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TRANSFORMATION OF TOMATO VARIETY RIO GRANDE WITH DROUGHT RESISTANT TRANSCRIPTION FACTOR GENE *ATAF1* AND ITS MOLECULAR ANALYSIS

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Abstract

Tomato (*Solanum lycopersicum* L.) being an important vegetable is cultivated and used throughout the world. It not only contributes in fulfilling the basic nutritional requirements of the human body but also has many health benefits due to its rich biochemical composition. However, its production at large scale is hampered by many limiting factors such as biotic and abiotic stresses. Among the different abiotic stresses, drought poses drastic impact on tomato yield. Drought stress is genetically regulated by many transcription factors that not only regulate the stress responsive mechanism but also facilitate the growth and development of tomato plants. *NAC* is an important stress related transcription factor genes family, and the *ATAF1* gene, a member of this family, is involved in ABA signaling and stress response. In this study, tomato variety Rio Drande was transformed with drought resistant *ATAF1* gene *via Agrobacterium* mediated gene transformation method. The *ATAF1* gene was first cloned in the pK7WFG2 vector having kanamycin selectable marker and then it was introduced in the *Agrobacterium tumefaciens* strain *GV3101* through heat shock method. The tomato cotyledon and hypocotyl ex-plants of variety "Rio Ggrande" were cultured on callus induction medium (MS + 2.5 mg/L IAA + 2 mg/L BAP). The calli were then infected with *Agrobacterium tumefaciens* strain *GV3101* containing *ATAF1* gene and selection was carried out on the kanamycin selectable medium (MS + 100 mg/L Kan), and were regenerated on MS medium with 1 mg/L IAA + 1 mg/L BAP. Out of 216 putative transformed calli, 13 calli were able to regenerate on the selection medium. Of the 13 calli, three transgenic tomato plantlets were recovered, and these were confirmed through PCR analysis for the presence of 432 bp fragment of *ATAF1* gene. The transformation protocol reported here can be used to generate drought resistant tomato plants in future.

Abbreviations: ABA= Abscisic acid, ATAF1= Arabidopsis thaliana activating factor 1, BAP= 6-Benzylaminopurine, CIM= Callus induction medium, IAA= Indole-3-acetic acid, Kan= Kanamycin, Mg= Milligram, RM= Regeneration medium, µl= Microliter

Key words: *Agrobacterium tumefaciens*, drought stress, NAC transcription factor *ATAF1*, plant transformation, Rio Grande, tomato.

Introduction

Tomato belongs to the family Solanaceae, or "night shed" family, & it is a diploid crop with basic chromosome number (2n = 24). It ranked second in production and nutritional importance after potato worldwide. In Pakistan, tomato is cultivated on a large agricultural land with 60307 hectares having a production of 575923 tonnes (FAOSTAT, 2016). It has a significant share in Gross Domestic Product of the country. Tomato is a rich source of protein, vitamins and minerals along with the considerable amount of fibers and has a high amount of antioxidants such as vitamin C (Zhu *et al.*, 2018).

Tomato faces several abiotic stresses, of which drought is the most common one (Farooq *et al.*,, 2009), due to tomato's drought sensitive nature. Water demand during tomato initial vegetative growth phase and at fruit formation stage is very crucial and its deficiency can leads to very severe effects (Foolad *et al.*, 2003; Ashraf, 2010). Tomato growth and yield strongly depend upon water availability as its fruit comprised of 90% of water (Cao *et al.*, 2007). Water deficiency affects plant severely during all the stages of development as reported in the literature. It has been reported that drought stress causes a loss in the yield up to 25% during vegetative phase (25%), 43% in flowering phase and 52% in fruiting phase (Nuruddin *et al.*, 2003). It is also known that decrease in photosynthesis and stomatal closure is due to the lower water content (Zgallaï *et al.*, 2005).

Plant's response towards drought not relies on the single factor but it works in the cascade. When expose to water stress, plants regulate the gene expression which can be examined by changes in the morphological features (Ashraf, 2010). During initial osmotic stress under drought stress, plant response morphologically as observed in the form of modification in the root system. The roots penetrate deep in the soil to cover large area for the availability of the water and to maintain proper signaling pathways between root and shoot (Jones *et al.*, 2009). Plant response to drought stress cannot be easily and quickly elaborated *via* single plant

response mechanism as the plant response both at physiological and genetic levels. Signaling channel depends on the severity of the stress which directs the plant to respond by either morphological alteration or gene regulation (Blum, 2005). The role of abscisic acid (ABA) in the normal plant growth is very important under stressful conditions (Hu *et al.,* 2012; Narusaka *et al.,* 2003). Under the extreme environmental stress conditions such as drought and/or salinity the ABA regulate the stomatal conductance to maintain the osmotic level (Yoshida *et al.,* 2006).

Traits like drought are controlled by multiple genes, where environmental variations play a key role (Sivasakthi *et al.,* 2018). Drought stress effects on the plant can be controlled by developing tolerance as well as by improving the management practices. Management measures are costly in terms of time, energy and money. So to achieve this goal different methodologies are screening, selection, conventional breeding, molecular breeding and genetic engineering (Farooq *et al.,* 2009). Even though the tolerance mechanisms are complex and interlinked genetically and also sometimes has potential negative impacts, a great progress through the genetic transformation has been made to obtain the stress tolerant plants (Athar *et al.,* 2010).

TFs play a significant role in the regulation of genes by acting on the *cis*-regulating elements. The role of TFs has not been entirely elucidated but these factors play a crucial role in the regulation of stress responsive genes. There are certain TFs like C-binding elements/ dehydration responsive elements (*CBF1/DREB*) and abscisic acid-responsive element binding (*AREB*) which elevate the expression of drought responsive genes. Jaglo-Ottosen *et al.,* (1998) have reported the elevated expression of freeze tolerant genes when *CBF1* was introduced into the *Arabidopsis.* Others researchers have also reported the improved drought stress tolerance by using this CBF1 transcription factor (Gilmour *et al.,* 2000). The effects of overexpression of cold and drought responsive genes in rice with the incorporation of *OsDREB1* has been studied (Zhang *et al.,* 2009). Another transcription factor genes family known as NAC, is also used for the production of transgenic crops tolerant to biotic and abiotic stresses. Previously, it has been reported that three NAC gene family in the *Arabidopsis* plant were responsible for the up-regulation of drought responsive genes (Tran *et al.,* 2004). Takasaki *et al.* (2010) have also validated the NAC family genes expression in rice which enhance the tolerance of rice plant to drought with considerable encouraging results. *ATAF1* gene, a NAC transcription factor gene regulate ABA signaling and stress response (Jensen *et al.,* 2010; Wu *et al.,* 2009). In addition, *ATAF1* regular expressed in the different organs of plant and regulate stomatal conductance (Lu *et al.,* 2006). Previous studies suggest that the removal of *ATAF1*gene leads to the weak response to the ABA signaling both under stress and normal growth conditions (Jensen *et al.,* 2008). The over expression of *ATAF1* gene suggests the strong response to stress environment by the accumulation of ABA (Wu *et al.,* 2009). *ATAF1* Mode of action of ATAF1 gene has been studied in the yeast two hybrid system which interacts with sucrose non-fermenting 1 (SNF1) protein and thus channelize the plant response towards the stress conditions (Baena-González *et al.,* 2007; Jossier *et al.,* 2009). In plant, the interaction between the *ATAF1* transcription factor and its target genes of ABA biosynthesis pathway validates the promising plant response towards the stress environment by enhancing the response toward ABA signaling (Baena-González *et al.,* 2007).

