IMAM KHOMEINI INTERNATIONAL UNIVERSITY
IJGPB
Iranian Journal of Genetics and Plant Breeding
Print ISSN : 2251-9610
Online ISSN : 2676-346X

# Study of the presence of DREB1/ CBF gene family in *Viola tricolor*

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**ABSTRACT INFO**

**Research Paper**

**Received: 23 Aug 2022**

**Accepted: 21 Jan 2023**

**ABSTRACT**

Transcription factors are known as factors having the ability to simultaneously activate several genes in plant and animal genomes. Because of their significance in responding to environmental stresses like salinity, drought, temperature changes, etc., a group of these transcription factors known as DREB1/ CBF (Dehydration-Responsive Element Binding Protein 1/ C-repeat binding factor) gene family has been extensively studied in various plant species. In this research, considering the particular and well-stablished role of the genes of this family in cold stress in plants, *Viola tricolor* (wild pansy), as a cold resistant species, was chosen to identify this gene family. For this purpose, viola plants in the multi-leaf stage were gradually transferred to a -8 °C chamber and subjected to subfreezing treatment for 18 h. Using bioinformatics analysis based on NCBI database, the initial identification of the AP2/ ERF (APETALA2/ Ethylene-Responsive Factor) conserved region in the genes of the members of this family was carried out and the consensus sequence was used for primer design. The sequencing result revealed considerable sequence similarity in this region with genes found in other plant species. Thus, as predicted, this work established the presence of the DREB1 gene family in *V. tricolor*. The closest species to *V. tricolor* was also determined by drawing the phylogenetic tree based on the identified region. The gene expression pattern analysis revealed a significant increase in expression of this gene under cold stress conditions.

***Key words:*** CBF/DREB1 gene family, *Viola tricolor*, Transcription factors, Subfreezing stress, Gene expression, Phylogenetic tree.

**How to cite this article**:
Nezami E., and Deljou A. (2021). Study of the presence of DREB1/ CBF gene family in *Viola tricolor*. *Iranian Journal of Genetics and Plant Breeding,* 10(2): 1-12.
**DOI: 10.30479/IJGPB.2023.17723.1322**

## INTRODUCTION

During the growing season, plants are constantly influenced by abiotic environmental factors such as freezing, heat, high salinity, and drought. Plants use a variety of complex regulatory mechanisms to cope with abiotic stresses, including physiological, chemical, and molecular changes (at the transcriptional, post-transcriptional, and post-translational levels) (Guy, 1990; Chinnusamy *et al*., 2003; Zhu *et al.*, 2007; Winfield *et al.*, 2010; Wang *et al.*, 2013; Le *et al.,* 2014; Ritonga and Chen, 2020).

Low temperatures are among the most important and determining non-biological factors in plant growth in various geographical areas, which can be considered as a major impediment to the development of the plant's full potential in these areas (Najeeb *et al.,* 2021; Goering *et al.,* 2021; Adhikari *et al.,* 2021). Plant growth and survival under such stressful conditions are the result of a variety of complex functions at the cellular and subcellular levels. Meanwhile, genes play a key role, whose performance in low temperature conditions in temperate region plants is cold acclimation (Levitt, 1980). This adaptation is known to be caused by biochemical and physiological changes such as increased solute levels, changes in membrane lipid composition, and secondary metabolites accumulation (Guy, 1990).

Different genes respond to cold by altering the expression and cellular RNA content (transcriptome) (Guy *et al.*, 1985). Therefore, studying transcriptional changes during cold acclimation and identifying regulatory genes associated with low temperature can help to improve our understanding of the mechanisms of tolerance to low temperatures and freezing in various species. Furthermore, research on the molecular regulatory mechanisms under cold stress will be very beneficial in improving plant cold tolerance.

Many studies have been conducted in recent decades to investigate changes in the proteome and cellular metabolites under cold stress conditions, leading to the identification of many genes that are active/ inactive or altered, upon plant exposure to different temperatures (Thomashow, 1994, 1999; Neilson *et al.*, 2010; Rodziewicz *et al.*, 2014; deljou *et al.*, 2016). Accordingly, several gene regulatory networks related to cold stress in various plant species have also been reported (Dietz *et al.*, 2010; Alisoltani *et al.*, 2015; Kidokoro *et al.*, 2022).

In studies of changes in gene expression levels during cold stress, a special group of proteins has been identified that are simultaneously induced by cold and develop low temperature tolerance. These proteins belong to a set of genes, which are activated concurrently by the product of a key upstream gene known as transcription factors. This is basically related to the existence of similar response elements or cis-elements in the upstream region (promoter) of genes involved in a common process (Baker *et al*., 1994). These elements, through common and similar sequences, are a site for identification by transcription factors and subsequent activation of the relevant gene or genes (Maruyama *et al.*, 2004; Xu *et al.,* 2011). In this regard, one of the most important gene families encoding transcription factors related to cold stress in various plant species has been identified as DREB1/CBF, the protein product of which binds to the C-repeat responsive element in the promoter of COR (Cold responsive/ regulated genes) through its conserved AP2/ERF domain (Stockinger *et al.*, 1997). The response element is actually a common regulatory element known as CRT (C-repeat) or LTRE (Low-temperature response element), which is 5 nucleotides long and its common sequence is typically reported as CCGAC. This element's association with drought resistance has already been discovered, so it was dubbed the dehydration response element (DRE). To date, CRT/ LTRE / DRE element has been identified like the transcription factors of the CBF gene family, in various higher plants, including tropical and temperate species (Sharoni *et al.*, 2011; Charu and Manjo, 2011; Kumar *et al.*, 2018). CBFs and COR genes, along with another gene known as ICE (Inducer of CBF Expression), which plays the role of inducing and regulating these genes upstream of CBFs, create a signaling pathway that alleviates cold stress (Thomashow, 1999; Shu *et al.,* 2017; Li *et al.,* 2020; Tang *et al.,* 2020).

Initially, 3 genes of this family were identified in Arabidopsis, named *CBF1*/ *DREB1B*, *CBF2*/ *DREB1C* and *CBF3*/ *DREB1A* (Stockinger *et al.*, 1997, Gilmour *et al.*, 1998; Liu *et al.*, 1998; Medina *et al.*, 1999). According to the conducted studies, in terms of sequence and structure, these three genes and their corresponding protein sequences differ only marginally from one another. (Medina *et al.*, 1999; McKhann *et al.*, 2008; Vazquez-Hernandez *et al.*, 2017; Heo *et al.*, 2018; Salvo *et al.*, 2021). The percentage of sequence homology between these factors has been reported to be around 85% and their identification feature is AP2 domain. Structurally, the proteins generated by all 3 genes have a nuclear localization signal (NLS) sequence, DNA binding domain (AP2 domain), and

acidic region that can play a role in the interaction with other inducible proteins under adaptation conditions and cause expression of COR regulon (Kanaya *et al.,* 1999). The induction of DREB1s is thought to be the first step in the cold-response transcriptional cascade, which results in the expression of a large number of cold-inducing genes and subsequently the production of proteins involved in cold stress response and cold tolerance. Therefore, explaining the expression mechanisms of cold-induced DREB1 can be very important (Kidokoro *et al.,* 2017*).*

Today, DREB transcription factors in *Arabidopsis thaliana* fall into six subgroups, A-1 to A-6 (DREB1 to DREB6) based on structural characteristics (Sakuma *et al.,* 2002). While this designation refers directly to the role of these genes in hydration (drought) stress, the role of these transcription factors, in addition to low temperature conditions, in salinity, osmotic stress and heat has also been identified so far (Stockinger *et al.,*1997; Shinwari *et al.,* 1998; Haake *et al.,* 2002; Dubouzet *et al.,* 2003; Agarwal *et al.,* 2006; Qin *et al.,* 2007; Nakashima *et al.,* 2009; Dietz *et al.,* 2010; Fujita *et al.*, 2011). The *CBF4* / *DREB1D* gene, for example, is involved in plant drought tolerance, whereas its homologue, the *DREB1A* gene, responds to cold stress (Haake *et al.,* 2002). It has been reported that *DREB2C* is involved in heat tolerance rather than drought tolerance (Lim *et al.*, 2007). Despite their high sequence homology, the functional diversity of different DREB genes necessitates functional and molecular study of members of this gene family to comprehend the regulatory mechanisms involved in the expression of these genes.

CBF transcription factors in *A. thaliana*, which are rapidly expressed in response to cold and drought stress, have been studied more than any other plant. Other frost-tolerant species expression patterns of CBF and COR genes have been shown to be similar to Arabidopsis, and the central regions of the CBF genes are highly conserved in all of them (Heo *et al.,* 2018).

The importance of identifying and utilizing genes effective in viola survival under biotic and abiotic stresses due to the widespread use of this plant in various fields, has been considered by experts. In nature, viola is seen as an ornamental plant in a variety of colors. Medically, viola species, especially *V. tricolor*, have a long history in the treatment of various diseases such as epilepsy, shortness of breath, and skin diseases (Grieve, 1931). In industry, this species is used in the production of yellow, green and green-blue colors (Breverton, 2011). This plant contains an antimicrobial and anti-HIV compound called cyclotid, which is used in the treatment of cancer today (Tang, *et al.,* 2010).

The presence of CBF family genes in the *V. tricolor* genome, as a cold and frost resistance plant, has been demonstrated in this study by identifying the conserved domain in these genes. To date, this is the first study of CBF-type transcriptional factors in *V. tricolor.* The current study sought to determine the existence of CBF family genes in the *Viola tricolor* genome by finding the conserved AP2 domain. This study, along with similar studies on violas, could improve the understanding of the evolution of cold stress-related genes in *V. tricolor*. Identification of these types of genes and their key roles in different species has always been a means to achieve plants with better performance. It has been reported that Arabidopsis plants have become more resistant to low temperatures by overexpression of this gene (Liu *et al.,* 1998; Kasuga *et al.,* 1999).

## MATERIALS AND METHODS

### Plant preparation

*V. tricolor* seeds were provided from Iran Bazr Company of Tehran. In the spring, plants were grown under greenhouse conditions (16 hours light and 8 hours dark at 25- 35 °C, proper humidity and ventilation) in pots with appropriate soil (a mixture of sand, gravel, and animal manure). The plants reached the multi-leaf stage after 4 weeks, and the leaves were utilized for DNA and mRNA extraction. For RNA extraction, the plants were gradually transferred to a chamber with -8 °C and subjected to subfreezing treatment for 18 h. The slow drop in temperature creates circumstances for redistributing water to plant tissues and prevents ice formation inside cells, which occurs seldom in nature.

### Looking for conserved regions in NCBI-registered sequences

First, 5 DREB1 gene family-related nucleotide and protein sequences (DREB1A, DREB1B, DREB1C) that were registered in the NCBI database for a number of different plant species, were randomly retrieved (Table 1). To determine the region/ regions with the highest level of conservation, nucleotide and protein alignment was performed among these sequences. For this purpose, Mega 7 software was employed. The region with the highest degree of similarity among the five sequences was chosen. This region (consensus sequence) was used to examine and confirm the identified sequence using the BLASTx alignment tool in the NCBI database.

**Table 1.** Accession number of nucleotides and protein sequences of DREB1 genes identified in 5 different species, used for the initial identification of the conserved region among these sequences.

| Number | Genus and species | Accession number (Nucleotide) | Accession number (Protein) |
|---|---|---|---|
| 1 | *Gossypium barbadense* | KR233252.1 | ALG39399.1 |
| 2 | *Actinidia arguta* | MW419298.1 | UPY89935.1 |
| 3 | *Dimocarpus longan* | MN504651.1 | QIB03113.1 |
| 4 | *Sabal palmetto* | DQ497730.1 | ABF59736.1 |
| 5 | *Actinidia deliciosa* | MH018601.1 | AYM54798.1 |

**DNA extraction and PCR**

Using Oligo analyzer software, a pair of primers (F: 5′- GCGGGGAGGAAGAAGTTCC -3′ و R: 5′- CCACGCCGAGTCAGCGAA -3′) were designed based on a consensus sequence. Genomic DNA was extracted from viola leaves using the CTAB method and employed for PCR using designed primers. The amplified region was predicted to be 220 nucleotides long, based on the consensus sequence. The PCR product was evaluated by electrophoresis on a 2% agarose gel (w/v) stained with safe stain, in 1 X TBE buffer.

In order to check the result of the previous PCR reaction on DNA, the second PCR reaction was prepared from the synthesized cDNA, using the same primer pair and with the same programme.

Before PCR, cDNA was diluted 10-fold and 2 µl of it was used in the PCR mixture with a final volume of 20 µl. The amplified sequence was then sent to the Codon Genetic Company in Tehran for final confirmation of the identified region through two-directional sequencing.

**RNA extraction and cDNA synthesis**

Total RNA was extracted from the leaves of cold-treated plants at the multi- leaf stage using the RNX-Plus extraction kit and the instructions provided by SinaClone company. After RNase-free DNase I treatment and ensuring genomic DNA removal, the quality and quantity of the extracted RNA were evaluated with the NanoDrop spectrophotometer and on agarose gel. cDNA was synthesised using the Sinnaclon First Strand cDNA Synthesis Kit (SinaClone) according to the manufacturer's instructions, using 1 µg total RNA.

**Phylogenetic relationships**

In order to investigate the phylogenetic relationships, 27 sequences with low E-values (after removing duplicates) were chosen by using local alignment (BLASTn) of the sequences obtained from the NCBI database. Aligned sequences were downloaded and used for global alignment using Mega 7 software and the associated phylogenetic tree was made.

Similarly, BLASTp of the protein sequences obtained from the NCBI database was used to select 49 low E-value sequences (after removing duplicates). These sequences were downloaded and used in the Mega 7 programme for global alignment. The corresponding phylogenetic tree was then created using Mega 7 software.

Maximum Likelihood statistical method with a phylogenetic test based on the Bootstrap method was used to draw trees. The number of iterations of Bootstrap was considered to be 500.

**Amino acid sequence deduced from the 220-nucleotide**

The amino acid sequence of the 220-nucleotide sequenced region was acquired using the EXPASY database (https://web.expasy.org/translate/). Then, by aligning this sequence with the 49 sequences used to construct the phylogenetic tree in Mega 7 software and in accordance with the amino acid sequences reported for the AP2 domain in other CBFs, the 220- nucleotide region was analyzed to identify this domain in the newly identified CBF.

**Gene expression quantitation using RT-qPCR**

CBF gene expression in *V. tricolor* leaves in response to subfreezing stress was assessed using real-time quantitative transcription-PCR (RT-qPCR) analysis. Gene-specific primers were designed by Oligo Analyzer software. The primer sets (gene-specific primers and control gene primers) were initially tested by standard PCR and in addition to optimizing the PCR reaction, the synthesis and purity of cDNAs were ensured (Table 2). The amplification fragment (amplicon) size was investigated by electrophoresis on a 2% agarose gel. Subsequently, after dilution with distilled water (1: 10 ratio), the synthesized cDNA, was used for RT-PCR reaction using SYBR® Green Master Mix (SinaClone) and gene-specific primers, according to the protocol of SinaClone company.

The reaction condition was prepared according to the protocol recommended by the manufacturer (360 s at 95 °C, 40 cycles of 95 °C for 15 s, 61 °C for 20 s and 72 °C for 45 s). Reactions were performed using the

**Table 2.** 18S and CBF gene primers utilized in real-time PCR reactions.a

| Primers | Genes | | | |
|---|---|---|---|---|
| | 18s | Annealing (˚C) | CBF | Annealing (˚C) |
| Forward | CAACCATAAACGATGCCGACCAG | 64.6 | GGGAGGAGGAAGTTCAGGGA | 62.5 |
| Reverse | TTCAGCCTTGCGACCATACTCC | 64 | CGCCCCCATCTCCGCCGT | 65.2 |
| Length of product | 150bp | | 147bp | |

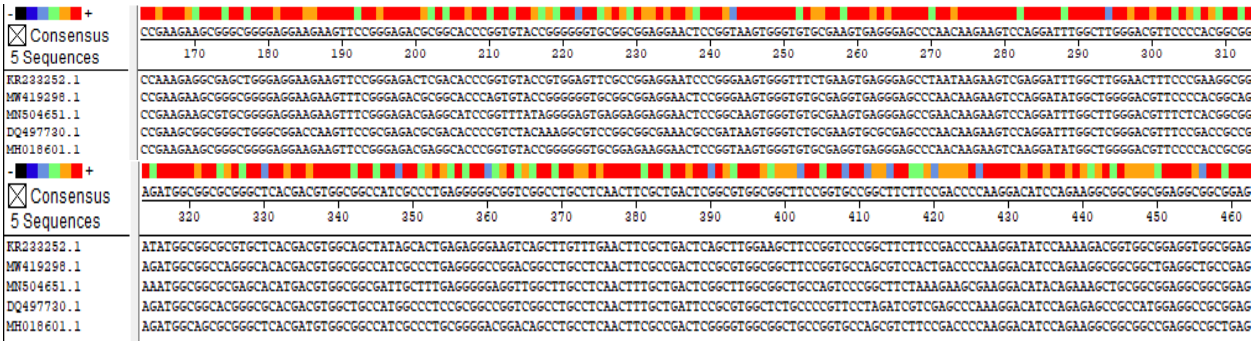


**Figure 1.** Multiple alignment of 5 nucleotide sequences of DREB1 from various species by Lasergene- MegAlign software to determine the conserved region in this gene family. These sequences were taken at random from the NCBI. The consensus sequence is shown above the aligned sequences.

LightCycler 96 system (Roche). Each 20 µl reaction mixture contained 7 µl DEPC-treated water, 2 µM of each primer, 10 µl SYBR® Green Master Mix and 1 µl cDNA diluted 10-fold. Dissociation curve was obtained by heating the amplicon from 65 to 97 °C. Data were generated from 4 independent biological replicates. Melting curve analysis were performed on the PCR products. Relative target gene expression levels were normalized to 18S reference gene. REST (Relative Expression Software Tool) software was used to calculate the amount of changes in this CBF gene expression.

## RESULTS AND DISCUSSION

### Determining the region with the highest level of conservation

By comparing the identified regions in both nucleotide and protein alignments, it was found that these two regions overlap. The identified region was 300 nucleotides and 100 amino acids long, which had the highest level of protection among the 5 desired sequences.

A consensus sequence was obtained from the multiple alignment of the desired nucleotide sequences by Lasergene- MegAlign software (Figure 1). Alignment of this sequence with the BLASTx tool in the NCBI database revealed that a region of 180 nucleotides (60 amino acids) in this sequence corresponds to a conserved domain called AP2 in the DREB1 gene family (Figure 2). This alignment also confirmed the conservation and presence of the consensus sequence in DREB1s known from other plant species.

### Identification of the conserved domain in the *V. tricolor* genome

The PCR reaction on DNA of the *V. tricolor* showed the presence of the desired region of CBF gene with a length of about 220 nucleotides on a 2% agarose gel (Figure 3). The PCR result from DNA was then confirmed by the PCR product from cDNA by amplifying a region of a similar length, on a 2% agarose gel (Figure 3).

The alignment of the sequence obtained from sequencing, with the 300 nucleotide region obtained through bioinformatics analysis, revealed a high degree of similarity and conservation (Identity=82%, E-value=$6e^{-61}$) in this region in DNA of *V. tricolor* (Figure 4).

In this way, it can be stated that DREB1 family genes are also present in *V. tricolor*, which is not surprising given the plant high resistance to low temperatures, and even below zero.

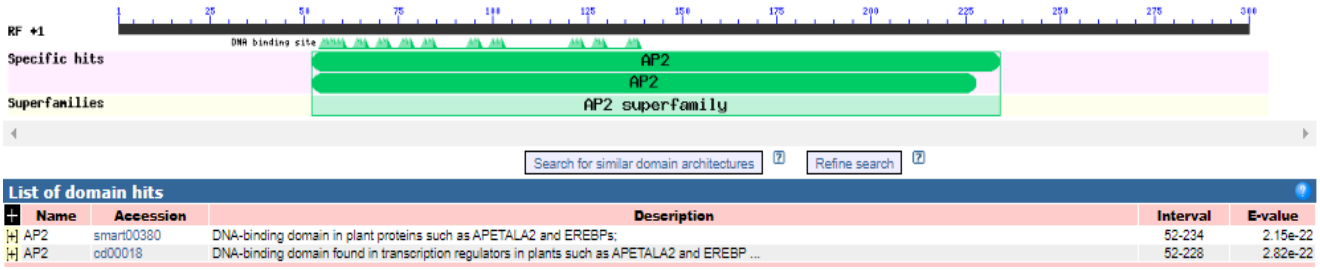


**Figure 2.** The BLASTx search result of the consensus sequence, showed this region with a high degree of conservation in the genes identified in other plant species, part of which is related to the conserved AP2 domain in DREB1 proteins.

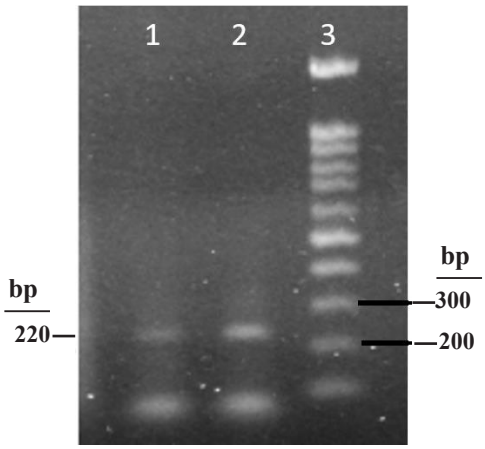


**Figure 3.** Gel electrophoresis of the PCR products of genomic DNA and cDNA of CBF gene/sequence. The wells show 1- PCR product on cDNA, 2- PCR product on DNA and 3- 100 bp sizing Marker, respectively.

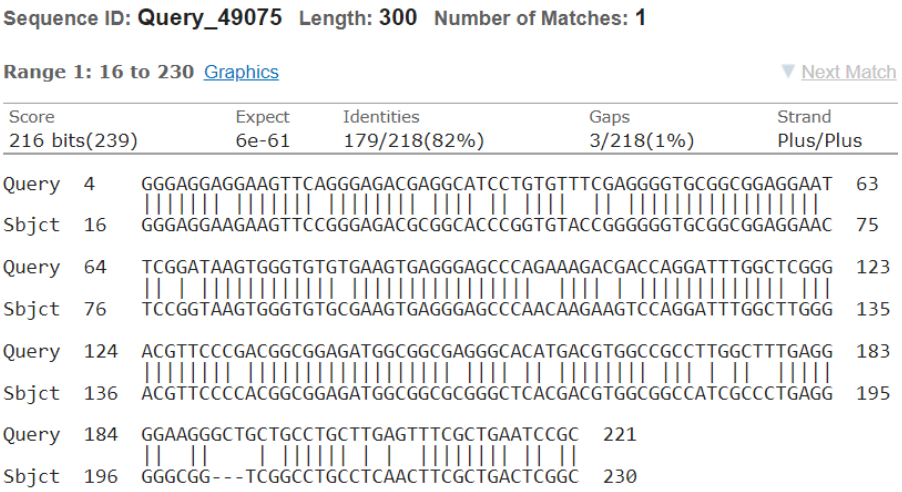


**Figure 4.** Alignment of the initial predicted 300 nucleotide sequence, with the sequence obtained from PCR product sequencing on DNA and cDNA of CBF gene, showed a high similarity in this region with CBF sequences in other plant species.

**Phylogenetic analysis of identified sequence**

The drawn phylogenetic tree revealed the lowest distance or, in other words, the highest similarity of the 220- nucleotide sequence of the CBF gene known in *V. tricolor* with the *CBF4b* gene of *Populus euphratica* (White Poplar). The sequence related to CBF of *V. tricolor* is assumed to be VT Viola tricolor CBF to distinguish it from other sequences. Bootstrap

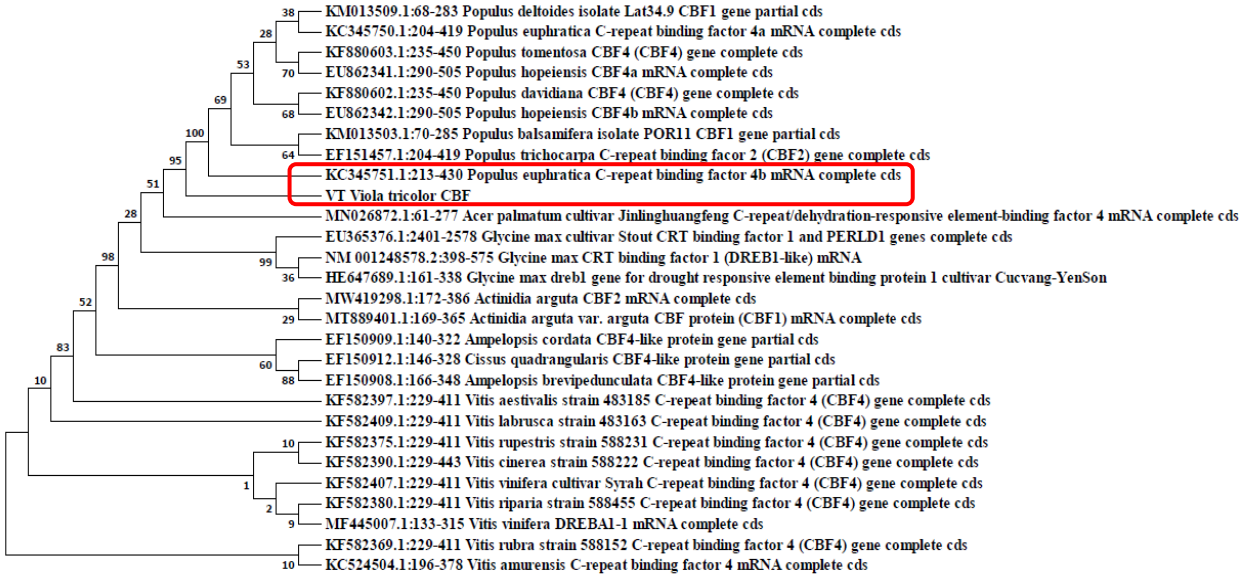


**Figure 5.** Phylogenetic tree based on the nucleotide alignment between the 220 nucleotide sequence identified in the *V. tricolor* genome with 27 CBF gene sequences of different plant species: The highest similarity was observed with *CBF4b* gene sequence of *Populus euphratica* species.

values are displayed on each node with 500 repetitions (Figure 5). Likewise, at the protein level, the greatest similarity was observed with the transcription factor DREB1D/ CBF4 of *Mangifera indica* (Mango) species (Figure 6).

**Determination of AP2 domain position**

The result of multiple alignment in Mega 7 programme determined the 60- amino acid domain of AP2 (Figure 7). High conservation in this region was observed among 50 sequences, including the AP2 sequence identified in the *V. tricolor* genome. Furthermore, by comparing and analyzing the sequence of this domain in viola, it was found that the two amino acids valine and glutamic acid, which are located at positions 14 and 19, respectively, have been protected during evolution (Figure 7). According to the studies, these amino acids are crucial for DREB proteins to bind to DRE cis-acting elements (Sakuma *et al.,* 2002).

**Analysis of gene expression**

CBFs are expressed in various tissues including carpel, cotyledon, flower pedicel, guard cells, hypocotyl, plant embryos, roots, shoot apex, stamen, stem, vascular leaves (Zhao *et al.,* 2016). The developmental stages of these genes expressed in the plant include the stages of germination, two leaves visible stage, flowering stage, petal differentiation and expansion stage, etc. (Novillo *et al.,* 2007).

In this study, after extraction of RNA from leaves of *V. tricolor* at multi-leaf growth stage and evaluating the quality and quantity of RNA samples extracted by agarose gel and NanoDrop spectrophotometer (Figure 8, Table 3), the final volume ($V_2$) for cDNA synthesis and its use for the Real-Time PCR reaction, was calculated using data from the NanoDrop and the equation $C_1V_1=C_2V_2$, for each sample, according to the initial concentration ($C_1$), the final concentration ($C_2$=1000 ng/µl) and the initial volume ($V_1$=1 µl), (Table 3).

It has been observed that in Arabidopsis, CBFs expression levels rise 15 min after being exposed to low temperatures and remain elevated for the next 1 to 2 h (Gilmour *et al.,* 1998). In a study conducted by Kindgren *et al.* (2018) on 2-week old cold-treated seedlings of Col-0 (3 h at 4 °C), they reported an upregulation in the expression of CBF genes by 100-400 fold (Kindgren *et al.,* 2018).

In this research as well, the analysis of gene expression data and examining the changes in CBF expression compared to the control using REST software (Pfaffl *et al.,* 2002), revealed that the CBF genes expression increased 52.619-fold in leaf tissues during the multi-leaf stage, under freezing stress (18 h at -8 °C) (Figure 9). These findings show that after applying the stress, gene expression is significantly (roughly 52.619 fold) higher than it was in the control group.

Based on the findings of this study and considering the proven role of the CBF gene family in relation

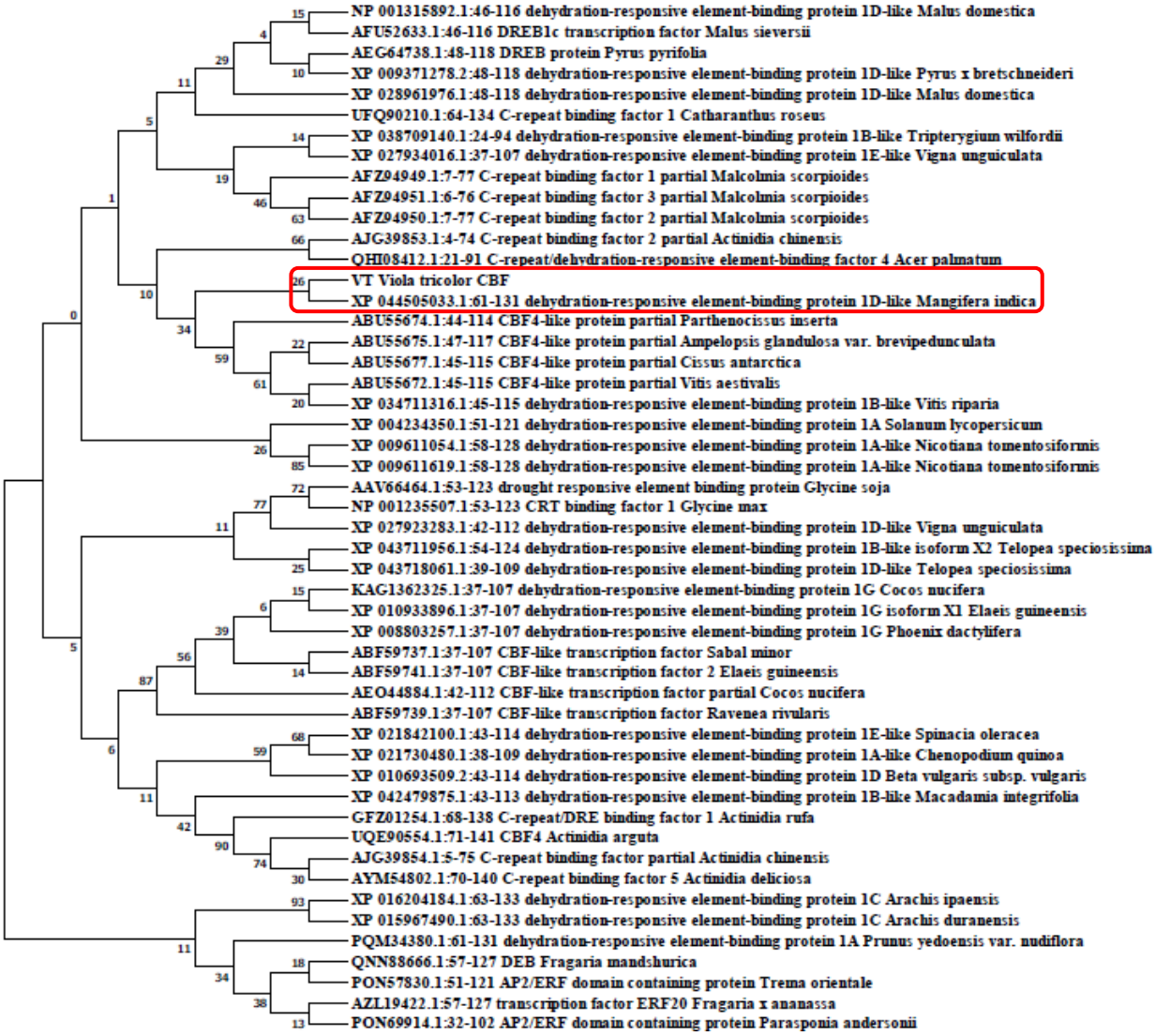


**Figure 6.** Phylogenetic analysis of the amino acid sequence of the 220 nucleotide region identified in *V. tricolor* with the protein sequence of CBF family genes in 49 different plant species: The lowest evolutionary distance was observed with DREB1D transcription factor of *Mangifera indica* species. The sequence related to CBF of *V. tricolor* is assumed to be VT Viola tricolor CBF to distinguish it from other sequences. Bootstrap values are displayed on each node with 500 repetitions.

to cold stress, it can be claimed that one of the main reasons for the resistance of various viola species to low temperatures is the high expression of these genes in cold stress conditions, and they can be used to induce resistance in cold-sensitive plants.

## ACKNOWLEDGMENTS

We appreciate the help of the agents and employees of Bu- Ali Sina University's greenhouse, who helped us prepare the seeds and grow plants in the greenhouse.

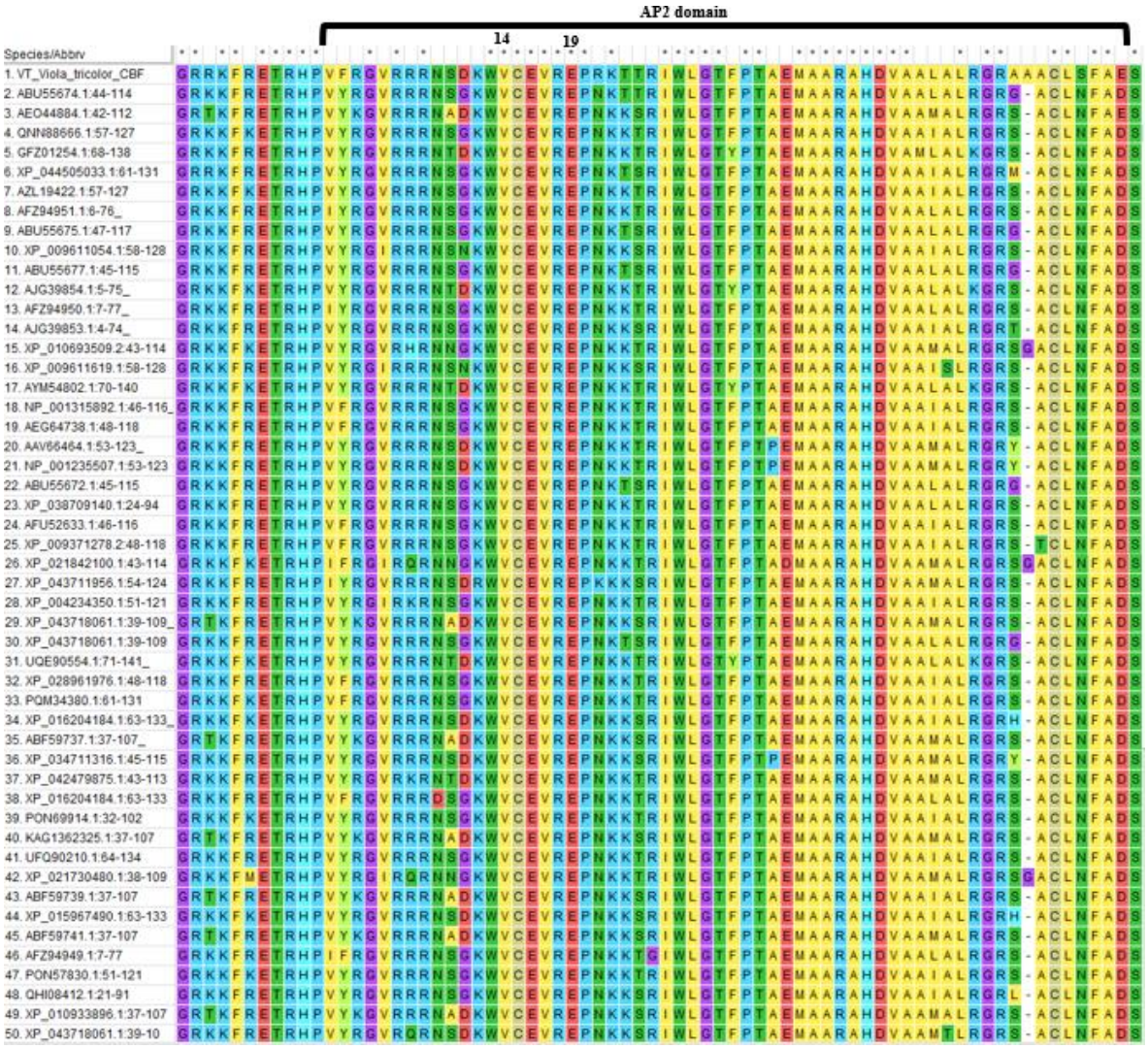


**Figure 7.** Multiple alignment of 49 CBF sequences from different species to determine the region of DNA-binding domain (AP2) in CBF found in *V. tricolor* (Line No.1): this region is 60 amino acids long and exhibits high conservation among these 50 species. Amino acids 14 and 19 in the AP2 sequence, which are essential for its binding to DNA, are conserved among all these species. An asterisk (*) indicates positions which have a single, fully conserved amino acid.

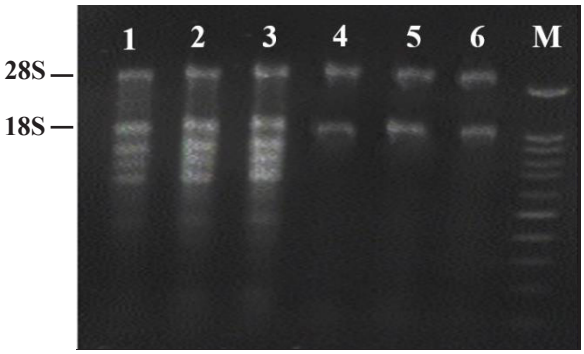


**Figure 8.** Evaluation of the quality of total RNA extraction on 1% gel electrophoresis. 1, 2 and 3: control samples; 4, 5 and 6: treated samples; M: 100 bp sizing marker.

**Table 3.** The quantity and quality of total RNA extracted were determined using NanoDrop spectrophotometer. The final volume ($V_2$) for cDNA synthesis was calculated by considering the final concentration ($C_2$) of 1000 ng/µl and the initial volume ($V_1$) of 1 µl, for each of the 8 samples using the equation $C_1V_1=C_2V_2$. $C_1$: Initial concentration.

| Samples | Number | $C_1$ (ng/µl) | $V_2$ (µl) | The absorbance ratio 260/280 |
|---|---|---|---|---|
| Control | 1 | 1389.2 | 0.72 | 1.92 |
| | 2 | 1367.8 | 0.73 | 1.87 |
| | 3 | 1209.9 | 0.827 | 1.96 |
| | 4 | 1256.4 | 0.796 | 1.85 |
| Treated | 5 | 1067.3 | 0.937 | 1.87 |
| | 6 | 836.3 | 1.2 | 1.91 |
| | 7 | 971.9 | 1.03 | 1.90 |
| | 8 | 802.6 | 1.246 | 1.81 |

| Parameter | Value |
|---|---|
| Iterations | 2000 |

| Gene | Type | Reaction Efficiency | Expression | Std. Error | 95% C.I. | P(H1) | Result |
|---|---|---|---|---|---|---|---|
| CBF | TRG | 1.0 | 52.619 | 37.472 - 67.883 | 32.391 - 100.978 | 0.022 | UP |
| 18S | REF | 1.0 | 1.000 | | | | |

**Interpretation**

CBF is UP-regulated in sample group (in comparison to control group) by a mean factor of 52.619 (S.E. range is 37.472 - 67.883).

CBF sample group is different to control group. P(H1)=0.022

**Figure 6.** The analysis of CBF gene expression data at P- value of ