 DWD in each except in H86 and D86

### Table 4: Super oxide radical in different tomato genotypes

| Events   | Super oxide radical ($\Delta_{540}$ min$^{-1}$ mg$^{-1}$ protein) |
|----------|---------------------------------------------------------------|
| 0 Days   | 07 Days | 14 Days | 21 Days |
| H86      | 0.490 ± 0.031$^a$ | 0.822 ± 0.043$^a$ | 1.434 ± 0.118$^a$ | 2.285 ± 0.135$^a$ |
| D86      | 0.239 ± 0.011$^b$ | 0.319 ± 0.022$^b$ | 0.432 ± 0.023$^b$ | 0.939 ± 0.071$^b$ |
| ZT1      | 0.245 ± 0.012$^b$ | 0.264 ± 0.031$^c$ | 0.358 ± 0.033$^c$ | 0.822 ± 0.061$^{bc}$ |
| DZ1      | 0.201 ± 0.005$^c$ | 0.226 ± 0.014$^d$ | 0.295 ± 0.008$^d$ | 0.728 ± 0.061$^c$ |
| DZ2      | 0.226 ± 0.021$^b$ | 0.239 ± 0.024$^c$ | 0.314 ± 0.023$^{cd}$ | 0.753 ± 0.041$^c$ |
| DZ3      | 0.220 ± 0.008$^{bc}$ | 0.245 ± 0.003$^c$ | 0.339 ± 0.011$^c$ | 0.734 ± 0.083$^c$ |
| DZ4      | 0.220 ± 0.012$^{bc}$ | 0.276 ± 0.016$^c$ | 0.358 ± 0.022$^c$ | 0.804 ± 0.063$^{bc}$ |
| DZ5      | 0.195 ± 0.007$^c$ | 0.213 ± 0.009$^d$ | 0.314 ± 0.012$^{cd}$ | 0.716 ± 0.053$^c$ |

The results are mean ± SE of triplicate measurements. Means followed by the same letter along same row are not significantly different ($P \leq 0.05$), according to Duncan’s multiple-range test.

Fig. 1 a-d Effect of increasing water deficit on activities of key antioxidants in tomato leaves. a – ascorbate peroxidase (APX); (b) – catalase (CAT); (c) glutathione reductase (GR); (d) – superoxide dismutase (SOD). The data are mean of three replicates ± SE. Those followed by different letters within similar water-stress treatment are significantly different according to Duncan’s multiple-range test at $P > 0.05$. Bar indication from left to right for each individual treatments: (1) H86 (non-transgenic), (2) D86 (AtDREB1A), (3) ZT1 (BcZAT12), (4) DZ1 (AtDREB1A X BcZAT12-1), (5) DZ2 (AtDREB1A X BcZAT12-2), (6) DZ3 (AtDREB1A X BcZAT12-3), (7) DZ4 (AtDREB1A X BcZAT12-4), (8) DZ5 (AtDREB1A X BcZAT12-5)
in which after 21 DWD, it gets reduced. Highest SOD activity after 7 DWD was noted in DZ5 while it was lowest in H86. After 14 DWD, significant increase in SOD compared to control was noted in ZT1 (3.02-fold), while it was significantly lower in H86 (2.05-fold). Similarly, after 21 DWD, highest increase in SOD activity compared to control was noted in ZT1 (3.22-fold); however, it was decreased in H86 (0.89-fold) (Fig. 1d).

**Dehydroascorbate and monodehydroascorbate reductase activity**

Dehydroascorbate reductase activity increases up to 14 DWD in NT, ST, and DT, however, it gets reduced after 21 DWD. The highest value of 1.36-fold increase compared to control noted in ZT1 after 7 DWD, however under the similar condition, it was lowest in H86 (1.01-fold). Similarly, after 14 DWD, it was maximum in ZT1 with a 2.18-fold increase compared to control, while it was minimum in H86 (1.56-fold) compared to control. A reduction in DHAR noted after 21 DWD with significantly highest value in ZT1 (1.39-fold compared to control), while it was minimum in H86 (0.64-fold compared to control) (Fig. 2a). Continuous enhancement in MDHAR was recorded with rise in DWD, with maximum value after 21 DWD. Under control and 7 DWD condition, minimum values 3.06 and 4.20 µmol ascorbate oxidized min\(^{-1}\) mg\(^{-1}\) protein and maximum values, 6.40 and 11.06 µmol ascorbate oxidized min\(^{-1}\) mg\(^{-1}\) protein were noted, respectively, for H86 and DZ5. Similarly, after 14 DWD, maximum increase in MDHAR compared to control was recorded in DZ2 (3.12-fold) whereas minimum in H86 (2.08-fold). However, MDHAR was recorded maximum in DZ5 and while lowest in H86 at 21 DWD (Fig. 2b).

