Constitutive Overexpressing *At.*TC Improves Drought-mediated Oxidative Tolerance in Transgenic *Brassica Napus* L.

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Research Article

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Abstract

Background: Environmental stresses are the most important factors limiting crops production in worldwide. Tocopherol, belonging to family of vitamin E compounds, is an amphipathic antioxidants involved in oxidative responses. In the current study, we generated transgenic canola plants overexpressing Arabidopsis *VTE1* gene (*At.TC*) through *Agrobacterium tumefaciens* system.

Methods and results: The putative transgenic plants were successfully regenerated and acclimated in greenhouse conditions. The transcriptional activity of the *At.TC* gene was evaluated by RT-PCR. In addition, the relative gene expression analysis by qRT-PCR confirmed an increased expression pattern of the transformed gene in canola transgenic lines, with the highest level in R. Line1. Given the results, the transgenic plants, particularly H. Line1 and R. Line2 showed a lower lipid peroxidation compared to WTs under FC 30%. Moreover, two ROS scavenging enzymes including CAT and PPO were up-regulated in transgenic lines; however, no significant pattern was observed for Ascorbate Peroxidase. Also, the amount of leaf tocopherol was significantly more in all T1 lines under drought stress (FC 30%).

Conclusion: Taken together, here we successfully developed transgenic lines overexpressing *At.TC* gene constituently throughout the plant. The results confirmed that the generated transgenic plants are resistant to drought stress, thereby paving the way toward introducing canola plants to deal with the climate change and water shortage.

Introduction

Plants are mainly subjected to a variety of unfavorable conditions such as biotic and abiotic stresses in their natural habitats. Unlike animals, these organisms are sessile and have evolved wide range of mechanisms to deal with the applied conditions. Drought stress is a multidimensional stress which adversely causes plant growth by affecting the physiological, morphological, biochemical, and molecular functions in plants [1, 2]. Under prolonged drought conditions, the accumulation of reactive oxygen species are increased in leaves. The ROS molecules are significantly harmful for photosynthetic apparatus, by oxidation of cellular components, including proteins, lipids, and chlorophyll, and subsequently photosynthetic apparatus[2, 3]. To overcome this problem, plants have evolved two protective mechanisms over the time, i.e., enzymatic and non-enzymatic responses. It is well-established that tocopherols are the most important factors involving in non-enzymatic detoxification [4–6]. In fact, the most prominent proposed function of these components is protecting polyunsaturated fatty acids from peroxidation of lipid by quenching and scavenging various ROS [5].

Tocopherols, which are collectively known as vitamin E, are only produced by photosynthetic organisms and synthesized though a diverse range of biosynthetic enzymes [7, 8]. The biosynthetic enzymes are localized at the inner envelope of the plastids, where the production of the tocopherols are occurred. The precursors of tocopherols formation are derived from two pathways, i.e. plastidic methylerythritol phosphate (MEP) and shikimate pathways[7, 9]. It is proposed that the accumulation of tocopherol is mainly regulated by the activities and substrate specificities of some key enzymes such as tocopherol cyclase (TC) which also known as VTE1, homogentisate phytyltransferase (HPT), and two methyltransferase enzymes (VTE3 and VTE4) [7, 10]. The TC enzyme catalyzes a cyclization reaction with 2-methyl-6-phytyl benzoquinol (MPBQ) and 2,3-dimethyl-6-phytyl-1,4,-benzoquinol (DMPBQ), as the primary intermediates [8]. This reaction is considered as the first committed step in this pathway. Chemically, the TC enzyme plays a pivotal function in the formation of the chromanol ring structure of the tocopherols by adding an oxygen heterocycle generated next to the aromatic ring produced from homogentisate, which regulate their free radical scavenging function [10]. Therefore, any up-regulations regarding the TC gene can enhance the drought tolerance in the given plant.

In the past few years, attempts to genetically overexpressing the tocopherol biosynthetic genes in model plants in order to increase the accumulation of tocopherol and their diverse function have been reported [11, 12]. Genetic manipulation of genes encoding individual enzymes in this pathway such as γ-tocopherol methyltransferase (γ-TMT) [13, 14], TC [15, 16], and Homogentisate phytyl transferase (HPT) [17, 18, 15]. Kanwischer and co-workers demonstrated that the overexpression of TC/VTE1 enhance the tocopherols level by 7-folds [19]. Moreover, Harishi and colleagues transformed the TC and HPT genes into tobacco plant and reported increased level of vitamin E in transgenic plants [20]. Although some limited number of researches have been carried out regarding overexpressing TC gene in model plants, the function of this gene in drought resistance in canola has not been reported. Therefore, this project is aimed to generate transgenic tolerance canola plants to oxidative stresses stemming from water deficiency by overexpressing the *At.TC* gene. The transformation of the gene was carried out through *Agrobactrium transformation* system,

and the resulting explants were genetically evaluated by PCR, RT-PCR and qRT-PCR assays. Moreover, in order to evaluate the drought tolerance of the transgenic plants, we applied water shortage challenges and measured the activity of oxidative enzymes as well as their MDA and proline concentrations.

