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*Original paper*

# *Expression of OsDREB2A in Transgenic Tomato Improves Drought Tolerance*

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## Abstract

Dehydration responsive element binding (DREB) are important regulatory molecules which have a crucial role in abiotic stress tolerance. The productivity of tomato, as a drought-sensitive crop, is highly restricted by drought stress. The current study aimed at introducing the *OsDERB2A* gene into two tomato genotypes via *Agrobacterium*-mediated transformation system. Cotyledonary explants were pre-cultured for two days with *Agrobacterium* strain LBA4404 harboring pCAMBIA1301 with *OsDREB2A* driven by the constitutive promoter CaMV35S for transformation. Shoots were directly regenerated on MS medium containing 1 mg $l^{-1}$ zeatin and 1 mg $l^{-1}$ BAP, and in presence of 30 mg $l^{-1}$ hygromycin as selective agent. Only eight weeks were needed to regenerate transgenic tomato using this protocol. An $OD_{600}$ of 0.4 resulted in 64.3-76.9% transformation efficiency. Stable integration and expression of the *OsDREB2A* gene were confirmed in transgenic tomato using PCR and RT-PCR analyses, and drought tolerance of $T_0$ transgenic lines was confirmed by leaf disc assay in 300 mM mannitol. The superior biomass, photosynthetic pigments, free soluble sugars and proline accumulation of *OsDREB2A* transgenic lines over wild type in response to mannitol-stress revealed their enhanced drought tolerance and indicated that the constitutive expression of *OsDREB2A* might modulate the expression of other drought responsive genes.

**Keywords** Tomato, Drought tolerance, *OsDREB2A*, Transformation, *Agrobacterium*

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# Introduction

Drought and high temperatures are major limitations for growth and productivity of crops worldwide. Drought is expected to increase as the weather continues to become generally drier and warmer due to climate change (Raza et al. 2019). Tomato (*Solanum lycopersicum* L.) is a popular and economically important vegetable crop which contains important bioactive compounds including carotenoids (α- and β-carotene and lycopene), phenolic compounds (flavonoids and phenolic acids), vitamins (vitamin A and ascorbic acid) and the anti-oxidative lycopene (Chaudhary et al., 2018). Tomatoes have a high water need, consequently under drought stress, tomato plants suffer symptoms such as reduction in vegetative growth, plant height, leaf surface area and fruit size, thus, drought stress has a negative impact on the general growth and development of tomato plants, resulting in a severe drop in yield. (Krishna et al. 2019). Recent plant biotechnology involving gene transfer has showed remarkable advances in improving tolerance to environmental stresses by introducing certain foreign stress-related genes into tomato germplasm (Senapati 2016). Among these developments, one intriguing avenue is to use genes encoding transcription factors as key molecular targets to further up-regulate a wide range of stress-related genes in order to improve drought stress tolerance in plants. (Manna et al. 2021).

Drought negatively affects water balance, membrane permeability and biomass. Moreover, it significantly inhibits photosynthesis and enzyme activity causing metabolic perturbation (Fang and Xiong 2015). In response to decreased external water potential due to drought stress, plants may rely on osmotic adjustment as one of tolerance mechanisms. In this mechanism plants increase the accumulation of organic solutes working as compatible osmolytes or osmoprotectants to increase their internal osmotic potential. Among these solutes are soluble sugars and proline. Besides their role in osmotic adjustment and maintenance of turgor and water uptake, these osmoprotectants scavenge free radicals and maintain membranes and enzymes stability (El-Shafey et al. 2009; Fang and Xiong 2015; Singh et al. 2015). Several studies have used a leaf disc assay to assess drought tolerance of transgenic plants by estimating various physiological parameters, such as biomass, photosynthetic pigments content and the accumulation of osmolytes (Jha et al. 2013; Lim et al. 2016; Sharma et al. 2019).

DREBs transcription factors recognize the dehydration responsive elements/C-repeat elements (DRE/CRT) in the promoter region of targeted stress responsive genes under several abiotic stresses and activate the expression of those genes (de Paiva Rolla et al. 2014). Results of Sakuma et al. (2006) proved that the transcription factor DREB2A is involved in the expression of drought responsive genes. More recently, it has been found that overexpression of this transcription factor enhances the tolerance to drought, salinity and heat stress. In a study conducted by Sandhya et al. (2021), co-expression of DREB2A and APX in rice plants can improve drought tolerance by physiologically activating the anti-oxidative defense system to scavenge reactive oxygen species (ROS) generated under drought stress. Overexpression of *AtDREB2A CA* in sugarcane up-regulated genes involved in drought stress response, consequently enhanced drought tolerance (Reis et al. 2014). Similarly, overexpression of *Fraxinus pennsylvanica FpDREB2A* gene in *Robinia pseudoacacia* (Xiu et al. 2016) and the *OsDREB2A* from barley in flax (Tawfik et al. 2016) showed improved tolerance to drought stress. Wei et al. (2016) reported increased tolerance of DREB1B transgenic *Salvia miltiorrhiza* to drought stress without stunting growth. Huang et al. (2018) reported that overexpression of *OsDRAP*1 in rice resulted in maintaining water balance under drought stress.

Additionally, heterologous expression of the *AhDREB* transcription factor improved tolerance to salt stress without affecting growth rate in *Populus tomentosa* under greenhouse conditions (Guo et al. 2019). Overexpression of the GmDREB6 gene increased proline accumulation in genetically modified soybean plants that suggested that DREB6 could act as a promising candidate for improving salt tolerance in plants (Wang et al. 2017). Also, DREB2-type transcription factor in lily regulates and mediates heat stress response (Wu et al. 2018). Nishawy et al. (2015) reported that overexpression of *Citrus grandis* DREB gene in tomato leads to accumulation of primary metabolites.

*Agrobacterium*-mediated transformation is the most efficient system which has been used for transformation of tomato. However, protocols that have been described for tomato are genotype dependent. Among factors affecting the transformation efficiency, genotype and *Agrobacterium* density are the most effective ones (Shahriari et al. 2006; Gao et al. 2009; Senapati 2016). The present study aims at developing an efficient transformation protocol for the delivery and expression of the *OsDREB2A* gene into tomato using *Agrobacterium*-mediated transformation. The study aims also at investigating whether the expression of *OsDREB2A* enhanced drought tolerance in transformed tomato plants.

# Materials and Methods

***Plant materials***

Seeds of two tomato genotypes (accession numbers; 13163 and 12676) were supplied by the Egyptian National Gene Bank, Agriculture Research Center, Egypt and abbreviated here as E3 and F4 respectively. Seeds of both genotypes were sterilized and germinated on half-strength MS medium (Murashige and Skoog 1962) supplemented with 30 g $l^{-1}$ sucrose and 8 g $l^{-1}$ agar. Cotyledonary leaf of 10 days old seedling was used as explant (El-Shafey et al., 2017).

***Expression vector and Agrobacterium culture preparation***

*Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector pCAMBIA1301 was used for

transformation. The gene *OsDREB2A* (accession number on European Nucleotide Archive; BAS70561.1) was cloned in the vector under the control of constitutive promoter CaMV35S and terminated by the CaMV35S poly A signal. The whole cassette was cloned in *Hind* III sites (Figure 1). Single colony of *Agrobacterium* LBA4404, carrying the recombinant plasmid pCAMBIA1301-*OsDREB2A*, was inoculated in 20 ml liquid (LB) Lennox media (Bertani 1951), pH 7.0, supplemented with kanamycin (50 mg $l^{-1}$), streptomycin (50 mg $l^{-1}$) and rifampicin (25 mg $l^{-1}$). The culture was incubated at 28ºC with shaking at 200 rpm overnight. The culture was diluted to different optical densities ($OD_{600}$; 0.2, 0.4, 0.6, 0.8, 1.0) with LB medium containing the appropriate antibiotics and then 30 ml of the diluted culture was centrifuged at 4000 rpm for 10 min. The collected pellets of the *Agrobacterium* cells were re-suspended in 30 ml of liquid MS medium containing 30 g $l^{-1}$ sucrose, 200 mg $l^{-1}$ acetosyringone and 0.5 mg $l^{-1}$ Kn + 0.5 mg $l^{-1}$ IAA for cultivar E3 or 0.2 mg $l^{-1}$ IAA + 0.5 mg $l^{-1}$ BA for cultivar F4. Then the bacterial suspensions were incubated for 3 h at 28ºC with shaking at 200 rpm before infection.

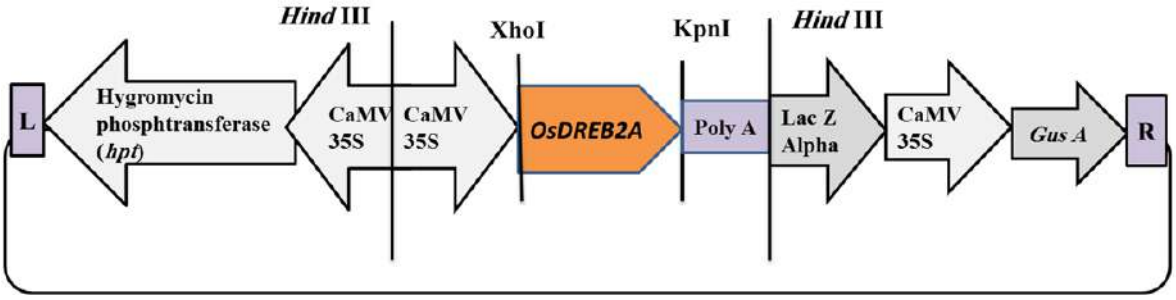


**Figure 1:** Diagrammatic representation of the binary vector (pCAMBIA1301) used for tomato transformation. The vector is harboring *OsDREB2A*gene driven by CaMV35S promoter, and poly A

***Transformation and direct regeneration***

Cotyledonary explants were pre-cultured on solid MS media supplemented with 30 g $l^{-1}$ sucrose and 0.5 mg $l^{-1}$ Kn + 0.5 mg $l^{-1}$ IAA for cultivar E3 or 0.2 mg $l^{-1}$ IAA + 0.5 mg $l^{-1}$ BA for cultivar F4. The pre-cultured explants incubated in the dark at 25 ± 2ºC for two days and then collected and submerged in *Agrobacterium* suspension for 30 min with occasional agitation. The infected explants were transferred to the co-cultivation medium containing the same components of pre-culture medium but with 100 mg $l^{-1}$ acetosyringone, and incubated in the dark for two days at 25±2ºC. The co-cultivated explants were washed five times with sterile half strength MS medium supplemented with 600 mg $l^{-1}$ cefotaxime and transferred to pre-selection medium composed of MS medium containing 30 g $l^{-1}$sucrose, 1 mg $l^{-1}$ BAP, 1 mg $l^{-1}$ Zn, 400 mg $l^{-1}$ cefotaxime, and 8 g $l^{-1}$ agar for three and then transferred to selective shoot induction medium containing the same components of pre-selection medium but with 250 mg $l^{-1}$ cefotaxime and 30 mg $l^{-1}$ hygromycine. Cultures on pre-selective and selective media were incubated at 25 ± 2ºC under 16/8h (light/dark) photoperiod and sub-cultured on fresh selective shoot induction media once after 3 weeks (Figure 2a). Transformation efficiency was calculated as number of explants with regenerated shoots on hygromycin selective media / total number of explants × 100.

***In vitro rooting and acclimatization***

The well-developed shoots (2-3 cm long) that regenerated on the selective regeneration medium were transferred to rooting medium containing ½ MS, 30 g $l^{-1}$ sucrose, 1 mg $l^{-1}$ IAA and 8 g $l^{-1}$ agar for root induction. The plantlets with well-developed roots (Figure 2b, 2c, 2d) were hardened and acclimatized in plastic cups for two weeks and then transferred to *ex vitro* conditions in net house. After another two weeks, the percentage of survival was recorded and the plants were transferred to peat moss for one month, and then planted in a mixture of sand and clay soil in a ratio of 3:2 in 25 × 25 cm pots (Figure 2e).


**Figure 2:** Regeneration and acclimatization of putative *OsDREB2A* transgenic tomato of genotype F4; **(a)** Direct shoot regeneration on selective shoot induction media, **(b)** Induction of roots from regenerated shoots after two weeks on rooting medium, **(c)** Rooted shoot, **(d)** Acclimatized putative *OsDREB2A*transgenic tomato and **(e)** Regenerated $T_0$ *OsDREB2A* transgenic lines in larger plastic pot after survival. Scale bar length is 1 cm.

***Molecular screening of transformants***

To confirm the integration of *OsDREB2A* in the putative transgenic plants, the acclimatized plants were analyzed by conventional PCR. Total genomic DNA was extracted using JET Plant Genomic DNA Purification Mini Kit (Thermo-Scientific Gene, USA) following manufacturer's instructions. The specific primers of *OsDREB2A* gene (DRE-forward; 5'-GAGTACCTC GAGATGGAGCGGGGGGAGGG-3' and DRE-reverse; 5'-GCAGCGGTACCGACTACTACTCTAATAGGAG-3') were used to amplify the transgene in the putative transformed and non-transformed genomic DNA. The PCR reaction was performed by Dream Taq Green PCR Master Mix (2X) (Thermo Fisher Scientific, USA). The recommended PCR reagents were used following manufacturer's instructions. The thermal cycler for *OsDREB2A* was programmed as follow; initial denaturation at 94ºC for 5 min, 35 amplification cycles (denaturation at 94ºC for 30 s, annealing at 56ºC for 45 s and extension at 72ºC for 2 min) and final extension 72ºC for 7 min.