In view of the devastating effects of drought, there is a need to develop drought resistant tomato plants. Plant transformation technology can develop drought resistant tomato for increased production (Gosal *et al.,* 2009). In this study, transformation of tomato variety Rio Grande with *ATAF1* gene was performed to optimize transformation protocol and also analyze the transgenic tomato plantlets at molecular level.

Materials and Methods

The current research work was performed at Biotechnology Laboratory of Environmental Sciences Department at COMSATS Institute of Information Technology, Abbottabad.

Tomato seeds collection: The seeds of tomato variety Rio Grande were kindly provided the seed bank of National Agriculture Research Center, Islamabad. Rio Grande is an open pollinated tomato variety, grows well in hot climate, well known for its tomato paste quality and was developed originally by University of California in 1994.

Surface sterilization: The seeds of the tomato variety Rio Grandi were surface sterilized before sowing on MS medium (Murashige & Skoog, 1962). Seeds were soaked in distilled water overnight and then treated with 70% ethanol for one minute followed by 7% (w/v) sodium hypochlorite (commercial bleach) for five minutes and after that the seeds were rinsed three times with the same volume of distilled water for five minutes each time. The seeds were dried with sterilized filter paper before culturing on MS medium. One liter MS medium was prepared by adding 4.44 gram of solid MS medium with vitamins (Bioworld) and 30 grams sucrose. pH was adjusted to 5.8 via NaOH and/or HCl by using a pH meter (Jenway 3505) and then eight grams of agar was added to the medium and autoclaved at 121°C for 20 minutes at 15 Psi pressure. The filter sterilized antibiotics were added to the medium in laminar air flow hood to avoid any contamination.

Seedlings preparation for transformation: The surface sterilized seeds of tomato variety Rio Grande were sown on ½ MS medium sterile polystyrene petri plates. Total 10 petri plates were used and each petri plate contained 10 seeds. These petri plates were placed in a growth chamber with 16/8 light, dark period respectively and 25 ±1°C temperature.

Media used for tomato tissue culture: For the induction of callus, the MS medium was supplemented with growth regulators Auxin (IAA) and Cytokinin (BAP) in various concentrations (Table 1). The calli induced from leaves explants, were put on the different regeneration media

(Table 1). The regenerated explants were subcultured after each 3 to 4 weeks and were then transferred to rooting medium (MS + 0.5 mg/L IAA). The selection medium consists of MS + 100 mg/L kanamycin.

Callus induction from tomato explant: Hypocotyls and leaf discs were cut in uniform size approximately, 1 cm in length from 2-3 weeks old *In vitro* grown, seedlings. These were put on CIM for 28 days.

Construct* containing gene of interest used in the current study*: The construct used in the current study, was kindly provided by Dr. Salma Balazadeh (Garapati *et al.,* (2015). In brief, this construct contains *ATAF1* gene with 35S promoter, 35S: *ATAF1*-GFP gene and selectable markers kanamycin and spectinomycin.

Competent *Agrobacterium GV3101* cells preparation: The competent cells of *GV3101* were prepared using the calcium chloride ($CaCl_2$) method. 1ml of *GV3101* from stock culture was inoculated into 50 ml of LB medium and was incubated at 28°C in shaking incubator for overnight. After 12 hours of incubation the OD at 600 nm wavelength was measured using a spectrophotometer. After measuring the optical density, it was centrifuged at 6000 rpm for 10 min at 4°C. The supernatant was discarded and the cells were resuspended in 25 ml of ice-cold 50 mM calcium chloride ($CaCl_2$) solution. The suspension was kept on ice for 10 min and was re-centrifuged at 6000 rpm for 10 min at 4°C. The supernatant was discarded again and the pellet was resuspended in 3 ml of 30% glycerol. Aliquots of 100 ul in 1.5 ml tubes were prepared and preserved at -80°C.

Transformation of *Agrobacterium tumefaciens GV3101* with pk7FWG2 plasmid: *Agrobacterium tumefaciens GV3101* was transformed with pk7FWG2 plasmid through heat hock method. For this, 100 ul of the competent cells were taken from -80°C freezer and inoculated in 1.5 ml eppendorf tube. The eppendorf tube was put on ice for 20-30 min and then added 50ng (1µl) of DNA (pK7FWG2). The tube with DNA and *GV3101* cells were incubated on ice for 10 min to thaw a competent cell. After 10 min of incubation the tube was transferred to a water bath at 42°C for 45 sec. The tube was put back on ice for 2 min to reduce damage to the cells. After that 1 ml of LB without antibiotics was added and the tube was incubated for one hour at 28°C. The resulted culture of 100 µl was then spread on solid LB plate with 100 mg/L of spectinomycin and incubated for overnight at 28°C. The transformation of the *Agrobacterium* was confirmed by colony PCR.

***Agrobacterium* mediated transformation of tomato:** The overnight grown culture of *Agrobacterium tumefaciens GV3101* was determined for the OD at 600 nm wavelength. A culture with OD of upto 0.8 was selected. The calli were infected with the *GV3101* for 15 to 20 minutes. To remove the excessive *Agrobacterium* the calli were blotted on sterile filter paper after infection. The explants were then cultured on co-cultivation medium for 48 hours and kept at 25 ± 2°C and low light intensity of 15 µmol m^2/s. The calli were transferred to selective medium after co-cultivation period of two days. The medium for co-cultivation was same as CIM.

Selection of transformed calli: The selection of transgenic calli was carried out on selective medium that contained 2 mg/L BAP, 1.5 mg/L IAA, 100 mg/L kanamycin and 200 mg/L cefotaxime. Only the transgenic calli were able to grow on this selective medium.

Regeneration of transgenic calli: After two weeks on selective medium the transgenic calli were transferred to regeneration medium which contains 100 mg/L kanamycin and 200 mg/L cefotaxime.

PCR analysis of transgenic tomato plantlets

DNA extraction from tomato leaves: DNA extraction was carried out according to CTAB method (Porebski *et al.,* 1997). Briefly, 250 mg of transgenic tomato leaves was taken and ground into the fine powder with pestle and mortar in liquid nitrogen. The sample was collected in the 1.5 ml sterile eppendorf tube to which 600 µl of CTAB extraction buffer was added and mixed gently. Then the tube was placed in the water bath for 30 min at 65°C. After incubation, 600 µl of chloroform isoamyl alcohol (24:1) was added and centrifuged at 11000 rcf for 15 min at 4°C. The supernatant was transferred to the fresh eppendorf and an equal volume of chloroform isoamyl alcohol was added. Again centrifugation was carried out at the same condition as mentioned earlier. The supernatant was collected in the fresh sterile eppendorf and an equal volume of chilled isopropanol was added and placed on the -20°C for overnight. Next day, the samples were centrifuged at 11000 rcf for 15 min at 4°C. The pellet was then washed with 70% ethanol and let it dried for two hours. The pellet was dissolved in double distilled water and placed at -20°C. The DNA quality was checked on 1% agarose gel.