![Fig. 2 a-c Effect of increasing water deficit on activities of key antioxidants in tomato leaves.](image-url)

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![Fig. 2 a-c Effect of increasing water deficit on activities of key antioxidants in tomato leaves.](image-url)
Guaicol peroxidase activity

Guaicol peroxidase activity was highest in DZ5 and minimum in H86 under controlled conditions. It gets reduced in H86 and D86, while increases in ZT1, DZ1, DZ2, DZ3, DZ4 and DZ5 after 7 DWD. Again it increases in NT, ST, and DT after 14 DWD, the increase compared to control was highest in DZ3 (2.07-fold) whereas lowest for H86 (1.41-fold). After 21 DWD, it reduces in all, the maximum reduction compared to control was recorded in H86 (1.16) and minimum in DZ2 (1.98) (Fig. 2c).

Ascorbate

Under control condition, the value of dehydroascorbate was highest in DZ2 (0.693 mg g⁻¹ DW) while it was lowest in H86 (0.277 mg g⁻¹ DW). After 7 DWD, the dehydroascorbate increases however again decreases after 14 DWD and the values were lower than the values under 7 DWD, however, still, it was higher than the value under control conditions. At the end of maximum 21 DWD, the value in fold change was highest in DZ4 (3.40) and lowest in H86 (1.20) as compared to control (Table 5). Reduced ascorbate after 7 DWD increases maximum in case of DZ5 (1.05–2.97 mg g⁻¹ DW), while it increases minimum in case of H86 (0.577–0.959 mg g⁻¹ DW) (Table 5). However, after 14 DWD, reduced ascorbate was highest 2.57 mg g⁻¹ DW in DZ5. Although, after 21 DWD, the reduced ascorbate recorded increase order D86 > DZ2 > DZ5 > DZ3 > DZ1 > H86 > DZ4 > ZT1 (Table 5). Compared to control, total ascorbate increases highest by 3.47-fold in DZ2 after 7 DWD. However, it gets reduces after 14 DWD, and the highest fold value as compared to control was 3.56 (Table 6). After maximum DWD of 21 days, results in increase of total ascorbate compared to control, with a maximum increase of 4.29-fold (DZ4) and a minimum of 2.19-fold (H86).

Glutathione

Under control condition, highest glutathione recorded in DZ5 (0.666 µg g⁻¹ FW) however, it was lowest in case of H86 (0.312 µg g⁻¹ FW). After 7 DWD, it increases maximum by 1.47-fold in DZ3 compared to value under control condition, similarly after 14 DWD, maximum

**Table 5** Dehydroascorbate and reduced ascorbate in different tomato genotypes

| Events | Dehydroascorbate (mg g⁻¹ DW) | Reduced Ascorbate (mg g⁻¹ DW) |
|--------|-----------------------------|-----------------------------|
|        | 0 Days | 07 Days | 14 Days | 21 Days | 0 Days | 07 Days | 14 Days | 21 Days |
| H86    | 0.591 ± 0.023b | 1.570 ± 0.121c | 0.880 ± 0.037c | 1.720 ± 0.129bc | 0.802 ± 0.033c | 2.021 ± 0.123c | 1.730 ± 0.121d | 2.290 ± 0.178ab |
| D86    | 0.495 ± 0.017d | 1.880 ± 0.119ab | 0.730 ± 0.031d | 2.230 ± 0.154a | 1.120 ± 0.097a | 2.090 ± 0.145c | 1.720 ± 0.158d | 1.590 ± 0.123d |
| ZT1    | 0.547 ± 0.033c | 1.970 ± 0.125a | 0.956 ± 0.041b | 2.130 ± 0.137a | 0.927 ± 0.052b | 2.656 ± 0.151ab | 2.420 ± 0.188a | 1.880 ± 0.119b |
| DZ1    | 0.694 ± 0.053a | 1.780 ± 0.138ab | 1.120 ± 0.038ab | 1.560 ± 0.119bc | 0.896 ± 0.038bc | 2.240 ± 0.171b | 1.890 ± 0.111c | 2.230 ± 0.176ab |
| DZ2    | 0.609 ± 0.055b | 1.630 ± 0.132c | 1.020 ± 0.07b  | 1.880 ± 0.125b | 0.969 ± 0.067b  | 2.521 ± 0.118ab | 2.270 ± 0.145b | 2.190 ± 0.166b |
| DZ3    | 0.651 ± 0.043ab| 1.980 ± 0.131a | 0.873 ± 0.091c | 2.220 ± 0.152a | 0.958 ± 0.056b  | 2.448 ± 0.121ab | 2.090 ± 0.177bc | 1.810 ± 0.135c |
| DZ4    | 0.684 ± 0.033a | 1.740 ± 0.112b | 1.240 ± 0.081a | 2.290 ± 0.138a | 1.052 ± 0.099  | 2.969 ± 0.143a  | 2.570 ± 0.192a | 2.430 ± 0.155a |
| DZ5    | 0.591 ± 0.027b | 1.570 ± 0.119c | 0.880 ± 0.028c | 1.720 ± 0.183bc | 0.802 ± 0.05a   | 2.021 ± 0.151c  | 1.730 ± 0.115c | 2.290 ± 0.187ab |