Reverse transcriptase PCR (RT-PCR) was performed to analyze the stable expression of the gene. The transgenic lines from E3 and F4 that gave positive results with the conventional PCR were selected for RNA extraction. RNA was extracted from fresh leaves as described by Chomczynski and Mackey (1995). Samples of RNA were visualized by resolving on 1.5% agarose gel. For RT-PCR, cDNA was first prepared from 2µg total RNA following the instructions of Sensi-FAST cDNA synthesis kit (Bioline, USA). The cDNA from the transgenic and wild type plants was amplified by primers of *OsDREB2A* (*DRE*-forward; 5'-ATTGCTCCGTGCA AGTGAGGAAG-3' and *DRE*-reverse 5'-ATCTCAG CCACCCACTTACCC-3'). ). The thermal cycler program was; 5 min denaturation step at 94ºC, 35 amplification cycles (30 s denaturation at 94ºC, 45 s annealing at 53ºC and 2 min extension at 72ºC) and 7 min final extension at 72ºC. The amplified fragments were separated on a 1.5 % agarose gel.

***Molecular screening of transformants using leaf disc assay and physiological analysis***

To assess the potential of *OsDREB2A* transgene in enhancing drought tolerance in transgenic tomato, leaf discs were cut from leaves of putative $T_0$ transformants (L1, L2 and L3) and wild type (WT) plants after 6 weeks from *ex vitro* growing plants. Leaf discs were floated on 10 ml ½ MS basal salts supplemented with mannitol (0 and 300 mM) and incubated for 5 days at 25±2ºC and 16 h light/8 h dark photoperiod. Then, biomass (fresh weight; FW and dry weight; DW) and water content, as percent of dry weight, were measured (Lim et al. 2016). Photosynthetic pigments content was estimated following the protocol of Lichtenthaler and Buschman (2001). The content of chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoids was expressed as mg $g^{-1}$ fresh weight. Total soluble sugars were extracted from the dry samples in 80% ethanol, and their concentration were estimated using anthrone reagent (Maness 2010). The concentration of soluble sugars was calculated from glucose standards curve and expressed as mg glucose equivalent $g^{-1}$ dry weight. Proline was extracted from the fresh leaf discs in 3% aqueous salphosalicylic acid, estimated using ninhydrin reagent according to the protocol of Bates et al. (1973) and expressed as mg $g^{-1}$ fresh weight.

***Statistical Analysis***

Data were subjected to One Way Analysis of Variance (ANOVA) using the software SPSS (version 16). The significant differences among samples of each genotype were determined by Duncan's multiple range tests at $p = 0.05$. All values were expressed as means ± SE. Two Way Analysis of Variance was also performed to determine the extent at which the difference between and within genotypes and *Agrobacterium* densities is due to interaction between these two variables. The data of leaf disc assay were performed in triplicates, where each replicate is a petri-dish containing 6 leaf discs.

# Results and discussion

***Regeneration and selection of transformed shoots***

Screening different concentration of hygromycin revealed that 30 mg l-1 was the lethal dose that was sufficient to kill the non-transformed cells. Consequently, that concentration (30 mg l-1 hygromycine) was used in selective media. The optical density of *Agrobacterium* induced a profound effect on tomato transformation efficiency, and both genotypes (E3 and F4) showed great variations in transformation efficiency depending on the bacterial density. E3 genotype showed a relatively higher transformation efficiency compared with those of F4 (Table 1). At $OD_{600}$ 0.4, both genotypes scored the highest transformation efficiency; about 76.9% of the explants of genotype E3 and 64.3% of the genotype F4 regenerated putative transgenic shoots. The $OD_{600}$ 0.2 also revealed relatively high regeneration frequency, 61.2% and 57.6% of the explants of E3 and F4 respectively regenerated transgenic shoots. At $OD_{600}$ 1.0, the frequency of regeneration markedly decreased due to over-growth of *Agrobacterium*.

The number of regenerated shoots/explant is generally proportional to the percentage of regenerated explants Meanwhile, at $OD_{600}$ 0.4, 3.42 and 2.76 shoots were counted for the genotypes E3 and F4 respectively (Table 1). The regeneration of transgenic shoots on explants of genotype E3 took place after 8-10 weeks while regeneration of genotype F4 transgenic shoots was observed within 6-8 weeks on the same medium. The Two-Way ANOVA analysis of data showed that each of genotype and bacterial optical density independently had a significant effect on transformation efficiency, while their interaction effect was non-significant on the investigated criteria (Table 2).

***In vitro rooting and acclimatization of transformants***

Root induction began within 2-3 weeks after subculture on rooting medium. Root induction from F4 genotype shoots was more frequent than genotype E3 (Table 3). About 68.7% of shoots that regenerated from genotype F4 developed roots compared with 56.8% in case of genotype E3. Moreover, genotype F4 showed

higher root number and root length. Concerning the survival of acclimatized plants, genotype F4 produced stronger and healthier plants than genotype E3. About 58.6% of genotype F4 Putative transgenic plants survived during acclimatization versus only 40% for genotype E3.

**Table 1:** Effect of *Agrobacterium* suspension's optical densities (OD $_{600}$) of 0.2, 0.4, 0.6, 0.8 and 1.0 on transformation efficiency and number of regenerated shoots/explant directly regenerated from cotyledonary explants of E3 and F4 genotypes after 8 weeks from the culturing.

| Genotypes | | Transformation efficiency | Shoot number/explant |
|---|---|---|---|
| **F4** | **0.2** | 57.6 ± 2.9$^{c}$ | 2.21 ± 0.39$^{ab}$ |
| | **0.4** | 64.4 ± 1.9$^{c}$ | 2.76 ± 0.39$^{bc}$ |
| | **0.6** | 46.7 ± 2.1$^{b}$ | 1.77 ± 0.40$^{ab}$ |
| | **0.8** | 39.0 ± 3.0$^{ab}$ | 1.16 ± 0.29$^{a}$ |
| | **1** | 31.9 ± 2.0$^{a}$ | 1.12 ± 0.35$^{a}$ |
| **E3** | **0.2** | 61.2 ± 3.5$^{c}$ | 2.71 ± 0.43$^{bc}$ |
| | **0.4** | 76.9 ± 4.3$^{d}$ | 3.42 ± 0.40$^{c}$ |
| | **0.6** | 56.7 ± 4.1$^{c}$ | 1.38 ± 0.33$^{a}$ |
| | **0.8** | 48.0 ± 2.0$^{b}$ | 1.62 ± 0.41$^{ab}$ |
| | **1** | 36.1 ± 2.8$^{a}$ | 1.37 ± 0.59$^{a}$ |

Values are means ± SE. Values followed by at least one similar letter are not significantly differed at $p \leq 0.05$

**Table 2:** Interaction effect of genotype and optical density (OD $_{600}$) of *Agrobacterium* suspension on transformation efficiency (TE) of tomato and shoot number/explant regenerated on hygromycin selective media.

| | Shoot number/explant | | TE | |
|---|---|---|---|---|
| **Source** | **df** | **F** | **df** | **F** |
| **Genotype** | 1 | 1.28$^{NS}$ | 1 | 17.05$^{S}$ |
| **OD $_{600}$** | 4 | 8.04$^{S}$ | 4 | 49.12$^{S}$ |
| **Genotype* OD $_{600}$** | 4 | 0.46$^{NS}$ | 4 | 0.91$^{NS}$ |

TE; Transformation efficiency, NS; Non-significant, S; Significant at $p \leq 0.001$

**Table 3:** Rooting and survival of transgenic plantlets of tomato E3 and F4 genotypes

| Genotypes | Rooting (%) | Root length (cm) | Root number | Survival (%) |
|---|---|---|---|---|
| **E3** | 56.8 | 7.4 ± 0.25 | 4.9 ± 0.23 | 40.0 |
| **F4** | 68.7 | 8.8 ± 0.27 | 7.4 ± 0.34 | 58.6 |

Values are means ±SE.

***Molecular screening of transgenic lines***

PCR amplification using specific primers for *OsDREB2A* gene and electrophoresis of amplified PCR product revealed the expected size of 825 bp for *OsDREB2A* (Figure 3a) in the transgenic lines (L1, L2 and L3) of both genotypes. At the same time, no amplicons were detected in the wild type plants. Similarly, the RT-PCR analysis confirmed the integration and stable expression *OsDREB2A* gene after transgenic plants survival. Electrophoresis for the amplified cDNA revealed the expected size 250 bp (Figure 3b) for *OsDREB2A* gene. *OsDREB2A* gene was found in all the tested transgenic plants.

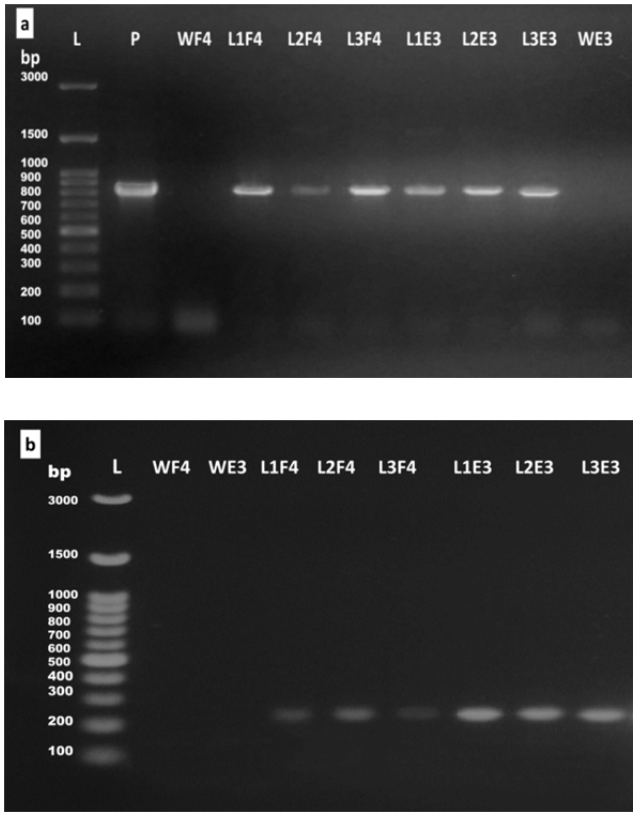


**Figure 3: (a)** Electrophoretic separation of PCR amplified *OsDREB2A* (825 bp). **(b)** RT-PCR analysis of *OSDREB2A* (250 bp). (P); plasmid used in transformation, (WF4 and WE3); wild type of genotype F4 and E3 respectively, (L1F4, L2F4 and L3F4) ; transgenic lines of genotype F4, (L1E3, L2E3 and L3E3); transgenic lines of genotype E3 and (L); 3000 bp DNA ladder.

***Leaf disc assay and biomass production by transgenic lines***

Since the transgenic lines of genotype F4 scored higher survival rate and showed healthier and stronger plants than those of genotype E3, they were used in the leaf disc assay. A significant difference in biomass was revealed between the leaf discs of WT plants and transgenic lines in response to mannitol-stress. Under non-stressful conditions, the highest FW and DW were scored by the transgenic line L1, while the lowest ones were scored by L3 (Figure 4a and 4b). Although the fresh and dry weights of the WT were significantly reduced by 48% and 30.8% of control, those of the transgenic lines were non-significantly affected by mannitol-stress. Even in L3 that exhibited a significant reduction in FW, the percentage of reduction in its biomass was obviously less than that observed on WT plants. In contrast to WT leaf discs, those of the transgenic lines could maintain their water content under drought stress (Figure 4c).

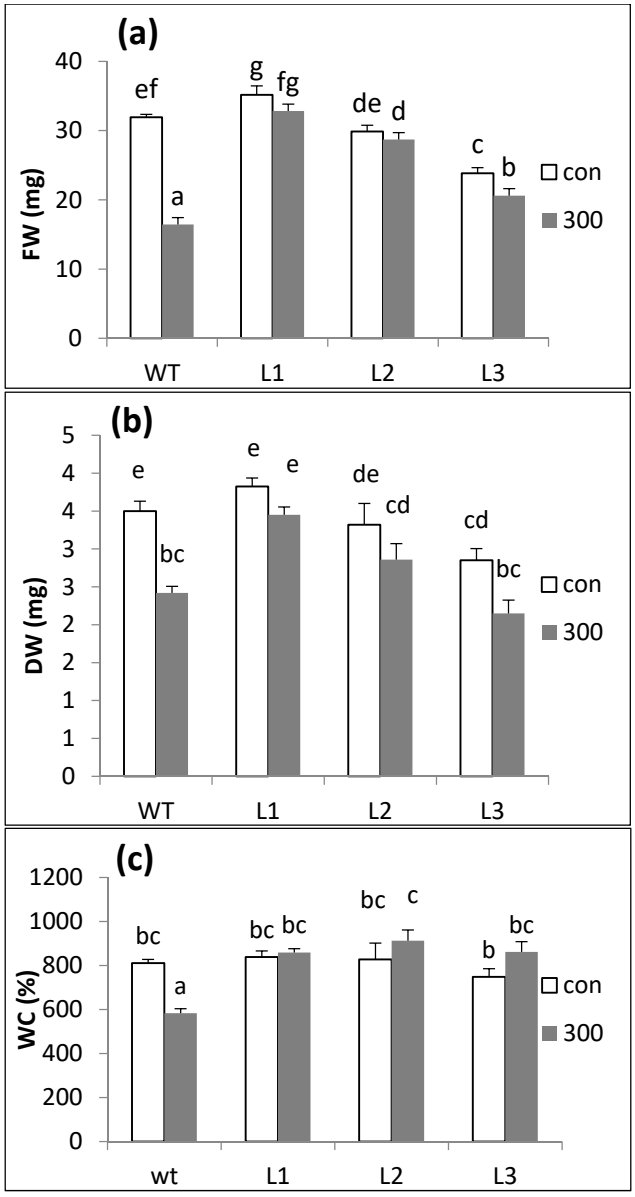


**Figure 4:** Effect of mannitol (0.0 and 300 mM) on leaf disc **(a)** fresh weight (FW), **(b)** dry weight (DW) and **(c)** water content (WC) of wild type (WT) plant and transgenic lines L1, L2 and L3. Values are means ±SE. Bars with at least one similar letter are not significantly different at p = 0.05.

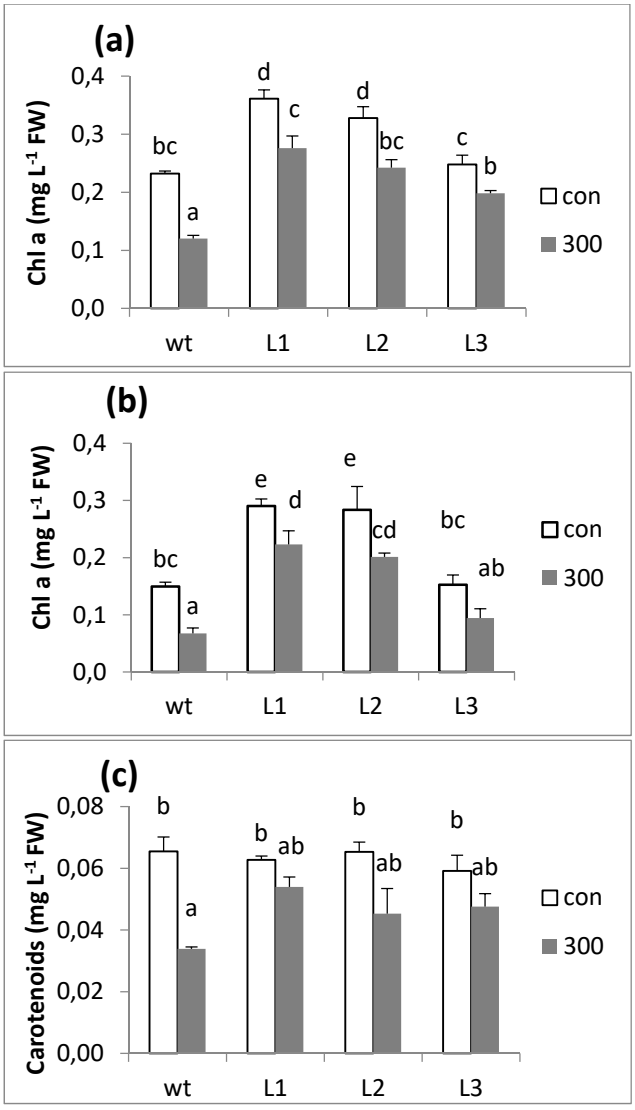


**Figure 5:** Effect of mannitol (0.0 and 300 mM) induced drought stress on **(a)** chlorophyll a (Chl a), **(b)** chlorophyll b (Chl b), **(c)** carotenoids contents in leaf discs of wild type (WT) plant and transgenic lines L1, L2 and L3. Values are means ±SE. Bars with at least one similar letter are not significantly different at p = 0.05.

***Photosynthetic pigments content***

The chlorophyll content of the transgenic lines was higher than those of WT plant under both non-stressful and stressful conditions (Figure 5a and 5b). Drought stress decreased contents of Chl a and Chl b in both WT and transgenic plants. However, its impact on those of WT plants was much more injurious. For example, Chl a and Chl b of WT leaf discs were dramatically declined by 48.2% and 54.8%, while those of line L1 were reduced by only 23.5% and 23.1% below respective control in response to osmotic stress. Regarding the content of carotenoids (Figure 5c), the harmful effect of drought seemed significant only on carotenoids of WT. While this variable decreased to less than half of control in WT, it was nonsignificantly changed in transgenic lines under drought stress.

***Total soluble sugars and proline content***

All transgenic lines contained a higher content of soluble sugars than WT plants under non-stressful conditions (Figure 6a). On exposure to osmotic stress, transgenic lines maintained their higher content or even accumulated much more soluble sugars, while WT plants non-significantly changed their own sugar content relative with respective control. Although there were no significant differences among WT and transgenic plants concerning proline content in absence of stress (Figure 6b), the transgenic lines augmented much higher proline over the respective control in response to mannitol (300 mM) than WT plant.

An *Agrobacterium*-mediated transformation system, for the *OsDREB2A* gene driven by CaMV35S constitutive promoter, was developed for two genotypes (E3 and F4)

of tomato. The association between the efficiency of transformation and the genotype of tomato has been reported for other cultivars and no standard protocol for tomato transformation can be generalized (Ellul et al. 2003; Sun et al. 2006; Khoudi et al. 2009; Sun et al. 2015; Van et al. 2019). The increment of bacterial density above $OD_{600}$ 0.4 negatively affected the viability of the explants and subsequently the transformation efficiency. Above $OD_{600}$ 0.4, the explants became gradually black, soft and finally lost their regeneration capacity. A direct relation between the transformation efficiency and the optical density of the *Agrobacterium* was also reported by Sharma et al. (2009). The current results also agree with those reported by Juan et al. (2015), who confirmed that above $OD_{600}$ 0.4, tomato adventitious bud differentiation rate significantly decreased. The effect of optical density of *Agrobacterium* suspension on the number of regenerated shoots/explant was also linked to transformation efficiency. The highest shoot number on selective media was scored by explants infected with *Agrobacterium* at $OD_{600}$ 0.4, indicating that $OD_{600}$ 0.4 is optimal for transformation for both genotypes. Each of the genotype and optical density independently exerted a significant effect on transformation efficiency, but there was no significant interaction between them. This may indicate that the proper bacterial concentration for transformation is associated with other factors rather than genotype, which may be bacterial strain, growth rate or experimental conditions. The current transformation protocol showed higher transformation efficiency (76.9% and 64.3% in genotype E3 and F4 respectively), expressed as number of explants with regenerated shoots on selective media / total number of explants × 100, relative to those reported in previous studies on *Agrobacterium*-mediated transformation of tomato. Sun et al. (2006) reported that transformation efficiency exceeded 40% of the explants, while Qiu et al. (2007) found that the best transformation efficiency, 20.87%, was obtained when they incubated cotyledon explants with *Agrobacterium* at $OD_{600}$ = 0.2. Recently, Sun et al. (2015) developed a protocol for tomato (cv. Hezuo 908) with 40% transformation efficiency.