PCR analysis of the transgenic plantlets: The Polymerase Chain Reaction was carried out for the detection of desire gene in the transformed tomato leaves. The reaction mixture was prepared of 10ul in 200ul of PCR tube containing 1 µl of forward primers, 1 µl reverse primers, 1 µl of DNA sample (tomato leaf), 5 µl of PCR master mix (Bio Basic) and 2 µl of nuclease free water. Denaturing step was carried out at 94°C for 4 minutes, followed by 36 cycles of 30 second at 94°C; 30 seconds at 56°C for annealing of primers and 30 seconds at 72°C for amplification of *ATAF1* gene. After completion of 36 cycles the reaction was left at 72°C for 4 min. The *ATAF1* gene was amplified with *ATAF1*-Forward: 5´-GTTG TTTACGGCGACGAAATC-3´ and *ATAF1*-Riverse: 5´-TAAAACGGTCTCGTGTTGCCA TAA-3´ primers.

To analyze PCR product, 8µl of PCR product was mixed with dye and loaded into the well of the agarose gel and was run at 100 voltage for 45 min. The amplicons of 432 bp size were detected under the UV light using gel documentation system.

Phylogenetic studies of *ATAF1* gene: To see the evolutionary relationship of the *ATAF1* gene in different crops, phylogenetic studies were performed. *ATAF1* DNA and protein sequences were retrieved using BLAST bioinformatics tools of NCBI. These sequences were aligned using CLUSTALW software and finally phylogenetic tree was constructed using an on-line available bioinformatics tool phylogeny.fr (http://www.phylogeny.fr).

Results

Insertion and confirmation of *ATAF1* gene in plasmid pk7FWG2: In pk7FWG2 vector two selectable marker genes; Kan (kanamycin resistant gene) and Spec (spectinomycin resistant gene) (Fig. 1) for selection of transformants are present. As kanamycin and spectinomycin are plant and bacterial selectable marker genes respectively. So their selection was made on kanamycin and spectinomycin selective media. The presence of *ATAF1* gene was confirmed by PCR. The desire gene *ATAF1* was about 432 bp fragment which was observed on 1.5% gel under UV light using gel documentation system (Fig. 2).

Screening and confirmation of transformed strain *Agrobacterium tumefeciens* (*GV3101*): First, selection of transformed *Agrobacterium tumefacien* (GV3101) with pk7WG2 plasmid was done on spectinomycin selective medium. After that colony PCR was done for further confirmation and desire gene product *i.e.,* 432 bp *ATAF1* gene was observed (Fig. 3).

Callus induction from tomato explants: To optimize the best medium for callus induction for tomato explants, four different types of callus induction media (CIM) were used which are mentioned in Table 1. The best callus induction medium from two different explants sources *i.e.,* leaf discs and hypocotyls was selected for callogenesis. The results for callogenesis were recorded in each callus induction medium (CIM) where the best results were recorded on CIM_2 (Table 2).

Leaf discs showed better response towards callogenesis than hypocotyls. In each CIM 50 explants were cultured and the percent calli were calculated. Although all the CIM responded, but compared to others CIM, CIM_2 showed better results for callus induction (68%). The callogenesis percentage on CIM_3 was noted as 52% while in CIM_1 and CIM_4 calli formation were noted as 42% and 46% respectively. The percentage of total calli induced and the necrotic calli are given in Table 2.

For hypocotyls, 50 hypocotyls explants were cultured on different callus induction media as given in Table 2. CIM_2 showed better response towards callogenesis (42%) than other callus induction media. The formation of calli in other media *i.e*., CIM_1, CIM_3 and CIM_4 were 26%, 34% and 38%, respectively. The percentages of callogenesis from hypocotyls are given in Table 2.

Table 1. Different callus induction and regeneration media used in the current research.

CIM	Composition (mg/L)	RM	Composition (mg/L)
CIM_1	MS + 2.5 IAA	RM_1	MS + 5 BAP, 1 IAA
CIM_2	MS + 2.5 IAA + 2 BAP	RM_2	MS + 1 BAP + 1 IAA
CIM_3	MS + 0.2 IAA + 0.2 GA_3	RM_3	MS + 2 BAP + 0.2 IAA, 0.2 GA_3
CIM_4	MS + 3 BAP + 1 GA_3	RM_4	MS + 3 BAP, 2 IAA + 1 GA_3

CIM: Callus induction medium, RM: Regeneration Medium

Table 2. Callus induction from cotyledon leaf and hypocotyl explants on different media.

Medium	Nos of explant	Calli (%)	Necrotic calli (%)	Healthy calli (%)
	Cotyledon leaf disc explants			
CIM_1	50	21 (42)	6 (12)	15 (30)
CIM_2	50	34 (68)	4 (8)	30 (60)
CIM_3	50	26 (52)	5 (10)	21 (42)
CIM_4	50	23 (46)	5 (10)	18 (36)
	Hypocotyl explants			
CIM_1	50	13 (26)	5 (10)	8 (16)
CIM_2	50	21 (42)	0 (0)	21 (42)
CIM_3	50	17 (34)	2 (4)	15 (30)
CIM_4	50	19 (38)	5 (10)	14 (28)

Table 3. Regeneration of explants on different regeneration media.

Medium code	No. of explants		Regenerated plantlets		% Of regenerated plantlets	
	Cotyledons	Hypocotyls	Cotyledons	Hypocotyls	Cotyledons	Hypocotyls
RM_1	15	15	9	6	60	40
RM_2	15	15	13	8	87	53
RM_3	15	15	7	5	46.7	33
RM_4	15	15	9	6	60	40

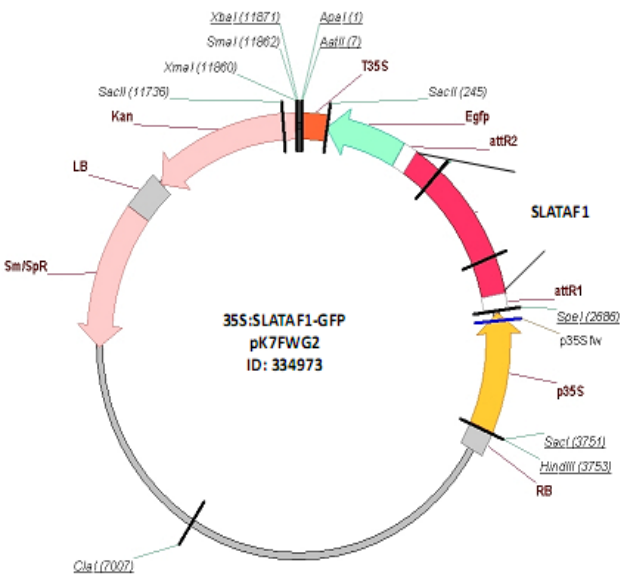


Fig. 1. Map of vector pK7FWG2 adapted from Garapati *et al.*, (2015). Fig. 1 is very poor could not publish