The results are mean ± SE of triplicate measurements. Means followed by the same letter along same row are not significantly different (P ≤ 0.05), according to Duncan’s multiple-range test

**Table 6** Total ascorbate in different tomato genotypes

| Events | Total Ascorbate (mg g⁻¹ DW) |
|--------|-----------------------------|
|        | 0 Days | 07 Days | 14 Days | 21 Days |
| H86    | 1.880 ± 0.112b | 3.930 ± 0.223c | 3.550 ± 0.229c | 4.120 ± 0.299d |
| D86    | 1.854 ± 0.131b | 5.400 ± 0.332bc | 5.190 ± 0.37b | 6.220 ± 0.432c |
| ZT1    | 1.833 ± 0.119b | 5.790 ± 0.392b | 5.220 ± 0.323b | 7.380 ± 0.571b |
| DZ1    | 2.000 ± 0.145a | 6.380 ± 0.161ab | 5.460 ± 0.273b | 7.390 ± 0.387b |
| DZ2    | 2.115 ± 0.121a | 7.340 ± 0.532c | 6.880 ± 0.412a | 7.890 ± 0.541ab |
| DZ3    | 2.146 ± 0.133a | 5.960 ± 0.232b | 6.430 ± 0.297a | 7.980 ± 0.612a |
| DZ4    | 1.938 ± 0.127ab | 6.900 ± 0.321ab | 6.730 ± 0.354a | 8.330 ± 0.622a |
| DZ5    | 2.240 ± 0.167a | 7.320 ± 0.312a | 6.810 ± 0.335a | 8.770 ± 0.572a |

The results are mean ± SE of triplicate measurements. Means followed by the same letter along same row are not significantly different (P ≤ 0.05), according to Duncan’s multiple-range test
2.02-fold increase compared to control was recorded in DZ2 and after 21 DWD, highest 1.49-fold increase compared to control was recorded for ZT1 (Table 7). The total GSH increases with an increase in DWD in NT, ST and DT, except in H86 after 21 DWD which reduces slightly compared to the value after 14 DWD. As in glutathione, the highest GSG was recorded in DZ5 under control, 7, 14, and 21 DWD condition with respective values of 0.857 µg g⁻¹ FW, 1.52 µg g⁻¹ FW, 2.05 µg g⁻¹ FW, and 2.57 µg g⁻¹ FW, however, it was lowest in H86 under the similar condition with respective values 0.40 µg g⁻¹ FW, 0.542 µg g⁻¹ FW, 0.884 µg g⁻¹ FW and 0.855 µg g⁻¹ FW (Table 7). Total glutathione increases after 7, 14 and 21 DWD in NT, ST and DT compared to control, maximum total glutathione recorded in DZ5 under control, and under all the drought treatments viz; 7, 14 and 21 DWD, with respective values 1.60 µg g⁻¹ FW, 2.62 µg g⁻¹ FW, 4.07 µg g⁻¹ FW and 5.21 µg g⁻¹ FW, while it was least in H86 under the similar conditions with respective values 0.67 µg g⁻¹ FW, 0.85 µg g⁻¹ FW, 1.11 µg g⁻¹ FW, and 1.49 µg g⁻¹ FW (Table 8).