Although both genotypes scored their highest transformation efficiency and shoot number at the same optical density ($OD_{600}$ 0.4), the transgenic plants produced from F4 genotype required shorter time to regenerate and produced more healthy shoots. Moreover, F4 transgenic plants showed higher ability to survive after acclimatization that may be linked with their higher number of induced roots. PCR product confirmed the integration of *OsDREB2A* gene in transformed plants, while the wild type plants did not show the amplicon of this gene. The stable expression of *OsDREB2A* in PCR-positive plants was detected by RT-PCR analysis. All transgenic lines produced an amplification product of 250 bp for *OsDREB2A* gene. This approach was also used to confirm the integration and expression of the *CsZCD* and the *ICE1* genes in transgenic tomato plants (Qiu et al. 2007; Juan et al. 2015).

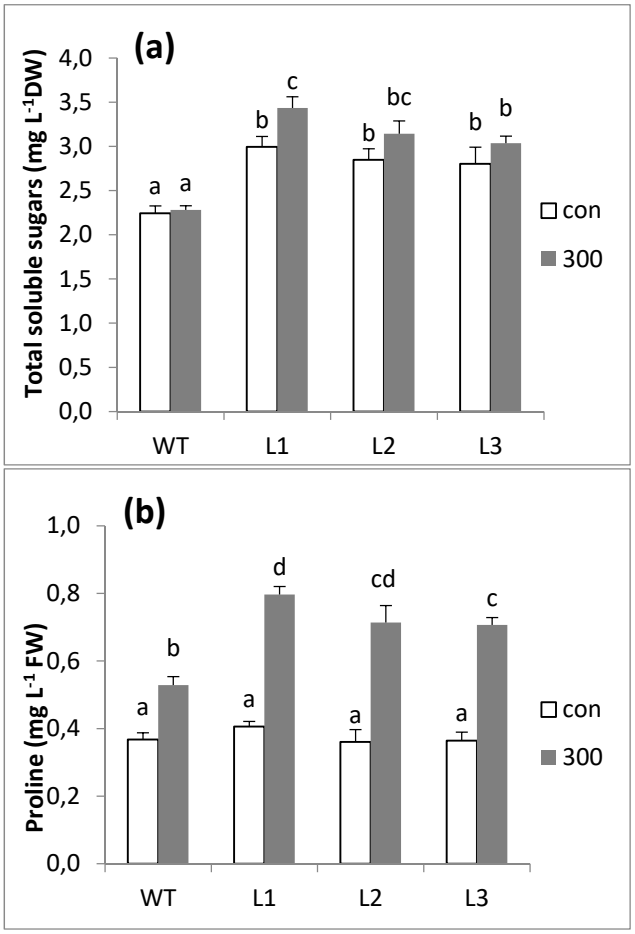


**Figure 6:** Effect of mannitol (0.0 and 300 mM) induced drought stress on **(a)** total soluble sugars and **(b)** proline contents in leaf discs of wild type (WT) plant and transgenic lines L1, L2 and L3. Values are means ±SE. Bars with at least one similar letter are not significantly different at p = 0.05.

In response to drought stress, WT leaf discs showed a dramatic decrease in their biomass; FW and DW, water content and Chl a, Chl b and carotenoids content. In contrast, fresh and dry biomass as well as the water content of *OsDREBB2A*-transgenic lines leaf discs non-significantly affected by drought compared with the normal conditions. Moreover these transgenic lines significantly enhanced the accumulation of soluble sugars and proline in response to drought stress. The non-significant change in fresh and dry biomass that detected in leaf discs of transgenic lines, particularly L1 and L2, as affected by drought indicates an enhanced tolerance of these transgenic lines compared with the significant loss of the WT discs. This enhanced tolerance was more obvious in L1, followed by L2 and then L3. This adaptable response of transgenic lines reflects the positive effect of *OsDREB2A* and its role in improvement of drought tolerance in transgenic lines. In addition, the transgenic lines recorded a higher percentage of water content, indicating a higher ability of retaining water as a strategy of adaptation to drought.

Photosynthetic pigments are generally regarded as biomarkers for the rate and efficiency of photosynthesis as chlorophylls (Chl a and Chl b) play the major role in photosynthesis. The current results are supported by similar findings by Fahad et al. (2017) and Sharma et al. (2019) reporting a decrease in fresh and dry weights under dehydration stress and decline in chlorophyll content of leaves that could be the result of pigment photo-oxidation and chlorophyll degradation. Under non-stressful condition, total chlorophyll content of transgenic plants was higher than that of WT plants, indicating the role of *OsDREB2A* in pre-preparation and adaptation of the transgenic plants to cope with the unfavorable condition. In the current study, although Chl a and Chl b in leaf discs of both WT and transgenic lines negatively affected by manitol, the loss in those of transgenic lines was much less obvious. That may indicate a more stability in photosynthetic membranes that in their turn lead to a more efficient photosynthesis and higher production of assimilates. Similar to the current results, transgenic *Salvia miltiorrhiza* expressing *AtDREB1B*, driven by CaMV35S or induced RD29A promoters resulted in higher content of chlorophyll and higher net photosynthetic rate under drought stress (Wei et al. 2016). This may explain why L3 line, the one that recorded the least content of photosynthetic pigments, exhibited the highest reduction in biomass among the investigated transgenic lines. The non-significant change in carotenoids content of transgenic lines relative to the marked loss in that of WT plants may reveal the positive role of carotenoids in enhancing the tolerance of transgenic plants. The constitutive expression of *OsDREB2A* may not directly affect carotenoids biosynthesis, nevertheless it may contribute preventing the damage of carotenoids that work as photoprotector and participate in protecting the transgenic lines against drought-induced oxidative stress.