Materials And Methods

Plant materials, bacterial strains and culture conditions

The seeds of canola (*Brassica napus* L.) cultivars including Hyola4815, and RGS003 were provided from the Seed and Plant Research Improvement Institute, Karaj, Iran.

In this study, the *Escherichia coli* DH5α strain was used to maintain the expression vector and the binary vector; additionally, *A. tumefaciens* LBA4404 strain was used to transform the plasmids into canola plants. The pBin19:*At.TC* plasmid [21] harboring the selective kanamycin resistant gene, CaMV35S promoter and NOS terminator was utilized to generate stably transformed plants (Fig. 1). The specific primers for amplification of *At.TC* gene were as follow: At.TCF 5'-GGTACCGCATATTTCTTCTTCTTCTTCCATTATGG-3' and At.TCR (5'-GGATCCCAGACCCGGTGGCTTGAAGAAAGG-3', which underlined sections are restriction site for BamHI and Asp 718, respectively.

The utilized bacteria were cultured in the (Lauria-Bertani) LB medium containing 10 gl^{-1} trypton, 5 gl^{-1} yeast extract, 10 gl^{-1} NaCl.

The canola seeds were sterilized using 70% ethanol and hypochlorite 2.5%, and then cultured in 1/2MS medium. Cotyledon segments (0.7 to 1cm) were cut from 7-day-old seedlings.

Agrobacterium mediated transformation and regeneration

After cloning, the pBin19:*At.TC* plasmid was extracted from *E. coli* via alkaline lysis method according to the protocol [22]. The concise vector confirmed by PCR transformed into *A. tumefaciens* LBA4404 strain through shock transformation by using liquid nitrogen, and then cultured at LB medium supplemented with rifampicin (50 mgl^{-1}) and kanamycin antibiotics (100 mgl^{-1}) for 48 h. Similarly, PCR performed for confirmation with aforementioned primers. A single *Agrobacterium* colony cultured in liquid LB supplemented with kanamycin (100 mgl^{-1}) and rifampicin (50 mgl^{-1}) on a shaker at 180 g and 28°C until reaching the optimum OD (0.6 at 650 nm) for transformation. Cotyledons from one week seedlings were used as explant to transformation (Fig. S1A) [23].

Afterward, the explants were transferred into a media containing 500 mgl^{-1} cefotaxime to remove the *Agrobacterium* contamination, and 1 mgl^{-1} 6-benzylaminopurine and 0.2 mgl^{-1} 1-Naphthaleneacetic acid (NAA) hormones to directly shoot initiation. After one week, the explants were transformed into shoot-initiation medium with 2 mgl^{-1} BAP, 0.2 mgl^{-1} NAA, and 0.05 mgl^{-1} GA3 supplemented with cefotaxime (250 mgl^{-1}) and kanamycin (25 mgl^{-1}) antibiotics. The subculture into new medium was carried out every 2 weeks. After 6 weeks, the induced shoots were transferred into elongation MS medium containing 0.5 mgl^{-1} BAP, 3 mgl^{-1} GA3 and 2 mgl^{-1} zeatin riboside, with 50 mgl^{-1} kanamycin and similar content of cefotaxime. The seedlings were then transformed to a half-strength MS medium supplemented with 0.4 mgl^{-1} NAA for 1-2 weeks for root induction. All the aforementioned cultures were kept at 25±2 ºC under a 16/8-h (light/darkness) photoperiod conditions.

The rooted explants were transferred to small cups with autoclaved perlite and peat moss (1:1) and grown in a greenhouse under 12/12-h light/dark with a full light intensity of 200-300 µmol m-2s-1 at 25/22 ºC (day/night). After obtaining T0 generation seeds, they were assayed on selective MS medium supplemented with 50 µg/mL Kan to determine transgene inheritance and inserted gene copy number [24,25]. The mature and acclimatized canola plants were transferred to pots and kept in greenhouse at a photoperiod of 16/8 h (light/darkness).

Confirmation of transgene in canola plants

The genomic DNA of the transgenic and non-transgenic canola plants were extracted from fresh leaves (0.1-0.15 g) using CTAB method [26]. The quality of the extracted DNA was evaluated by 1% gel electrophoresis. The specific primers amplifying the *At.TC*

gene (Accession No. AT4G32770) was designed by PrimerQuest and validated via Oligo Analyzer tool. The forward and reverse primer sequences were:

At.TCF 5'-GGTACCGCATATTTCTTCTTCTTCTTCCATTATGG-3' and At.TCR (5'-GGATCCCAGACCCGGTGGCTTGAAGAAAGG-3'.

The PCR assay was run with the following program: pre-denaturation at 94 °C (5 min) followed by 40 cycles of denaturation at 94 °C (1 min), annealing at 57 °C (45 s), and elongation at 72 °C (1 min) with final extension stage at 72 °C for 10 min. The PCR products were then visualized by 1% gel electrophoresis in TAE buffer.

Analysis of RNA expression in transgenic plants

In order to evaluate the transcript abundance of the *At.TC* gene, the mRNA level of this gene was assessed using a Quantitative real-time PCR (qRT-PCR) assay. For this purpose, total RNA was extracted from the transgenic and non-transgenic plants by the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The quality and quantity of the extracted RNA were analyzed via 1% gel electrophoresis and NanoDrop spectrophotometer (BioTek, EPOCH, USA), respectively. The isolated RNA was revers-transcribed to cDNA using the Reverse Transcriptase (Invitrogen) as described by the company. The primers utilized in the RT-PCR for measuring gene expression were as follow: 5'- GGAAGTTCTTCGAGGGATGG-3' and 5'-GACAAACTCTGCCGAAATGC-3'. In this study, the Actin gene (Accession No. At2g29960) was also selected as an internal reference gene. The specificity of the designed primers and synthesized cDNA was analyzed by conducting a RT-PCR assay.

The qRT-PCR was carried out using a BioRad system with the fluorescent dye SYBR®Green Master Mix (Ampliqon, Denmark). The PCR was run at 95°C for 15 min, followed by 35 cycles at 94°C 30 s, 58°C for 20, 72°C for 15 s. In addition, dissociation curve was obtained by slowly increasing the temperature from 65°C to 95°C (0.5°C per second) to check the specificity of the amplification. In this study, three technical replicates were also considered for each sample. Baseline and threshold cycle (Ct value) were automatically calculated with default parameters. The relative expression of the gene was measured using Livak method ($2^{-\Delta\Delta Ct}$) [27].

Drought treatment conditions

The transgenic canola plants over-expressing *At.TC* gene as well as control samples were cultured in small pots containing farmland soil, leaf mold and sand at the greenhouse of Plant Breeding and Agronomy (Tehran University, Iran). The two-week old plants (two/three leaf stages) were then subjected to drought stress at different concentrations including 30%, 50%, 70% and 90% of Field Capacity based on factorial in completely randomized design (CRD) with three replication. We choose two control, Hyola4815 and RGS003 separately for each cultivar transgenic line. After subjecting the treatment, the plant leaves were harvested and maintained at -80^{o}C. The growth of the plants was also closely monitored for morphological changes.

Malondialdehyde

Lipid peroxidation in canola transgenic and control plants was measured by quantification of malondialdehyde (MDA) as described by with slight modifications [28]. To this end, 500 mg powder of the samples was mixed with 10 ml trichloroacetic acid (TCA), and the suspension was centrifuged at 12000 g for 10 min at 4°C. Then, 1 ml of the supernatant was homogenized with 4 ml of thiobarbituric acid (TBA) 0.5%, and vortexed for 1 min. The samples were maintained on water bath at 95°C for 1 h and then transferred on the ice bath. After 20 min, the mixtures were centrifuged at 12000 g for 10 min at 4°C. Subsequently, 300 µl of the resulting supernatant was collected and used for determination of MDA by using a spectrophotometer at 523 nm wavelength. The concentration of the MDA was measured based according to the C = D / L equation; where D refers to density and E is equal to molar differentiation factor.

Chlorophyll and carotenoid determination

In order to evaluate the alternations in photosynthetic pigments, chlorophyll and carotenoid content of the studied plants were measured [29,30]. 400 mg of fresh leaves powder was mixed with 1 ml acetone 80%, vigorously vortexed, and maintained in dark and room temperature for 30 min. Afterward, the mixtures were centrifuged at 12000 g for 15 min at 4 ^{o}C. The debris were discarded and the supernatants were kept at refrigerator at 4^{o}C. Again, 1 ml of acetone 80% was added to the suspension and the above-mentioned processes were exactly repeated. This replication was carried out for five times until the entire content of chlorophyll and carotenoid was separated from the leaf tissue. Finally, the resulting supernatant was subjected to the plate reader and the absorbance of chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids (Car) were red at 470, 645, and 663 nm, respectively. The concentration of the pigments were also measured using the following equations:

Chl a= [12.7 (OD_{663}) - 2.69 (OD_{645})]* V/1000 * W

Chl b= [22.9 (OD_{645}) - 4.68 (OD_{663})]* V/1000 * W

Total chl= [20.2 (OD_{645}) - 2.69 (OD_{663})]* V/1000 * W

Total carotenoids= (1000 (OD_{470}) – 1.9* chl a– 63.14 * chl b)/ 214

Where V and W indicate volume of the extract and weight of leaf segments, respectively.

Enzymatic antioxidant activities

It is well-established that during drought stress, the activity of some key enzymes increase significantly. Herein, we measured the total extractable activities of, Polyphenol oxidase , Catalase (CAT), and Ascorbate Peroxidase (APX) based on the method established by [31-33], respectively. Activity were estimated through absorbance in 420, 240, and 290 nm for PPO, CAT, and APX, respectively, using Shimadzu UV-160 spectrophotometer.

Vitamin E measurement

In this regard, the sample and standard solution were analyzed by HPLC after injecting 20 µl [34,35]. The separation was achieved on a Ncleosil-C_{12} column (125 × 4mm, 5 µm, Umzam-Crustal-200, England) with a simple isocratic elution using mobile phase (n-hexane, Diethyl ether, and acetic acid at a ratio of 70:29:1 v/v) at a flow rate of 0.8 mL/min. Methanol was used as diluent. The column oven temperature was 30 °C and column effluent was monitored at 300 nm. The specific tocopherols form achieved through comparing the retention times with standards within the linear range of the calibration.

Statistical analysis

Statistical difference between transgenic and control plants were analyzed following tukey range test at a probability level of $P < 0.01$ in SAS 9.4 software.

Results

Transformation of canola plants

After cloning the pBin 19: *At.TC* vector into Agrobacterium LBA4404 strain, the presence of the *At.TC* gene (1485 bp) was validated within the vector by PCR assay (Fig. 2A). In addition, the recombinant Agrobacterium was confirmed before on LB medium containing rifampicin (50 mgl^{-1}) and kanamycin antibiotics (100 mgl^{-1}), respectively. The canola plant transformation was carried out with the Agrobacterium possessing pBin 19:*At.TC* construct.

Regeneration of canola transgenic plants

A. tumefaciens-infected cotyledons were cultured on shoot initiation medium. After ten days, approximately 70-80% of the explants showed shoot initiation (Fig. S1b). Then, the explants were sub-cultured on the elongation media for four and two weeks, respectively. The results showed that 60% of the shoots were proliferated to plantlets (Fig. S1C). In order to induce roots, all of the regenerated shoots were transferred to the root-induction medium containing 0. 2 NAA hormone. Finally plantlets transferred into greenhouse were acclimated after 2 weeks (Fig. S1D).

Molecular indication of the putative transgenic plants

The confirmation of the transgenic canola plants harboring *At.TC* gene was performed using PCR assay and specific primers. The PCR pattern validated the presence of the expected 1137 bp length, which confirm the transgenic nature of the canola plants (Fig. 2B). Accordingly, the pBin:TC construct was successfully delivered to the canola plants through the *A. tumefaciens* system with an acceptable efficacy. In addition, the Hyola 4815 (20%) cultivar showed the highest transformation efficiency than RGS003 cultivar (10%).

Transcription abundance of the transformed *At.TC* gene

After checking the integrity of the isolated RNA, cDNA synthesis was carried out. Firstly, the presence and expression of the transformed gene was proved using reverse transcription PCR. The results indicated to the acceptable expression (a sharp band) of the *At.TC* gene in transgenic canola cultivars. The obtained gel electrophoresis also indicated to the specificity of the applied primers, which amplify the specific gene with the exact length (Fig. 2C). Moreover, the SYBR® Green-based real-time qPCR experiment was carried out to evaluate the relative expression of the *At.TC* gene in the studied canola cultivars in mRNA level. Given to the obtained results, the *At.TC* gene was effectively transcribed in all the transgenic lines, i.e. H. Line1, 2 and R. Line1. Totally, the transcript abundance in transgenic T1 lines (H. Line 1, H. Line2, and R. Line3) were more than their controls under stress. On the other hand, the R. Line1 showed the highest expression level of *At.TC* (Fig. 2D).

Physiological and biochemical responses of wild-type and transgenic plants to drought stress

The potential influence of drought stress on both transgenic lines and non-transgenic or WT plants was evaluated by measuring MDA content, as an end-products of lipid peroxidation in bio-membranes (Fig. 3A). The results clearly indicate to the lower accumulation of MDA in transgenic lines i.e., R. line1 and H. Line 1, as compared to WT plants in FC 30%. Of which, the transgenic R. Line1 and H. Line1 showed considerable decrease in lipid peroxidation as compared to their wild types. In contrast, no significant differences between H. Line2 and Hyola4815 (WT) was observed, which is probably due to the genomic location or DNA methylation that is stochastic event. Overall, the lower MDA concentration in canola plants overexpressing the *At.TC* gene indicates that lipid peroxidation was lower in the transgenic canola plants than in the non-transgenic ones, which demonstrate that the *At.TC* gene contribute to decreased lipid peroxidation in canola.

Chlorophyll and carotenoid content

According to results, chlorophyll a content had not significantly changed, but total chlorophyll and chlb, and carotenoid contents varied between and controls. Although chl b was little higher in Hyola 4815 and RGS003 as controls compared to T1 lines under stress, total chl and carotenoid showed similar trend between controls and T1 lines under three stress (Fig. 3 B,C and D).

Biochemical activity of the ROS-scavenging enzymes

In the current study, we measured the biochemical activity of three important ROS-scavenging enzymes including PPO, APX and CAT. Given to the results, the activity of CAT enzyme was more in T1 lines compared to WTs under three levels of drought stress. In this case, R. Line1 showed the highest activity compared to other in FC 30% (Fig. 4A). Interestingly, the PPO activity was higher only

in T1 lines of Hoyla compared to Hyola 4815 (WT) under FC 30%, while there was no significant difference between R. Line 1 and RGS003 (WT) (Fig. 4B). In contrary, APX has showed higher activity in R. Line1 compared to RGS003, especially in FC 30%, which this has not changed significantly between Hyola4815 and H. Lines 1, 2 under stress Fig. 4 C).

Altogether, we have measured the tocopherol content of transgenic lines and wild type. Total tocopherol levels increased simultaneously along with drought treatment progress in both wild type and transgenic plants. Continuously, total tocopherol were more in all T1 lines in compare to wild types under three level of drought stress (Fig. 4D).

Table 1

Analysis of variance for the effect of drought stress on biochemical traits in transgenic lines.

Source	df	Mean Square							
		APX	CAT	PPO	Chl a	Chl b	Chl T	carotnoeid	MDA
Varity	4	1.08**	0.034**	0.003**	0.42^{ns}	0.0008**	0.00014**	0.0073**	0.043**
Treatment	3	18.05**	0.144**	0.004**	0.05^{ns}	0.001**	0.0014**	0.018**	0.14**
Varity*Treatment	12	0.189**	0.006**	0.0008**	0.91^{ns}	0.00003**	0.00054**	0.0030**	0.00011**
Error	40	0.018	00.00005	0.00001	0.0008	0.000001	0.00012	0.00004	0.000010
CV		4.59	7.20	7.10	3.69	7.14	3.08	3.97	2.71

Discussion

Manipulating the industrial crops such as *Brassica napus* L, which is sensitive in seed yield and quality to abiotic stress, is highly necessary to cope with global food demand. It has been demonstrated that tocopherols properly involved in quenching and scavenging ROS and lipid-soluble byproducts of oxidative stress [36,37]. For instance, each tocopherol is able to scavenge 120 free radical molecule like $^1O_2$, which adversely affect the growth and development of the plants. This study was aimed to investigate these compounds function as non-enzymatic antioxidant system in tolerance to drought-mediated oxidative stress. Successfully, *At.TC* gene was overexpressed in H. Line 1, 2, and R. Line1 which change these plants response to different level of drought stress. Antioxidant enzymes .i.e., APX and CAT simultaneously up-regulated in T1 lines with high content of total tocopherol (1.6 fold) compared to WTs, particularly in FC 30%. Overexpression of the biosynthetic genes encoding the enzymes involved in tocopherol biosynthesis have also been reported in maize [38], and rice [39]. Similarly, Woo et al [40] reported the high content of total tocopherol, and the antioxidant status activity in *Nt.TC*-transgenic rice compared to control under drought stress. In addition, *vte1* mutants of Arabidopsis showed high contents of ascorbate and glutathione which known as antioxidants to compensate tocopherol deficiency. In this regard, lipid peroxidation severely was increased in *vte1* mutant plants with ascorbate shortage under water stress indicating their cooperation in oxidative response. Overexpressing the endogenous *VTE1* gene in Arabidopsis not only increased the leaf tocopherol [19], but also heightened converting α- to γ- tocopherol, which approved by Zebierzak et al [41]. Li et al [42] showed that engineering the tocopherol pathway changed the glutathione-ascorbate cycle because tocopheroxyl resulted by tocopherol reaction with lipid peroxyl radicals linked to this cycle. In present study, no significant difference was observed between transgenic lines and controls for chla/b and carotenoids that was in consistent with Kanwischer et al [19]. Also, Havaux et al [43] concluded that *vte1* mutants of Arabidopsis had not sensitivity to light, but absence of xanthophyll in double mutant vte1 npq1 resulted PSII photoinhibition as well as lipid and pigment oxidation indicating that tocopherols could be functionally redundant and cooperate with xanthophyll to protect chloroplast from ROS and photooxidation.

Abbasi and co-workers reported that silencing homogentisate phytyltransferase, which is a determinative enzyme in tocopherol biosynthesis, considerably accelerated the senescence in *Nicotiana tabacum*. This was due to the lower accumulation of tocopherol in the tobacco leaves; thus, elevating the ROS production and concomitant photooxidative [17]. The transcription analysis using RT-PCR and qRT-PCR of the *At.TC* gene indicated to the activity of this gene in mRNA level. Additionally, the expression level of the transformed gene in the studied cultivars were also different. The R. Line1 was significantly up-regulated as compared to other lines; H. Line 1 and 2. This is probably due to the genomic differences between the studied ecotypes; although all the studied cultivars are

commercially known. Similarly, the MDA changes also supported the gene expression results, with the lowest dosage of lipid peroxidation in canola plants. Based on promoter analysis, *VTE1* had more elements classified into stress and phytohormone groups indicating diverse function of these gene in response to various abiotic stress [12]. Assessment of MDA, as a byproduct of oxidative damage to membrane lipids, in transgenic and WT plants showed that the accumulation of MDA significantly was lower in transgenic lines i.e., H. Line1 and R. Line 1 under severe drought (FC 30%). In accordance with this study, Liu et al [44] demonstrated that transgenic tobacco overexpressing At.TC significantly had lower MDA content under PEG-mediated drought stress.

To sum up, overexpressing the At.TC changed significantly the antioxidant system response to drought mediated oxidative stress in whole plant. In addition, VTE1 activity is important in tocopherol biosynthetic pathway, and also previous promoter analysis along with experiments proved this gene as responsive to abiotic stress. Therefore, it may be essential to investigate the co-regulatory network under stress, and applying new genome editing systems, such as CRISPR-Cas 9, effectively accelerated the advance plant breeding programs. Moreover, due to the increased rate of water shortage in global scale, engineering and providing a resistance canola line can profoundly improve the productivity of this oilseed crop.

Declarations

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Author contributions

The authors, Alireza Abbasi, Manijeh Sabokdast, and Sajjad Sobhanverdi contributed to the study conception and design. Material preparation, data collection and analysis were performed by Sajjad Sobhanverdi, Atefeh Majidi, Zahra Asghari Mollabashi, Hamid Bayat, and Mina Sadeghi Niaraki. The first draft of the manuscript was written by Sajjad Sobhanverdi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declaration

1. All authors approved this version to be published.
2. All materials and data clearly reported and are accessible.

Conflict of interest

There is no conflict of interest

Founding

Not applicable

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Figures

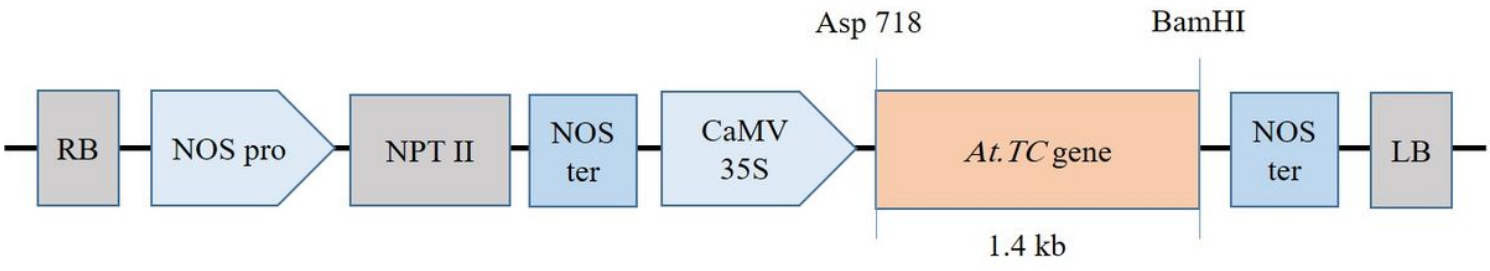

Figure 1

A schematic illustration of the pBin19 binary vector designed to express the At.TC gene in canola. The 1485 bp gene was cloned between cauliflower mosaic virus 35S promoter and NOS terminator in sense and antisense orientation. The enzymatic restriction

sites for both BamHI and ASP 718 are presented in the figure. RB: right boarder, NOS pro: nopaline synthase promoter, NPT II: Neomycin phosphotransferase, NOS ter: nopaline synthase promoter, CaMV 35S promoter, LB: left boarder.

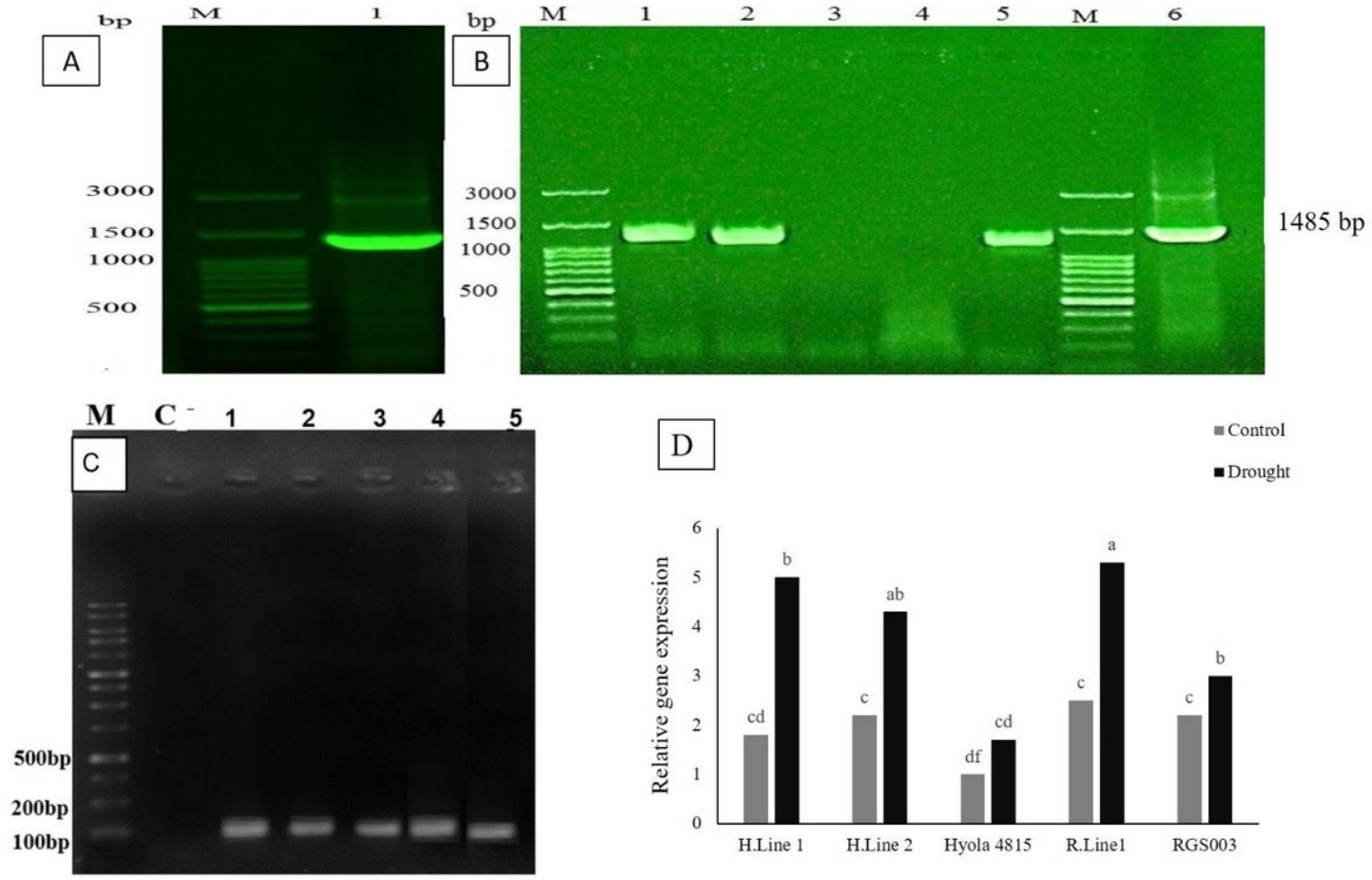

Figure 2

PCR and RT-PCR results. A. Amplification of the 1485 bp At.TC gene fragment cloned into Agrobacterium LBA 4404 (1), B. PCR-confirmed transgenic lines: 1-2 :transgenic Hyola 4815 lines (H. Line 1 and 2), 5: transgenic RGS003 line (R. line1), 3 and 4 are WT plants, 6 : pBin 19:At.TC vector, M: 3000 bp DNA marker. (C) RT-PCR analysis showing mRNA expression pattern of the A.tTC transgenic lines including H. Line 1&2 (1, 2), Hyola4815 (3), R. Line (4), RGS003 (5), and C- is negative control, M refers to 250 bp DNA ladder. (D) The relative expression analysis of the A.tTC gene in three transgenic lines by using qRT-PCR. The Actin gene was considered as internal control for measuring the relative transcription.

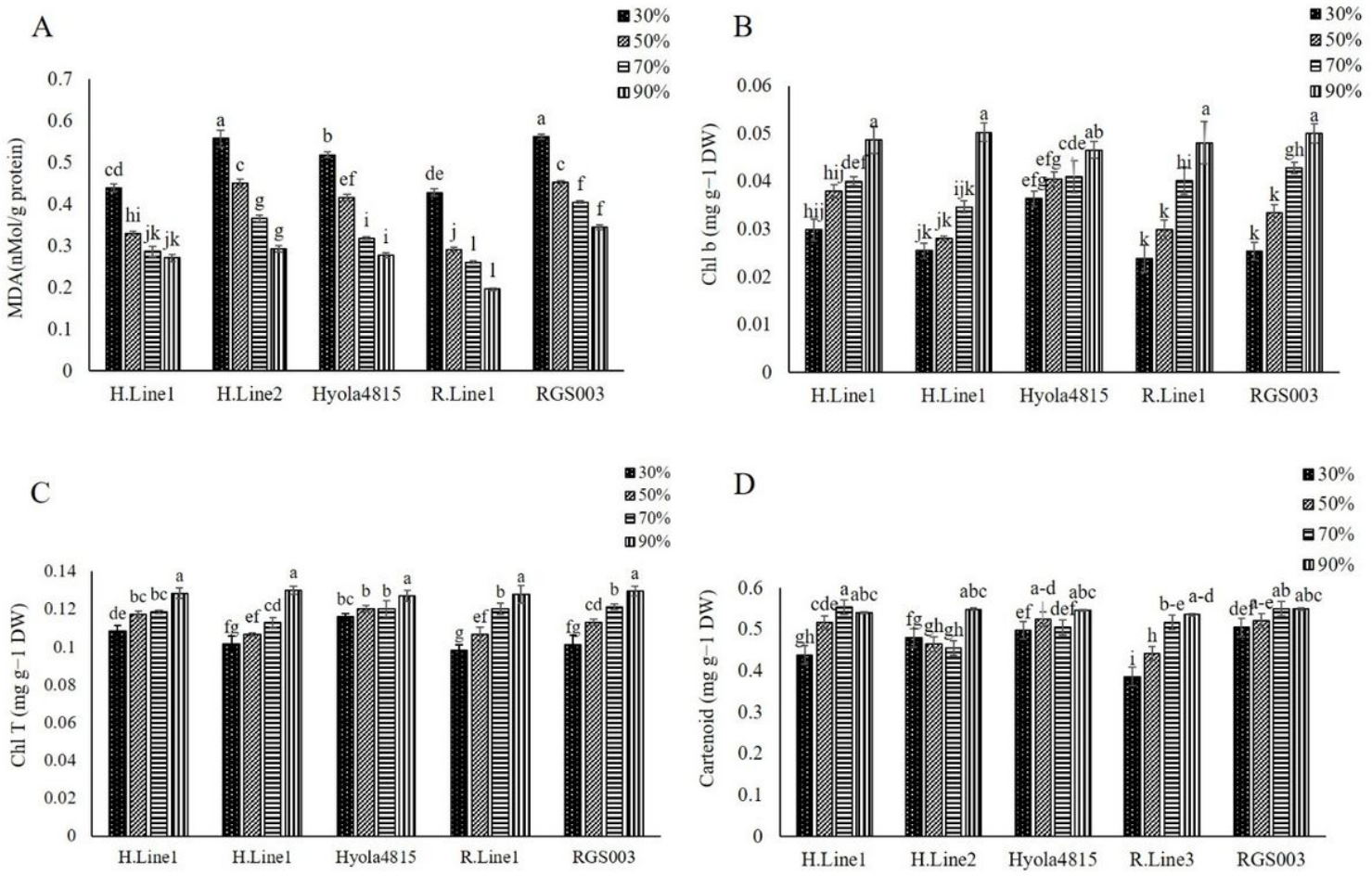

Figure 3

MDA, Chlorophyll and carotenoid content of transgenic and WTs of Canola under drought and control stress. A: MDA, B: chlorophyll b, C: Total chlorophyll, D: carotenoid.

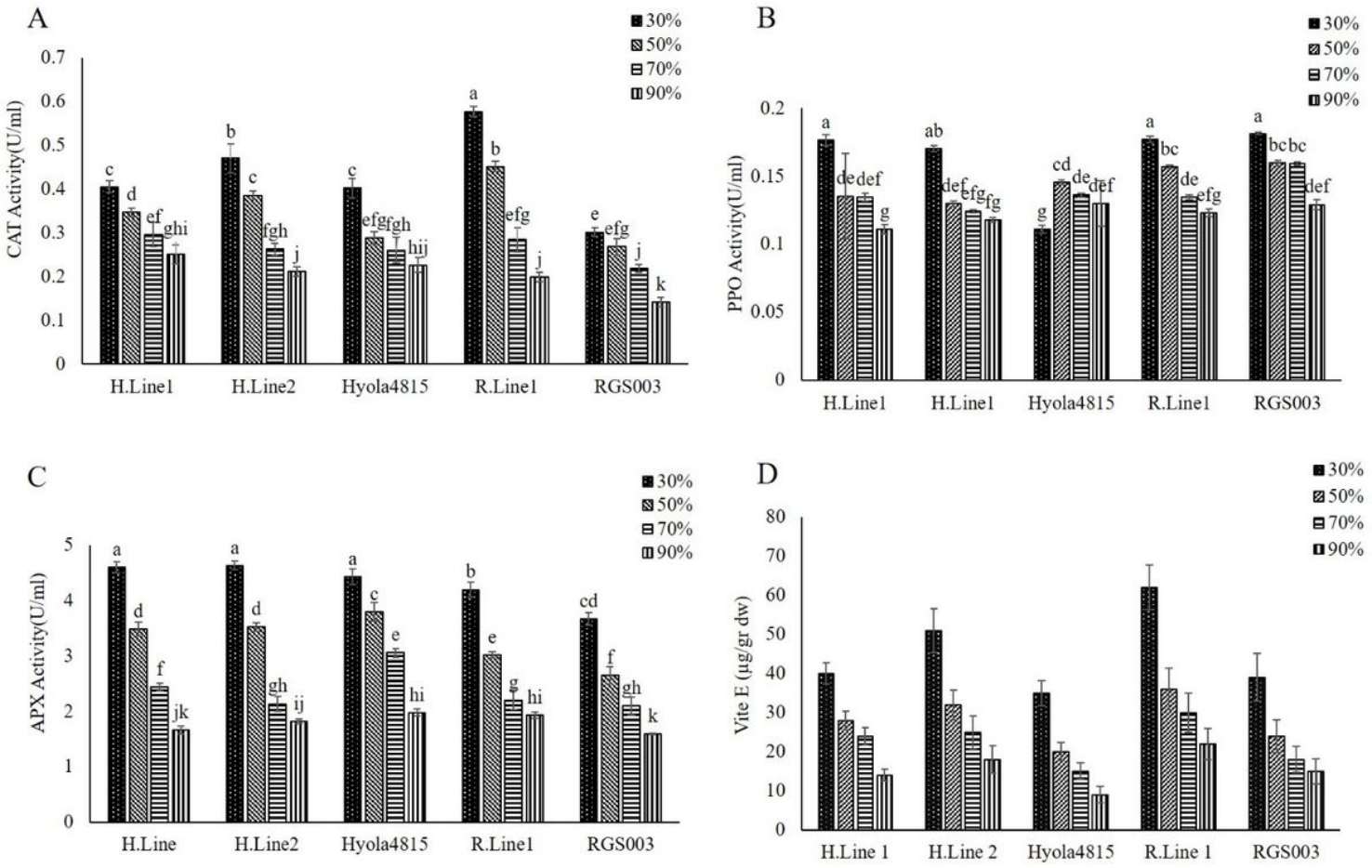

Figure 4

Effect of drought stress on Polyphenol oxidase (PPO), Catalase (CAT), Ascrobat peroxidase (APX), and tocopherol content in transgenic and non-transgenic lines of Brassica napus L. A: CAT, B: PPO, C: APx, and D: Tocopherol.

Supplementary Files

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