### Table 7 Oxidized glutathione and reduced glutathione in different tomato genotypes

| Events | Oxidized Glutathione (GSSG) (µg g⁻¹ FW) | Reduced Glutathione (GSH) (µg g⁻¹ FW) |
|--------|---------------------------------------|--------------------------------------|
|        | 0 Days                  | 07 Days                | 14 Days               | 21 Days               | 0 Days                  | 07 Days                | 14 Days               | 21 Days               |
| H86    | 0.312 ± 0.017d          | 0.262 ± 0.015c         | 0.231 ± 0.014c        | 0.347 ± 0.015d        | 0.401 ± 0.025d          | 0.542 ± 0.023c         | 0.884 ± 0.035c         | 0.855 ± 0.033d         |
| D86    | 0.578 ± 0.023b          | 0.427 ± 0.025d         | 0.875 ± 0.064c        | 0.620 ± 0.036c        | 0.762 ± 0.047bc         | 1.010 ± 0.083d         | 1.682 ± 0.094c         | 2.160 ± 0.145c         |
| ZT1    | 0.409 ± 0.012c          | 0.518 ± 0.033c         | 0.702 ± 0.029d        | 0.613 ± 0.041c        | 0.603 ± 0.035c          | 0.868 ± 0.041c         | 1.415 ± 0.088c         | 1.993 ± 0.165d         |
| DZ1    | 0.566 ± 0.023b          | 0.712 ± 0.053b         | 1.115 ± 0.089p        | 0.787 ± 0.051b        | 0.848 ± 0.043c          | 1.343 ± 0.071c         | 1.932 ± 0.135ab        | 2.407 ± 0.171ab        |
| DZ2    | 0.529 ± 0.033bc         | 0.715 ± 0.032b         | 1.072 ± 0.078ab       | 0.740 ± 0.029bc       | 0.783 ± 0.037b          | 1.302 ± 0.081c         | 1.840 ± 0.154ab        | 2.307 ± 0.125b         |
| DZ3    | 0.596 ± 0.041b          | 0.881 ± 0.063a         | 1.158 ± 0.098a        | 0.807 ± 0.057a        | 0.823 ± 0.031a          | 1.301 ± 0.071c         | 1.903 ± 0.117ab        | 2.368 ± 0.144          |
| DZ4    | 0.572 ± 0.029b          | 0.752 ± 0.055b         | 1.128 ± 0.0894a       | 0.803 ± 0.033a        | 0.741 ± 0.042bc         | 1.452 ± 0.091b         | 2.015 ± 0.135ab        | 2.740 ± 0.153a         |
| DZ5    | 0.666 ± 0.045a          | 0.923 ± 0.063a         | 1.182 ± 0.112a        | 0.843 ± 0.057a        | 0.858 ± 0.047a          | 1.520 ± 0.081a         | 2.025 ± 0.118a         | 2.573 ± 0.147a         |

The results are mean ± SE of triplicate measurements. Means followed by the same letter along same row are not significantly different (P ≤ 0.05), according to Duncan’s multiple-range test.

### Number of fruit number and fruit weight

With an increase in DWD, total fruit number and fruit weight decrease in NT, ST, and DT plants. The highest fruit number recorded in DZ5 under control, 7, 14 and 21 DWD, while it was lowest in H86 under similar conditions. Similarly, total fruit weight was maximum in DZ5 under control, 7, 14, and 21 DWD, while it was minimum in H86 under similar conditions (Table 9).

### Relative gene expression

Relative expression of seven different antioxidant enzyme genes was measured under control, 7, 14 and 21 DWD through RT-PCR. Respective increase in APX gene expression was measured after 7, 14, and 21 DWD, and it was observed that a continuous increase takes place in NT, ST, and DT plants; however, it was higher in DT lines. After 7 DWD, it was maximum for DZ5 with 1.98-fold increase compared to control plants (Fig. 3a). Further, CAT expression was measured under the similar condition and it was observed that the CAT expression increases...
Table 9 Number of fruits and total fruit weight in wild, transgenic and double transgenic under 0, 07, 14 and 21 days of water deficit

| Events | Total fruit number | Total fruit weight |
|--------|-------------------|--------------------|
|        | 0 Days | 07 Days | 14 Days | 21 Days | 0 Days | 07 Days | 14 Days | 21 Days |
| H86    | 14.22 ± 0.65ab  | 12.25 ± 0.44b    | 7.22 ± 0.44c  | 5.21 ± 0.22c | 1142 ± 89d | 1004 ± 39d | 0552 ± 19d | 361 ± 12c |
| D86    | 15.12 ± 0.79a   | 13.78 ± 0.53ab   | 8.62 ± 0.59ab | 6.33 ± 0.45b | 1433 ± 45c | 1275 ± 55c | 0902 ± 56d | 623 ± 15d |
| ZT1    | 16.88 ± 0.46a   | 15.44 ± 0.78a    | 9.03 ± 0.63a  | 6.53 ± 0.51b | 1477 ± 33c | 1334 ± 47b | 1029 ± 37c | 655 ± 23c |
| DZ1    | 15.33 ± 0.39a   | 14.98 ± 0.88a    | 9.22 ± 0.44a  | 6.39 ± 0.39b | 1488 ± 55b | 1339 ± 68b | 1093 ± 77b | 677 ± 37b |
| DZ2    | 15.85 ± 0.77a   | 14.33 ± 0.91a    | 8.79 ± 0.22ab | 6.78 ± 0.22ab | 1502 ± 69c | 1348 ± 79b | 1104 ± 52ab | 723 ± 33ab |
| DZ3    | 15.35 ± 1.02a   | 14.67 ± 1.11a    | 8.63 ± 0.59ab | 6.93 ± 0.55ab | 1493 ± 63b | 1333 ± 53b | 1081 ± 26b | 678 ± 45b |
| DZ4    | 16.67 ± 0.66a   | 14.33 ± 0.73a    | 8.55 ± 0.41ab | 7.43 ± 0.51a | 1559 ± 71b | 1361 ± 38b | 1072 ± 51b | 653 ± 11c |
| DZ5    | 17.88 ± 0.97a   | 15.89 ± 1.05a    | 9.88 ± 0.53a  | 7.78 ± 0.44a | 1622 ± 33a | 1529 ± 55a | 1133 ± 22a | 764 ± 27a |