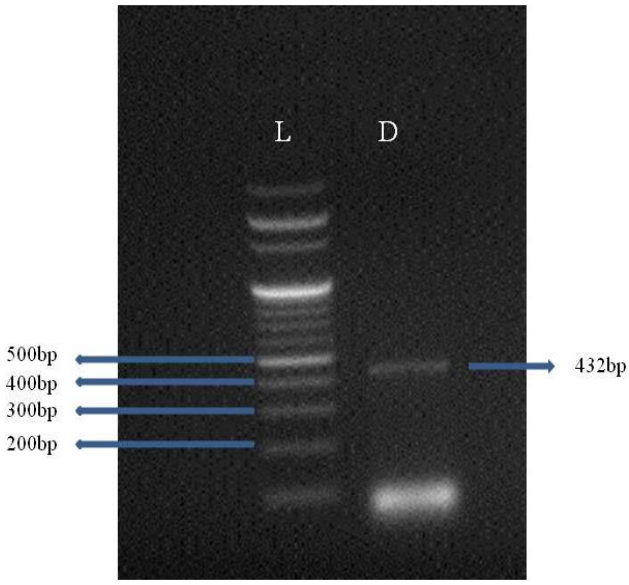


Fig. 2. PCR product of plasmid pK7WFG2 DNA; L: 1kb DNA ladder, D: 432 bp of *ATAF1* gene.

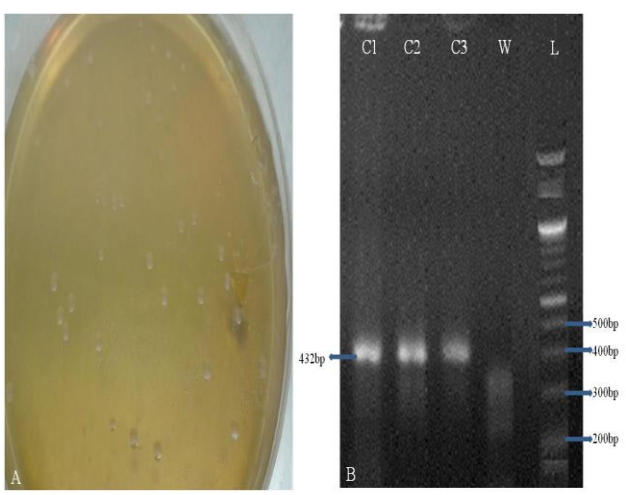


Fig. 3. Selection of transformed strain *Agrobacterium tumefaciens* (*GV3101*) on spectinomycin selective medium and colony PCR. A: *Agrobacterium tumefaciens* (*GV3101*) colonies on spectinomycin selective medium, B: Colony PCR product where C1, C2 and C3 are the transformed *Agrobacterium* colonies, W: non transformed *Agrobacterium tumefaciens* (*GV3101*) as negative control and L: 1kb ladder.

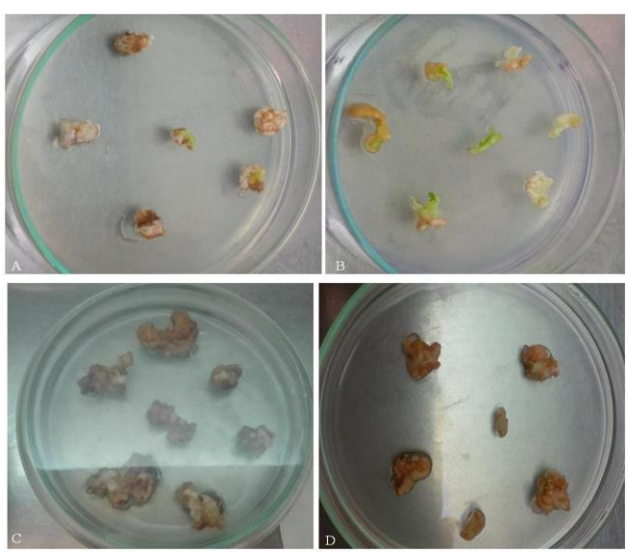


Fig. 4. The callus induction from hypocotyls and cotyledons explants. A: initiation of callus from cotyledons after 3 weeks, B: callus induction from hypocotyls explants after 3 weeks, C: after 5 weeks the callus induced from cotyledons explants, D: after 5 weeks the callus induced from hypocotyls explansts.

Regeneration of explants on different regeneration media: In order to optimize the regeneration medium, four different regeneration media (RM) were tried. These four different types of media contain growth hormones IAA and BAP in various concentrations. 15 explants were cultured ON each regeneration medium (RM). RM that showed optimum regeneration of explants was selected for regeneration of calli. RM_2 gave optimum regeneration rate on which 15 explants were cultured and 13 explants were regenerated (87%) for cotyledons and 53% for hypocotyls while RM_3 showed low regeneration which was 46.7% for cotyledons and 33% for hypocotyls and the other two media rm_1 and rm_4 gave 60% regeneration for cotyledons and 40% for hypocotyls (Table 3, Fig. 5).

Transgenic calli: Total 216 calli were infected with *Agrobacterium tumefaciens* (*GV3101*) and co-cultivated for two days in co-cultivation medium. After co-cultivation the calli were transferred to selection medium. Transgenic calli were put on selection medium containing 100 mg/L kanamycin. Only the transformed calli showed growth on selective medium Fig. 6). Out of 216 calli, only 13 calli (6%) showed growth on selective medium.

Regeneration of transgenic calli: A total of 216 calli were infected and put on RM2. Of these, 13 calli regenerated into plantlets. Of these 13 plantlets, 9 were from cotyledon and 4 from hypocotyls. The transgenic calli were transferred to regeneration medium (RM_2) after

two weeks on selective medium. The RM_2 contained 100 mg/L kanamycin and 200 mg/L cefotaxime antibiotics. Figure 6 shows the shoot induction of transgenic calli on RM_2. A transgenic plantlet is shown in Fig. 7.

PCR analysis of transgenic tomato plantlets: The DNA of transgenic tomato leaves and control tomato leaves was extracted for further confirmation of transgenic plantlets. The PCR products and 1kb DNA ladder were loaded on 1% agarose gel for 45 min at 100 voltage of electric field. After gel electrophoresis, the gel was studied under UV light using gel documentation system. The desire fragment of *ATAF1* gene which is about 432 base pairs can be seen in Fig. 8.

Phylogenetic study of *ATAF1* gene: Using the full length cDNA of *ATAF1* gene sequence (1317 bp) BLAST search was carried out to align the sequence with the sequence of *Solanum lycopersicum*, however, no significant similarity was found. The sequence was then aligned to other related species and the phylogenetic tree was made from the sequences. The phylogenetic tree shows that *ATAF1* (the *Arabidopsis thaliana* gene) sequence was more similar to *Capsella rubella* suggesting it as a close partner from an evolution point of view followed by *Thellungiella halophilia* and *Eutrema salsugineum* as a close relative. These later two species seem to be derived from *Brassica napus*, and then *Tarenaya hassleriana*. *Momordica charantia* seems to be the distant relative of the *ATAF1* gene from which all the other species are derived (Fig. 9).