The role of sugars and proline as osmolytes that regulate the osmotic adjustment is well documented. These solutes also work in providing membrane protection and scavenging toxic ROS under drought stress (Singh et al., 2015). The magnitude of soluble sugars accumulation in all transgenic lines was more significant than in WT plants. It is notable that the content of Chl a and Chl b as well as soluble sugars was obviously higher in transgenic plants than in WT even under non-stressful conditions. Increased soluble sugars in transgenic lines could be linked to accumulation of photosynthetic pigments indicating the enhanced drought tolerance and increased potential to manage its own tolerance strategies. An increase in proline content was observed in WT plants after mannitol stress. The *OsDREB2A*-transgenic lines showed higher levels of the accumulated free proline when compared with WT plants, but the variance in accumulation was non-significant among the investigated plants in normal condition. This indicates that genes responsible for proline metabolism may not be among the downstream drought responsive genes that may be up-regulated as a result of the constitutive expression of *OsDREB2A* in tomato. Nevertheless, the accumulated proline under drought stress might occur as a consequence of more efficient defensive mechanisms and more adaptability to stress by transgenic plants. Similarly, overexpression of *OsDREB2A* in transgenic soybeans increased the accumulation of soluble sugars and free proline that resulted in enhancement of salt tolerance (Zhang et al., 2013). The higher accumulation of soluble sugars and proline in transgenic lines compared with WT plants could be the reason of the enhanced maintenance of water content showed, in the current study, by transgenic lines in response to mannitol. It also may reveal a better management of these solutes that work as osmolytes and osmoprotectants conferring higher ability of osmotic adjustment and stability of macromolecules. These results support those found by Xiu et al. (2016) who reported that the over expression of *FpDREB2A* gene in *Robinia pseudoacacia* improved drought tolerance by inducing higher chlorophyll, sugars and proline contents in the transgenic plants. The accumulation of high content of chlorophylls, soluble sugars, and proline in response to drought indicate that the transgenic lines due to their ectopic expression of *OsDREB2A* acquired the ability to better utilize the defensive strategies that lead to improvement of their tolerance. All indicate also the regulatory role of *OsDREB2A* in fine-tuning the responses of the transgenic plants to drought and the expression of down-stream drought responsive genes. Recent advances in molecular biology enabled Mizoi et al. (2019) to explain that the stabilization and activation of DREB2A in response to heat stress comes via the inhibition of its negative regulatory domain (NRD) phosphorylation and results in enhance plant thermotolerance. The current findings is also congruent with the findings of Huang et al. (2018), where the overexpression of *OsDRAP1* in transgenic rice plants affect positively on maintaining water balance and vascular development under drought stress.

# Conclusion

A well-established *Agrobacterium*-mediated transformation system, for the *OsDREB2A* gene driven by CaMV35S constitutive promoter, has been developed for two genotypes of tomato, by incubating cotyledonary leaf explants in *Agrobacterium* suspension. The optimal *Agrobacterium* density ($OD_{600}$ 0.4) resulted in high transformation efficiency (76.9% for the genotype E3 and 64.3% for the genotype F4). An ectopic expression of *OsDREB2A* was confirmed in transgenic tomato by PCR and RT-PCR analysis. The *OsDREB2A* expression in transgenic tomato conferred drought tolerance as indicated by elevated amounts of photosynthetic pigments, soluble sugars and proline indicating a possible modulation of the expression of other drought responsive genes.

# References

1. Bates LS, Waldeen RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant and Soil 39:205-207.

2. Bertani G (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. Journal of Bacteriology 62:293-300.
3. Chaudhary P, Sharma A, Singh B, Nagpal AK (2018) Bioactivities of phytochemicals present in tomato. J Food Sci Technol 55: 2833–2849.
4. Chomczynski P, Mackey K (1995) Substitution of chloroform by bromo-chloropropane in the single-step method of RNA isolation. Analytical Biochemistry 225:163–164.
5. de Paiva Rolla AA, Carvalho JD, Fuganti-Pagliarini R, Engels C, Do Rio A et al. (2014) Phenotyping soybean plants transformed with rd29A: AtDREB1A for drought tolerance in the greenhouse and field. Transgenic Research 23: 75–87.
6. Ellul P, Garcia-Sogo B, Pineda B, Ríos G, Roig LA, Moreno V (2003) The ploidy level of transgenic plants in *Agrobacterium* mediated transformation of tomato cotyledons (*Lycopersicon esculentum* Mill.) is genotype and procedure dependent. Theoretical and Applied Genetics 106:231–238.
7. El-Shafey NM, Hassan N, Khodary SEA, Badr A (2017) Differential in vitro direct regeneration of tomato genotypes on various combinations of growth regulators. Biotechnology 16(4-6):155-164.
8. El-Shafey NM, Hassaneen RA, Gabr MMA, El-Sheihy O (2009) Pre-exposure to gamma rays alleviates the harmful effect of drought on the embryo-derived rice calli. Australian Journal of Crop Science 3:268–277.
9. Fahad S, Bajwa AA, Nazir U, Anjum SA, Farooq A, Zohaib A, Sadia S, Nasim W, Adkins S, Saud S, Ihsan MZ, Alharby H, Wu C, Wang D, Huang J (2017) Crop production under drought and heat stress: Plant Responses and Management Options. Front Plant Sci. 8:1147.
10. Fang Y, Xiong L (2015) General mechanisms of drought response and their application in drought resistance improvement in plants. Cell Mol Life Science 72(4):673-689.
11. Gao N, Shen W, Cao Y, Su Y, Shi W (2009) Influence of bacterial density during preculture on *Agrobacterium*-mediated transformation of tomato. Plant Cell, Tissue and Organ Culture 98:321–330.
12. Guo Q, Lu N, Sun Y, Lv W, Luo Z, Zhang H, Ji Q, Yang Q, Chen S, Zhang W, Li Y (2019) Heterologous expression of the DREB transcription factor AhDREB in *Populus tomentosa Carrière* confers tolerance to salt without growth reduction under greenhouse conditions. Forest 10(3):214.
13. Huang L, Wang Y, Wang W, Zhao X, Qin Q, Sun F, Hu F, Zhao Y, Li Z, Fu B and Li Z (2018) Characterization of transcription factor gene *OsDRAP1* conferring drought tolerance in rice. Frontiers in Plant Science 9:94.
14. Jha B, Mishra A, Jha A, Joshi M (2013) Developing transgenic *Jatropha* using the *SbNHX1* gene from an extreme halophyte for cultivation in saline wasteland. Plos One 8(8):e71136.
15. Juan JX, Yu XH, Jiang XM, Gao Z, Zhang Y, Li W, Yang G (2015) *Agrobacterium*-mediated transformation of tomato with the ICE1 transcription factor gene. Genetics and molecular research 14(1):597–608.
16. Khoudi H, Nouri-Khemakhem A, Gouiaa S, Masmoudi K (2009) Optimization of regeneration and transformation parameters in tomato and improvement of its salinity and drought tolerance. African Journal of Biotechnology 8:6068-6076.
17. Krishna R, Karkute SG, Ansari WA, Jaiswal DK, Verma JP, and Singh M (2019) Transgenic tomatoes for abiotic stress tolerance: status and way ahead. 3 Biotech 9:1-14.
18. Lichtenthaler HK, Buschman C (2001) Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy. In: Wrolstad, RE, Acree TE, An H, Decker EA, Penner MH, Reid DS, Schwartz SJ, Shoemaker CF, Sporns P (eds) Current Protocols in Food Analytical Chemistry, John Wiley and Sons, New York, F4.3.1-F4.3.8.
19. Lim MY, Jeong BR, Jung M, Harn CH (2016) Transgenic tomato plants expressing strawberry D-galacturonic acid reductase gene display enhanced tolerance to abiotic stresses. Plant Biotechnology Reports 10(2):105-116.
20. Maness N (2010). Extraction and analysis of soluble carbohydrates. Plant Stress Tolerance: Methods and Protocols, 639: 341-370.
21. Manna M, Thakur T, Chirom O, Mandlik R, Deshmukh R, Salvi P (2021) Transcription factors as key molecular target to strengthen the drought stress tolerance in plants. *Physiologia Plantarum* 172: 847– 868.
22. Mizoi J, Kanazawa N, Kidokoro S, Takahashi F, Qin F, Morimoto K, Shinozaki K, Yamaguchi-Shinozaki K (2019). Heat-induced inhibition of phosphorylation of the stress-protective transcription factor *DREB2A* promotes thermotolerance of *Arabidopsis* thaliana. Journal of Biological Chemistry *294*(3):902-917.
23. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15:473-497.
24. Nishawy E., Sun X, Ewas, M., Cheng Y et al. (2015) Overexpression of *Citrus grandis* DREB gene in tomato affects fruit size and accumulation of primary metabolites. Scientia Horticulturae 192:460-467
25. Qiu D, Diretto G, Tavarza R, Giuliano G (2007) Improved protocol for *Agrobacterium* mediated transformation of tomato and production of transgenic plants containing carotenoid biosynthetic gene *CsZCD*. ScientiaHorticulturae 112(2):172–175.
26. Raza A, Razzaq A, Mehmood SS, et al. (2019) Impact of climate change on crops adaptation and strategies to tackle Its outcome: A Review. *Plants (Basel)* 8(2):34.
27. Reis RR, da Cunha BA, Martins PK, Martins MT, Alekcevetch JC, Chalfun A Jr, Andrade AC, Ribeiro

AP, Qin F, Mizoi J, Yamaguchi-Shinozaki K, Nakashima K, CarvalhoJde F, de Sousa CA, Nepomuceno AL, Kobayashi AK, Molinari HB (2014) Induced over-expression of *AtDREB2A CA* improves drought tolerance in sugarcane. Plant Science 221(222):59–68.

28. Sakuma Y, Maruyama K, Osakabe Y, Qin F, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of an *Arabidopsis* transcription factor DREB2A, involved in drought responsive gene expression. The Plant Cell 18:1292–1309.
29. Sandhya J, Ashwini T, Manisha R, Vinodha M, Srinivas A (2021) Drought tolerance enhancement with co-Overexpression of DREB2A and APX in indica rice (Oryza sativa L.). American Journal of Plant Sciences 12(02):234-258.
30. Senapati S (2016) A review on research progress on in vitro regeneration and transformation of tomato. Annual Research & Review in Biology 9(6):1–9.
31. Shahriari F, Hashemi H, Hosseini B (2006) Factors influencing regeneration and genetic transformation of three elite cultivars of tomato (*Lycopersiconesculentum* L.). Pakistan Journal of Biological Sciences 9:2729-2733.
32. Sharma MK, Solanke AU, Jani D, Singh Y, Sharma AK (2009) A Simple and efficient *Agrobacterium*-mediated procedure for transformation of tomato. Journal of Biosciences 34(3):423-433.
33. Sharma V, Goel P, Kumar S, Singh AK (2019) An apple transcription factor, *MdDREB76*, confers salt and drought tolerance in transgenic tobacco by activating the expression of stress-responsive genes. *Plant Cell Rep* **38:**221–241.
34. Singh M, Kumar J, Singh S, Singh VP, Prasad SM (2015) Roles of osmoprotectants in improving salinity and drought tolerance in plants: a review. Reviews in Environmental Science and Bio/Technology 14(3):407–426.
35. Sun H, Sayaka U, Watanabe S (2006) A highly efficient transformation protocol for micro-tom, a model accession for tomato functional genomics. Plant Cell Physiology 47(3):426-431.
36. Sun S, Kang XP, Xing XJ, Xu XY, Cheng J, Zheng SW, Xing GM (2015) *Agrobacterium*-mediated transformation of tomato (*Lycopersiconesculentum* L. cv. Hezuo 908) with improved efficiency. Biotechnology & Biotechnological Equipment 29(5):861-868
37. Tawfik RS, Badr A, Sammour R, Ibrahim U, Matter M, Sakr M (2016) Improvement of flax drought tolerance using gene transfer. Plant Tissue Culture and Biotechnology 26(2):197-207
38. Van Eck J, Keen P, Tjahjadi M (2019) *Agrobacterium tumefaciens*-mediated transformation of tomato. Methods Mol Biol 1864:225-234.
39. Wang M, Zhuang J, Zou Z, Li Q, Xin H, Li X (2017) Overexpression of a *Camellia sinensis* DREB transcription factor gene (CsDREB) increases salt and drought tolerance in transgenic *Arabidopsis thaliana.* Journal of Plant Biology 60: 452–461.
40. Wei T, Deng K, Gao Y, Liu Y, Yang M, Zhang L, Zheng X, Wang C, Song W, Chen C, Zhang Y (2016) *Arabidopsis* DREB1B in transgenic *Salvia miltiorrhiza* increased tolerance to drought stress without stunting growth. Plant Physiology and Biochemistry 104:17-28.
41. Wu Z, Liang J, Zhang S, Zhang B, Zhao Q, Li G, Yang X, Wang C, He J and Yi M (2018) A canonical DREB2-type transcription factor in lily is post-translationally regulated and mediates heat stress response. Front. Plant Sci. 9:243. doi: 10.3389/fpls.2018.00243.
42. Xiu Y, Iqbal A, Zhu C, Wu G, Chang Y, Li N, Cao Y, Zhang W, Zeng H, Chen S, Wang H (2016) Improvement and transcriptome analysis of root architecture by overexpression of *Fraxinus pennsylvanica* DREB2A transcription factor in *Robinia pseudoacacia* L. 'Idaho'. Plant Biotechnology Journal 14(6):1456-69.