The results are mean ± SE of triplicate measurements. Means followed by the same letter along same row are not significantly different (P ≤ 0.05), according to Duncan’s multiple-range test.

Fig. 3 a–d Effect of increasing water deficit on expression of key anti-oxidative enzyme gene in tomato leaves. (a) ascorbate peroxidase (APX); (b) catalase (CAT); (c) glutathione reductase (GR); (d) superoxide dismutase (SOD). The data are mean of three replicates ± SE. Those followed by different letters within similar water-stress treatment are significantly different according to Duncan’s multiple-range test at P > 0.05. Bar indication from left to right for each individual treatments: (1) H86 (non-transgenic), (2) D86 (AtDREB1A), (3) ZT1 (BcZAT12), (4) DZ1 (AtDREB1A X BcZAT12-1), (5) DZ2 (AtDREB1A X BcZAT12-2), (6) DZ3 (AtDREB1A X BcZAT12-3), (7) DZ4 (AtDREB1A X BcZAT12-4), (8) DZ5 (AtDREB1A X BcZAT12-5).
maximum by 1.68, 1.92 and 2.38 as compared to control under 7, 14, and 21 DWD, respectively, for DZ5, DZ2, and DZ5, however, it was minimum for H86 under similar condition (Fig. 3 b). GR expression increases maximum by 2.22-fold in DZ1 under 7 DWD than control plants. However, under 14 DWD, it followed the trend DZ1 > D Z5 > DZ3 > ZT1 > DZ2 > D86 > DZ4. GR expression was highest after 21 DWD as compared control plants, with a maximum fold increase of 3.45 fold in DZ5 (Fig. 3 b). The expression of SOD increases under 7, 14 and 21 DWD compared to control plants, under 7 DWD, the expression level of SOD rises by 2.12-fold as compared to the respective value under control condition. Similarly, under 14 and 21 DWD, the DZ5 and DZ3 noted maximum with respective fold increases in SOD expression of 4.52 and 4.93 than control plants (Fig. 3 d). The rise in the expression level of DHAR and MDHAR genes was also recorded after 7, 14, and 21 DWD. After 7 DWD, the DHAR expression increases highest by 1.77-fold DZ1 as compared to control plant. Similarly, it was highest 2.12-fold under 14 DWD and 2.89-fold in DZ4 under 21 DWD, for the same plants, compared to control condition (Fig. 4 a). MDHAR gene expression was highest in DT line DZ4 with a respective fold increase of 3.13 compared to control, under 7 DWD. After 14 DWD, it was highest in DZ5 with respective fold increase of 3.47. Similarly, it increases maximum by 3.62-fold for DZ4, and DZ5, as compared to the fold change under water-deficit condition. After 7 DWD, POD expression level was highest in DT line DZ4 followed by DZ5 and DZ2 with respective fold change rise 2.45, 2.43 and 2.33. Similarly, after 14 and 21 DWD, it was noticed maximum

![Fig. 4](https://example.com/fig4.png)

**Fig. 4** a–c Effect of increasing water deficit on expression of key antioxidants in tomato leaves. a dehydroascorbate reductase (DHAR); (b) –monodehydroascorbate reductase (MDHAR); (c) guaiacol peroxidase (POD). The data are mean of three replicates± SE. Those followed by different letters within similar water-stress treatment are significantly different according to Duncan’s multiple-range test at $P>0.05$. Bar indication from left to right for each individual treatments: (1) H86 (non-transgenic), (2) D86 (AtDREB1A), (3) ZT1 (BcZAT12), (4) DZ1 (AtDREB1A X BcZAT12-1), (5) DZ2 (AtDREB1A X BcZAT12-2), (6) DZ3 (AtDREB1A X BcZAT12-3), (7) DZ4 (AtDREB1A X BcZAT12-4), (8) DZ5 (AtDREB1A X BcZAT12-5).
in DZ5 with respective values 2.44 and 3.82, followed by DZ3 with respective fold changes 2.33 and 3.67 as compared to control (Fig. 4c).

**Discussion**

In tomato, development of drought-stress tolerance varieties through breeding is very difficult due to lack of drought-stress tolerance gene/s in tomato gene pool and difficulties in introgression of available gene from its wild relatives (Krishna et al. 2019). To overcome this problem, many transgenic tomatoes have been developed using single transgene which generally act either ABA-dependent or ABA-independent pathway (Rai et al. 2012a, 2013b; Krishna et al. 2019). In present study, to increase drought-stress tolerance in tomato, we developed DT tomato using *AtDREB1A* and *BcZAT12* transgenic tomatoes. *AtDREB1A* genes work via ABA-independent pathway and have tolerance against drought, salt and cold (Rai et al. 2012b; Karkute et al. 2019; Muthurajan et al. 2021). *BcZAT12* works via ABA-dependent pathway and reported for drought-, heat- and salt-stress tolerance (Rai et al. 2012a, b; Shah et al. 2013; Li et al. 2018; Krishna et al. 2021). Thus, we developed double transgenic tomato plant having drought tolerance property for the sustainability of future agriculture. The prolonged exposure to drought stress causes over ROS production, which disrupts the metabolism of plants, prompts oxidative damage to the cellular components (Alexieva et al. 2001; Rai et al. 2012a, 2013a, b), and has significant involvement in decreased yield efficiency under stress conditions. APX, CAT, GR, SOD, DHAR, MDHAR, and POD are the crucial enzymatic antioxidants which perform a critical role in plants protection against severe damages caused due to oxidative stresses. Many reports suggested elevated antioxidant activities, in abiotic stress tolerance genes expressing transgenic plants (Krishna et al. 2019, 2021; Das et al. 2021; Muthurajan et al. 2021). Earlier reports presented the impacts on enzymatic and non-enzymatic antioxidant system under drought stress in *AtDREB1A* and *BcZAT12* transgenic plants (Rai et al. 2012a, 2013b), although, their combined impact was yet to be explored. Therefore, the present experiment was conducted to explore the variation in transgenic tomato plants antioxidant mechanisms harboring *AtDREB1A* and *BcZAT12* transcription factors. Additionally, the gene-stacking strategy paves the way to make transgenic plants capable to grow and survive better under abiotic stress conditions (Kudo et al. 2017), by the following (a) ionic and osmotic maintenance through homeostasis, (b) growth and cell division control, and (c) cellular repair through detoxification (Saijo et al. 2000). The study showed that, *AtDREB1A* and *BcZAT12* system might function mediated through protection of ionic and osmotic homeostasis. Increased expression of *AtDREB1A* and *BcZAT12* genes enhanced the quantity of GR, which most likely contributed to the detoxification of ROS, as *AtDREB1A* and *BcZAT12* pathway genes control GSH homeostasis, supporting survival and growth of plants even under the number of abiotic oxidative stresses in distinct species (Rai et al. 2013b; Rai et al. 2102a; Kudo et al. 2017; Liu et al. 2018). The two transcription factors additive overexpression is accepted to be an efficient approach for plant genetic engineering intended to elevate the tolerance against drought stress and improve plant growth (Krishna et al. 2021).

A detailed drought-stress resistance of tomato plants over-expressing *AtDREB1A* and *BcZAT12* separately and regulation of *AtDREB1A* and *BcZAT12* expression were conducted by Rai et al. (2012b, 2013b). In the present study, tomato plants over-expressing both *AtDREB1A* and *BcZAT12* jointly were compared for their drought tolerance and various biochemical parameters with respect to ST and NT plants. Expression levels of various antioxidants genes were elevated in transgenic plants compared to NT plants, although, appeared higher in DT plants with respect to the ST and NT plants, which showed their additive effects.

In the present study, the enhanced O−2 level recorded with rising days of drought stress in NT, ST, and DT lines emerged to be linked with time and severity of stress. The generation of O−2 demonstrated to be reliant on the level of stress, duration, plant age, and species (Rai et al. 2012a). The reduced superoxide anion generation is reminiscent of enhanced cell ROS homeostasis in DT tomato lines contrasted with ST and NT. In the present study, the drought stress-treated *AtDREB1A* and *BcZAT12* DT tomato plants showed significantly elevated CAT and SOD activity in comparison to their ST and NT corresponding plants, more specifically the DT lines DZ3 and DZ5. Under stress condition, SOD mediates the removal of superoxide radicals; it scavenges O−2 by catalyzing its dismutation. Earlier under drought stress, critical increment in SOD activity has been seen in a number of muskmelon genotypes with better drought tolerance capability (Ansari et al. 2017, 2018, 2019), and ST tomato plants over-expressing *BcZAT12* (Rai et al. 2012a) and *AtDREB1A* (Rai et al. 2013a), additionally drought-tolerant transgenic peanut (Bhatnagar et al. 2009), chrysanthemum (Hong et al. 2006), and Lolium perenne plants (Li et al. 2011) over-expressing *AtDREB1A*. In cherry tomato, enhanced activity of CAT reported particularly drought-exposed tolerant varieties (Bhatnagar et al. 2009), and ST plants over-expressing *AtDREB1A* and *BcZAT12* individually. Enhanced expression of CAT gene in transgenic tomato plants in reaction to heterologous over-expression of *AtCBF1* has been reported (Gill et al. 2013). Similar to CAT gene expression, under drought stress catalase, the activity was also higher in constitutively over-expressing DT plants of tomato comparatively with the ST and NT plants. In this
experiment, in addition to enhanced APX expression, increased APX activity was also recorded in DT and ST tomato plants exposed to different water-deficit conditions, however, it was higher in DT lines compared to ST and NT plants. When exposed to drought stress condition enhanced, the expression of APX has been reported in many plants, viz., tomato (Sánchez-Rodríguez et al. 2010; Rai et al. 2013b; Rai et al. 2013b), muskmelon (Ansari et al. 2017), and APX enhanced activity in ST AtDREB1A (Rai et al. 2013a) and BcZAT12 (Rai et al. 2012a) separately over-expressing. In the present study, drought-treated DT tomato lines showed significantly increased gene expression and activities compared to ST and NT plants, see in DT lines DZ5 which showed the highest gene expression and enzyme activities. An increased expression of GR was recorded in drought-stressed muskmelon (Ansari et al. 2017), and GR enzyme activity in rice (Sharma and Dubey 2005) and ST tomato (Rai et al. 2012b, 2013a) plants. In the present experiments, increased activities of DHAR up to 14 days and MDHAR up to 21 days of drought stress were observed in the DT tomato lines in comparison to counterpart ST and NT plants. Many reports are available which showed DHAR and MDHAR over-expressing transgenic plants exhibiting a higher AsA level (Yin et al. 2010). The elevated MDHAR activity is responsible for drought tolerance in ST tomato (Rai et al. 2012a, 2013a) and cherry tomato (Sánchez-Rodríguez et al. 2010). As reported earlier, AtDREB1A (Rai et al. 2012b) over-expressing transgenic tomato plants POD activity was random as it decreases initially, then increases and again decreases; instead of this, in BcZAT12 (Rai et al. 2012a), transgenic increase noted up to 14 days of water deficit then reduction observed after 21 days of water deficits; in the case of our DT lines, the results were similar to BcZAT12, but the level of POD was higher, this might be due to the additive effects of both genes (Rai et al. 2012a; Krishna et al. 2021). These enzymatic changes bolster the suggestion that AtDREB1A and BcZAT12 over-expressing in DT tomato plants ease stress-activated oxidative damages and adjust promptly to the drought stress condition. Observations supplement the knowledge about the proper adjustment of oxidative stress in ST plants over-expressing AtDREB1A and BcZAT12 gene, which is mostly controlled through the enzymes like SOD, CAT, APX, GR and POD (Rai et al. 2012a, 2013b; Kudo et al. 2017). It responds by neutralizing ROS to limit the harm, having the capability to donate electrons in various non-enzymatic and enzymatic responses, and furthermore goes about as an inherent substrate for numerous plant peroxidases (Gill et al. 2013). It is well recognized that amount of AsA enhances under drought conditions in plants; however, the buffering limit produced by AsA adds to resistance against the stress generated (Rai et al. 2012a). For instance, drought-resistant tobacco, poplar, and cherry tomato genotypes showed an increased leaf AsA and not withstanding this AtDREB1A and BcZAT12 (Rai et al. 2012a; Rai et al. 13a; Saxena et al. 2016) ST plants additionally indicated a higher measure of AsA which give plants improved resilience to oxidative stress (Sánchez-Rodríguez et al. 2010). The AsA redox framework comprises MDHA, DHA and L-ascorbic acid. An elevated proportion of reduced to oxidized AsA is required to combat cells ROS (Sánchez-Rodríguez et al. 2010). In this context, transgenic (DT) tomato plants enlisted increased AsA contrasted with the ST and NT plants, and seemed to have a more prominent ability to diminish ROS. In plants, glutathione is considered as important metabolite; moreover, its reduced-structure GSH accepted as a significant part for intra-cellular defense against ROS-generated oxidative stress. It additionally assumes a significant role in the development and advancement of plants, including differentiation, death, and senescence of the cell, also regulation of enzymes (Rai et al. 2012a; Krishna et al. 2019). Under drought stress, DT tomato lines displayed higher glutathione content contrasted with the ST and NT plants. GSH assumes a key function in the antioxidative protection pathway by recovering another potential water dissolvable antioxidant like AsA, by means of the AsA-GSH cycle (Gill and Tuteja 2010). The GSH and GSSG equilibria are the vital requirements in continuing the cellular redox state. Continuation of an enhanced reduced to an oxidized ratio of AsA and glutathione is critical for the appropriate diminishing of the ROS in cells (Choudhury et al. 2017; Miller et al. 2018). An increase in the AsA and glutathione levels in maize and wheat plants exposed to drought stress has been accounted (Nayyar and Gupta 2006; Rai et al. 2013b; Xu et al. 2015). In this experiment, drought stress-treated DT tomato lines exhibited distinctly enhanced activities of these enzymes than their ST and NT counterparts. In short, the results of the present study showed that AtDREB1A and BcZAT12 additive overexpression brought about a more prominent actuation of the segments of Halliwel Asada cycle under the conditions of drought stress in DT lines (Fig. 5). The amount of AsA, reduced AsA, glutathione, and GSH in the DT tomato lines demonstrated a positive interaction with ROS level. Accordingly, it might be reasoned that the upgraded oxidative stress resilience under drought stress condition was related to the additive expression of DT lines, which expanded the activities of these enzymes. These findings support that the enhanced antioxidant levels in ST added to bring down the ROS and a superior osmotic alteration prompting improved drought-stress resistance (Rai et al. 2012a). In the present investigation, the activities of the important antioxidant enzymes, were significantly higher in DT tomato lines, overall the DT tomato lines exposed to drought stress exhibited reduced oxidative stress comparatively the ST and NT plants. Under drought stress conditions in DT lines, the amount of enzymatic and non-enzymatic antioxidants was more prominent.
This demonstrates the DT tomato lines increased resistance against drought. The enhanced expression of genes linked with antioxidants may be an outcome of the additive expression of \textit{AtDREB1A} and \textit{BcZAT12} genes under drought stress presenting the DT tomato lines increasingly valuable for zones affected with drought stress. Drought-mediated diminution of leaf pigments is accepted as an indicator of oxidative stress which associates with the degradation of chlorophyll, photo-oxidation of pigment, or retardation in chlorophyll synthesis (Rai et al. 2012a). Similarly, in the present experiments, the photosynthesis pigments’ degradation was observed; however, it was lower in DT compared to the ST and NT plants.

Under drought stress, plants show lower fruit yield in tomato (Topcu et al. 2007; Jensen et al. 2010; Krishna et al. 2021) which might be attributed to delayed physiological processes (Cabello et al. 2009; Dasgan et al. 2018; Ibrahim et al. 2020). On the contrary, the present study revealed that double transgenic tomato lines recorded higher fruit yield as compared single transgenic (\textit{AtDREB1A} or \textit{BcZAT12} or NT) under different DWD. This increase in fruit yield may be attributed to normal physiological processes under water-deficit conditions. Some earlier studies reported that quality parameters, such as hardness, TSS, ascorbic acid, acidity, and color, get enhanced in tomato under water-deficit condition. Furthermore, reduced fruit size and low dilution from decreased water levels may result in reduced water transport within the plant system but not the photo-assimilates (Zegbe et al. 2006), thereby the improvement in quality parameters. Under drought stress conditions, double transgenic tomato lines from the present study showed increased fruit size.

Conclusion

Present findings suggest that \textit{AtDREB1A} and \textit{BcZAT12} stacked DT tomato line, more specifically, DZ3, DZ4, and DZ5 are increased tolerant to drought stress and able to survive better under drought stress condition with enhanced yield efficiency. It also enhanced significance the productivity under drought-affected area. The present study demonstrates that genetic engineering of the plant by employing approach
like gene stacking might be effective for transgenic plants generation which had better tolerance against drought stress and sustained yield (Fig. 6). Double transgenic lines with increased drought tolerance and sustained yield efficiency, might be useful in the cultivation of tomato in drought-prone areas, this will provide better food security and increased income for the farmers. Additionally, this study provides the path to develop more double transgenic lines targeting different abiotic and biotic stress factors.

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Author's contribution statement RK performed all the experiment and initial manuscript draft writing. WAA analyzed the data and helped in manuscript preparation. DKJ helped in data recording. MS developed the project idea and fund. AKS, RP, MS, and JPV edited the manuscript.

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Data availability All the data are presented in the manuscript and the materials used in this research work are with ICAR-Indian Institute of Vegetable Research (IIVR), Varanasi, India.

Code availability Not applicable.

Declarations

Conflict of interest All authors declare that there is no conflict of interest among the authors.

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