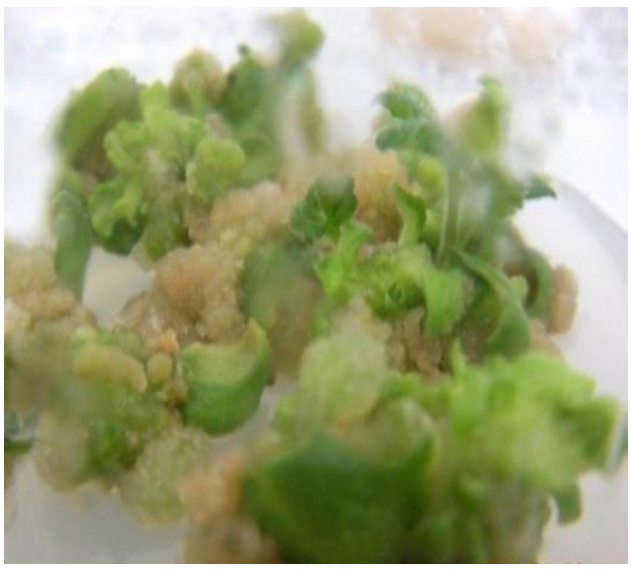


Fig. 5 Regeneration of non transgenic tomato explants on SRM_2 after 5 weeks of culturing on regeneration medium.

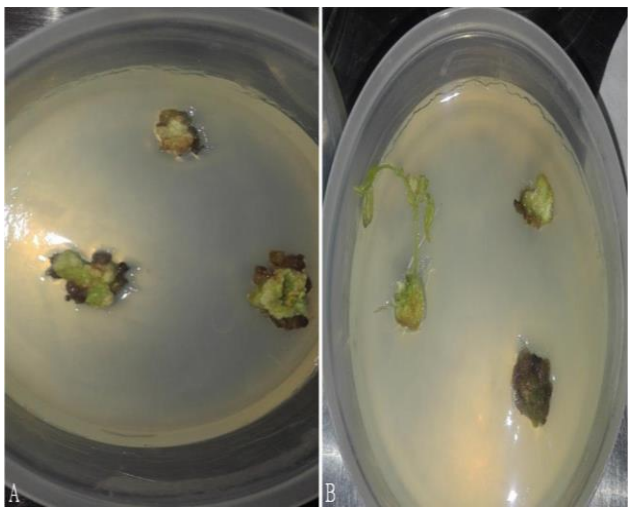


Fig. 6. The growth of transgenic calli on kanamycin selective medium. A: transgenic calli on kanamycin selective medium after two weeks, B: transgenic calli on kanamycin selective medium after 5 weeks.

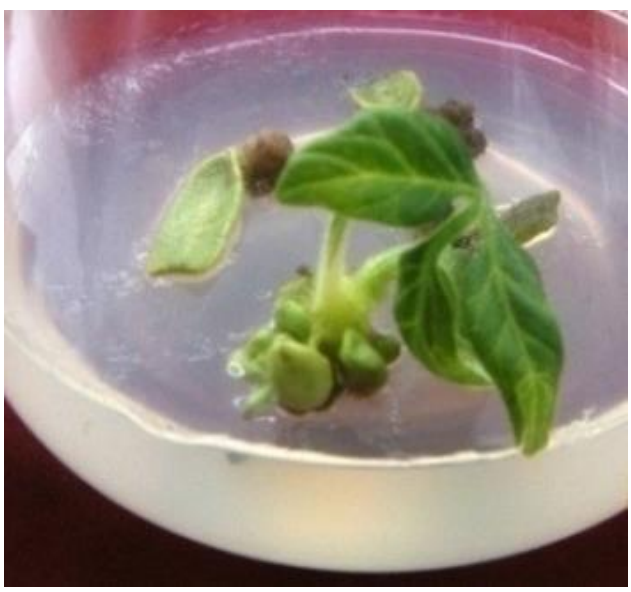


Fig. 7. Regeneration of transgenic plantlet of tomato explants after seven weeks on regeneration medium supplemented with 100 (mg/L) kanamycin and cefotaxime.

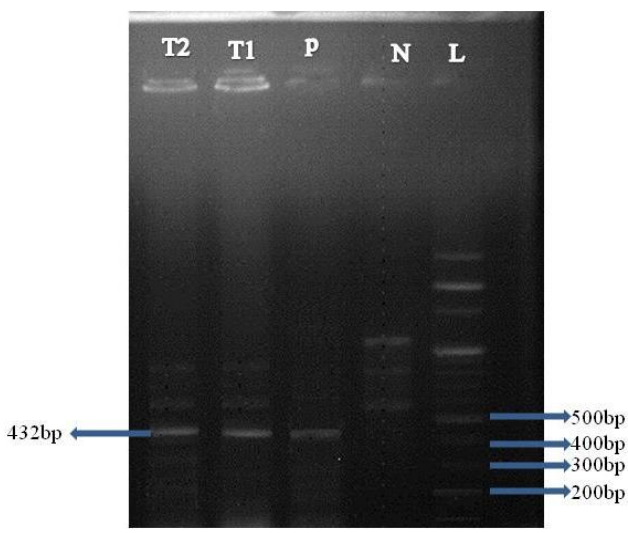
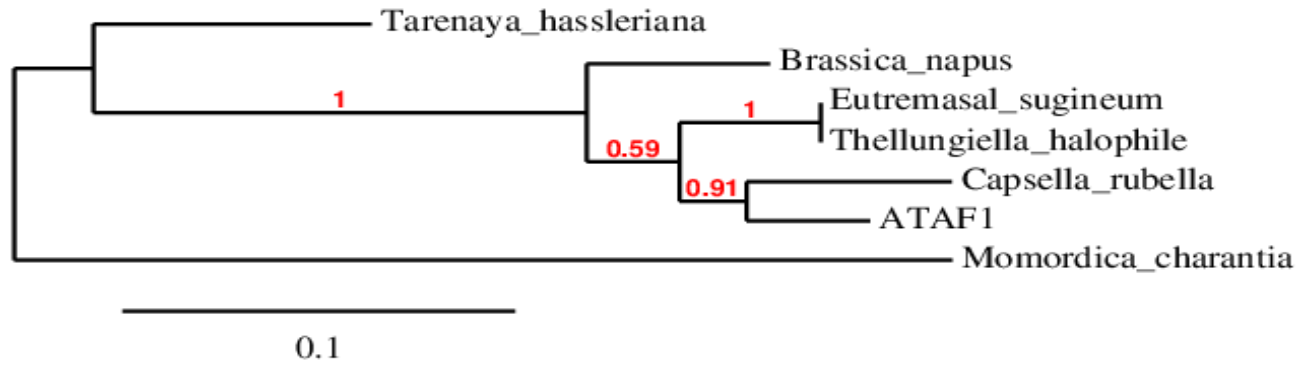
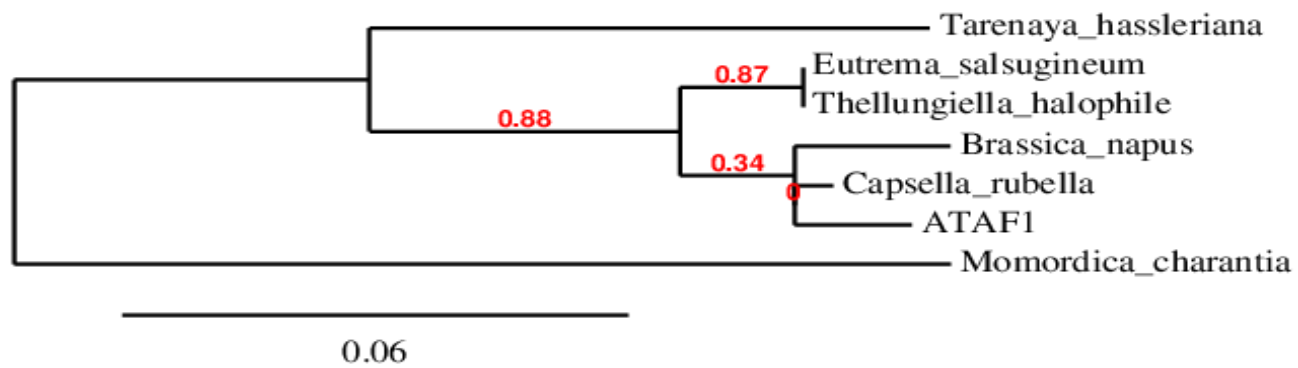


Fig. 8. PCR results of *ATAF1* gene, L: 1 Kb ladder of DNA, N: Non transgenic tomato leaf extracted DNA as negative control, P: plasmid pK7FWG2 DNA as positive control, T1 and T2: transgenic tomato leaf DNA. 432bp fragment confirmed the presence of *ATAF1* gene.



a. DNA



b. Protein

Fig. 9 Phylogenetic tree of *ATAF1* sequence. a. DNA based and b. protein based.

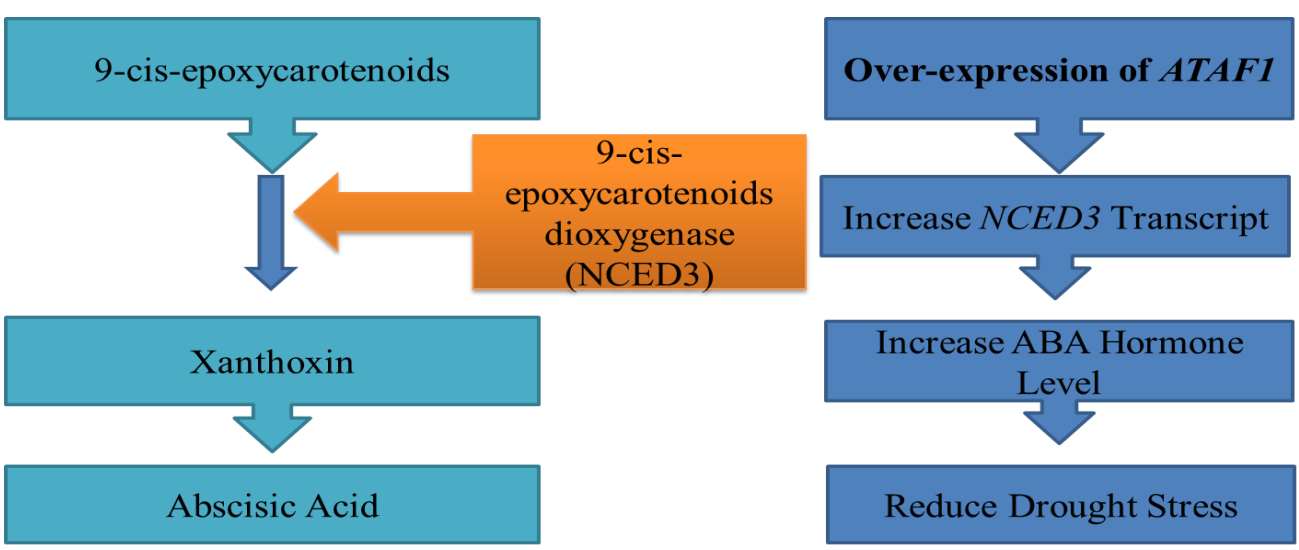


Fig. 10. Metabolic pathway of ABA production in plants. The over expression of *ATAF1* gene induce for increased ABA as a final product and thus help plants to overcome the drought stress.

Discussion

This study was conducted as a first step towards developing transgenic tomato variety "Rio Grande" containing *ATAF1* gene that world reduce the drought stress. *ATAF1* gene increases the production of abscisic acid and thus overcome drought stress (Fig. 10).

The morphogenetic responses of *In vitro* culture plants are affected in the culture medium by different components and therefore, evaluating their effects on plant callus induction and regeneration are essential (Elaleem *et al.*, 2009). The callus was initiated from cotyledons leaf and hypocotyls explants after 15-20 days in MS medium supplemented with cytokinin (BAP) and Auxin (IAA) in different combinations. The induction of callus was affected by the plant growth regulators and the types of explants used. Plant growth regulators auxin and cytokinin had a distinctive effect on both types of explants for the production of callus. Pal *et al.*, (2007) reported that *In vitro* production of callus is dependent on the exogenously supplied type of growth regulators as well as the endogenous concentration of growth regulators.

The cotyledons leaf explants showed good response towards the callogenesis than hypocotyls explants (Table 2). The highest number of calli have been obtained on MS medium containing 2 mg/L BAP and 2.5 mg/L IAA with 68% calli from cotyledons leaf explants and 42% calli from hypocotyls. Other researchers, Osman *et al.,* (2010), have reported hypocotyls as the best source for callus induction rather than cotyledon explant though they also used different growth hormone for callogenesis *i.e.,* NAA in various concentrations rather than BAP and IAA combination. They have reported 93% calli when used 2 mg/L NAA and 86% calli when used 0.5 mg/L NAA. While, other researchers have reported the cotyledon as the best source for callus induction using 2 mg/L of zeatin in combination with 0.1 mg/L of 3-indoleacetic acid (Khaliluev *et al.,* 2014). The reasons for this different response of the different explants towards callus induction may be the different growth hormones and also the tissue specific response of these explants. Cotyledons are the most active leaves and this may enhance their response towards callus induction when exposed to the optimum combinations and concentrations of growth hormones.

Different regeneration media with different combinations of growth regulators have been used in current study. As 1 mg/L IAA in combination with 1 mg/L BAP gave good results of regeneration (87%) on the other hand the medium containing 1 mg/L IAA with 5 mg/L BAP showed a decline in regeneration (60%) while MS containing 0.2 mg/L IAA, 2.0 mg/l BAPand 0.2 mg/L GA3 showed lowest number of calli (46.7%). *In vitro* plant regeneration by using leaf explants gave better results on MS medium containing zeatin along with IAA (Ahsan *et al.,* 2007). Osman *et al.,* (2010) reported that the maximum regeneration from the callus was observed (93%) in MS medium supplemented with TDZ in combination with 0.5 mg/L BAP. The current study supports that the IAA in combination with BAP leads to better plant regeneration (Table 3). The increasing BAP concentration enhanced the plant regeneration up to some degree but increased concentration also decreased the regeneration rate as reported by Mukta (2014).

Many factors affect the *Agrobacterium* mediated transformation efficiency. These factors could be the concentration of *Agrobacterium*, phenolic compounds, addition of selective agents, cocultivation duration, composition of media and plant genotypes (Cortina and Culiáñez-Macià, 2004; Qiu *et al.,* 2007). The current study showed that cotyledon leaf performed well for gene transformation in comparison to hypocotyls as out of 13 transgenic plants 9 were cotyledon leaf explants and the remaining 4 obtained from hypocotyls explants. Alatar *et al*., (2017) reported that the cotyledon leaf was found to be more efficient for transformation in tomato as compare to other explants due to its better regeneration ability. However, Sun *et al.,* (2015) reported slightly higher i.e. 70% regeneration frequency for hypocotyl explants as compared to 68% for that of cotyledon explants.

For the transformation 5 min infection did not elicit a positive response while with the increase in the infection period to the 15 to 30 min provide good transformants. Khoudi *et al.,* (2009) reported that the rate of transformation was increased by increasing the time of infection of explants. At 5 min infection time the rate of transformation was 15% when the infection time was increased upto 15 min the rate of transformation was increased to 67%. This supports that to get the transformed plantlets time of infection time plays a vital role.

Agrobacterium culture at the 0.8 OD_{600} showed good results for infection of tomato explants leading to the good amount of transformed plant with the use of appropriate dosage of antibiotic to inhibit the *Agrobacterium* proliferation in selective media. Qiu *et al.,* (2007) and John *et al*., (2014) also found that the most appropriate range for infection of explants through *Agrobacterium* was 0.6-0.8 OD_{600} and the *Agrobacterium* proliferation was controlled by antibiotic dosage but also inhibited the shoot induction when its dosage was increased. Park *et al.,* (2003) reported that the rate of transformation in cotyledon leaf explant was increased by various factors such as by increasing incubation time, increasing the optical density from 0.5-0.9 at 600 nm but the increase should be in optimal range as increase in incubation time or high amount of O.D could lead to poor transformation with the negative impact on plant regeneration.

For the primary selection of transgenic plants selective medium supplemented with the 50 mg/L and 100 mg/L kanamycin has been used. Both concentrations were good for the screening of positively transformed tomato plantlets. Cortina & Culiáñez-Macià (2004) reported that the primary selection of transgenic plants was carried out on the selective MS medium supplemented with 50 mg/L and 100 mg/L kanamycin. For the screening of transformed plants it is good to use the low dose of kanamycin as 50 mg/L because the high dose of kanamycin could degrade the plant proteins leading to disruption of normal plant growth (Cortina & Culiáñez-Macià, 2004).

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

References

Ahsan, N., S.H. Lee, D.G. Lee, M. Anisuzzaman, M.F. Alam, H.S. Yoon, M.S. Choi, J.K. Yang and B.H. Lee. 2007. The effects of wounding type, preculture, infection method and cocultivation temperature on the Agrobacterium-mediated gene transfer in tomatoes. *Ann. Appl. Biol.,* 151: 363-372.

Alatar, A.A., M. Faisal, E.M. Abdel-Salam, T. Canto and Q. Saquib. 2017. Efficient and reproducible *In vitro* regeneration of Solanum lycopersicum and assessment genetic uniformity using flow cytometry and SPAR methods. *Saudi J. Biol. Sci.,* 24: 1430-1436.

Ashraf, M. 2010. Inducing drought tolerance in plants: Recent advances. *Biotechnol. Adv.,* 28: 169-183.

Athar, H.R., A.R. Bhatti, N. Bashir, Z.U. Zafar, Abida and A. Farooq. 2010. Modulating infestation rate of white fly (*Bemicia tabaci*) on okra (*Hibiscus esculentus* L.) by nitrogen application. *Acta Physiol. Plant.*, 33: 843-850.

Baena-González, E., F. Rolland, J.M. Thevelein and J. Sheen. 2007. A central integrator of transcription networks in plant stress and energy signalling. *Nature*, 448: 938-942.

Blum, A. 2005. Drought resistance, water-use efficiency, and yield potential-are they compatible, dissonant, or mutually exclusive? *Aust. J. Agric. Res.*, 56: 1159-1168.

Cao, H.X., Z.B. Zhang, P. Xu, L.Y. Chu, H.B. Shao, Z.H. Lu and J.H. Liu. 2007. Mutual physiological genetic mechanism of plant high water use efficiency and nutrition use efficiency. *Colloids Surf. B Biointerfaces*, 57: 1-7.

Cortina, C. and F.A. Culiáñez- Macia. 2004. Tomato Transformation and Transgenic Plant Production. *Plant Cell Tissue Organ Cult.*, 76: 269-275.

Elaleem, K.G.A., R.S. Modawi and M.M. Khalafalla. 2009. Effect of plant growth regulators on callus induction and plant regeneration in tuber segment culture of potato (*Solanum tuberosum* L.) cultivar Diamant. *Afr. J. Biotechnol.*, 8.

FAOSTAT. 2016. http://www.fao.org/faostat/en/#data/QC

Farooq, M., A. Wahid, N. Kobayashi, D. Fujita and S.M.A. Basra. 2009. Plant drought stress: effects, mechanisms and management. *Agron. Sustain. Dev.*, 29: 185-212.

Foolad, M.R., P. Subbiah, C. Kramer, G. Hargrave and G.Y. Lin. 2003. Genetic relationships among cold, salt and drought tolerance during seed germination in an interspecific cross of tomato. *Euphytica*, 130: 199-206.

Gilmour, S.J., A.M. Sebolt, M.P. Salazar, J.D. Everard and M.F. Thomashow. 2000. Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol.*, 124: 1854-1865.

Gosal, S.S., S.H. Wani and M.S. Kang. 2009. Biotechnology and Drought Tolerance. *J. Crop Improv.*, 23: 19-54.

Hu, X., X. Wu, C. Li, M. Lu, T. Liu, Y. Wang and W. Wang 2012. Abscisic acid refines the synthesis of chloroplast proteins in maize (*Zea mays*) in response to drought and light. *PLOS ONE* 7, 49500.

Jaglo-Ottosen, K.R., S.J. Gilmour, D.G. Zarka, O. Schabenberger and M.F. Thomashow. 1998. Arabidopsis CBF1 overexpression induces *COR* genes and enhances freezing tolerance. *Science*, 280: 104-106.

Jensen, M.K., P.H. Hagedorn, M. De Torres-Zabala, M.R. Grant, J.H. Rung, D.B. Collinge and M.F. Lyngkjaer. 2008. Transcriptional regulation by an NAC (NAM–ATAF1, 2–CUC2) transcription factor attenuates ABA signalling for efficient basal defence towards *Blumeria graminis* f. sp. hordei in Arabidopsis. *Plant J.*, 56: 867-880.

Jensen, M.K., T. Kjaersgaard, K. Petersen and K. Skriver. 2010. NAC genes. *Plant Signal. Behav.*, 5: 907-910.

John, E., A. Maqbool and K.A. Malik. 2014. Optimization of *Agrobacterium tumefaciens* mediated transformation in *Populus deltoides*. *Pak. J. Bot*., 46: 1079-1086.

Jones, D.L., C. Nguyen and R.D. Finlay. 2009. Carbon flow in the rhizosphere: carbon trading at the soil root interface. *Plant Soil*, 321: 5-33.

Jossier, M., J.P. Bouly, P. Meimoun, A. Arjmand, P. Lessard, S. Hawley, H.D. Grahame and M. Thomas. 2009. SnRK1 (SNF1-related kinase 1) has a central role in sugar and ABA signalling in *Arabidopsis thaliana*. *Plant J.*, 59: 316-328.

Khaliluev, M.R., L.R. Bogoutdinova, G.B. Baranova, E.N. Baranova, P.N. Kharchenko and S.V. Dolgov. 2014. Influence of genotype, explant type, and component of culture medium on *In vitro* callus induction and shoot organogenesis of tomato (*Solanum lycopersicum* L.). *Biol. Bull*., 41: 512-521.

Khoudi, H., A. Nouri-Khemakhem, S. Gouiaa and K. Masmoudi. 2009. Optimization of regeneration and transformation parameters in tomato and improvement of its salinity and drought tolerance. *Afr. J. Biotechnol.*, 8.

Lu, P.L., N.Z. Chen, R. An, Z. Su, B.S. Qi, F. Ren, J. Chen and X.C. Wang 2006. A novel drought inducible gene, *ATAF1*, encodes a NAC family protein that negatively regulates the expression of stress-responsive genes in Arabidopsis. *Plant Mol. Biol*., 63: 289-305.

Mukta, F.A. 2014. Study of *In vitro* regeneration and transformation parameters for the development of transgenic Tomato (*Solanum Lycopersicum* L.). BRAC University.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 15: 473-497.

Narusaka, Y., K. Nakashima, Z.K. Shinwari, Y. Sakuma, T. Furihata, H. Abe, M. Narusaka, K. Shinozaki and K. Yamaguchi-Shinozaki. 2003. Interaction between two cis-acting elements, ABRE and DRE, in ABA-dependent expression of Arabidopsis rd29A gene in response to dehydration and high-salinity stresses. *Plant J.*, 34: 137-148.

Nuruddin, M.M., C.A. Madramootoo and G.T. Dodds. 2003. Effects of water stress at different growth stages on greenhouse tomato yield and quality. *Hort. Science*, 38: 1389-1393.

Osman, M.G., E.A. Elhadi and M.M. Khalafalla. 2010. Callus formation and organogenesis of tomato (*Lycopersicon esculentum* Mill, C.V. Omdurman) induced by thidiazuron. *Afr. J. Biotechnol*., 9: 4407-4413.

Pal, S.P., I. Alam, M. Anisuzzaman, K.K. Sarker, S.A. Sharmin and M.F. Alam. 2007. Indirect organogenesis in summer squash (*Cucurbita pepo* L.). *Turk. J. Agric. For.*, 31: 63-70.

Park, S.H., J.L. Morris, J.E. Park, K.D. Hirschi and R.H. Smith. 2003. Efficient and genotype-independent Agrobacterium-mediated tomato transformation. *J. Plant Physiol*., 160: 1253-1257.

Porebski, S., L.G. Bailey and B.R. Baum. 1997. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Mol. Biol. Rep.*, 15: 8-15.

Qiu, D., G. Diretto, R. Tavarza and G. Giuliano. 2007. Improved protocol for Agrobacterium mediated transformation of tomato and production of transgenic plants containing carotenoid biosynthetic gene *CsZCD*. *Sci. Hortic*., 112: 172-175.

Sivasakthi, K., M. Thudi, M. Tharanya, S.M. Kale and J. Kholova. 2018. Plant vigour QTLs co-map with an earlier reported QTL hotspot for drought tolerance while water saving QTLs map in other regions of the chickpea genome. *Bmc Plant Biol*., 18.

Sun, S., X.P. Kang, X.J. Xing, X.Y. Xu and J. Cheng. 2015. Agrobacterium-mediated transformation of tomato (*Lycopersicon esculentum* L. cv. Hezuo 908) with improved efficiency. *Biotechnol. Biotechnol. Equip*., 29: 861-868.

Takasaki, H., K. Maruyama, S. Kidokoro, Y. Ito, Y. Fujita, K. Shinozaki, K. Yamaguchi-Shinozaki and K. Nakashima. 2010. The abiotic stress-responsive NAC-type transcription factor *OsNAC5* regulates stress-inducible genes and stress tolerance in rice. *Mol. Genet. Genomics*, 284: 173-183.

Tran, L.S.P., K. Nakashima, Y. Sakuma, S.D. Simpson, Y. Fujita, K. Maruyama, M. Fujita, M. Seki, K. Shinozaki and K. Yamaguchi-Shinozaki. 2004. Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a Drought-Responsive cis-Element in the *early responsive to dehydration stress 1 Promoter*. *Plant Cell*, 16: 2481-2498.

Wu, Y., Z. Deng, J. Lai, Y. Zhang, C. Yang, B. Yin, Q. Zhao, L. Zhang, Y. Li, C. Yang and Q. Xie. 2009. Dual function of Arabidopsis ATAF1 in abiotic and biotic stress responses. *Cell Res*., 19: 1279-1290.

Yoshida, T., N. Nishimura, N. Kitahata, T. Kuromori, T. Ito, T. Asami, K. Shinozaki and T. Hirayama. 2006. ABA-Hypersensitive germination 3 encodes a protein phosphatase 2C (AtPP2CA) that strongly regulates abscisic acid signaling during germination among Arabidopsis Protein Phosphatase 2Cs. *Plant Physiol.,* 140: 115–126.

Zgallaï, H., K. Steppe and R. Lemeur. 2005. Photosynthetic, physiological and biochemical responses of tomato plants to polyethylene glycol-induced water deficit. *J. Integr. Plant Biol.,* 47: 1470-1478.

Zhang, G., M. Chen, L. Li, Z. Xu, X. Chen, J. Guo and Y. Ma. 2009. Overexpression of the soybean *GmERF3* gene, an AP2/ERF type transcription factor for increased tolerances to salt, drought, and diseases in transgenic tobacco. *J. Exp. Bot.*, 60: 3781-96.

Zhu, Z., Y. Zhang, J. Liu, Y. Chen and X. Zhang. 2018. Exploring the effects of selenium treatment on the nutritional quality of tomato fruit. *Food chem.,* 252: 9-15.

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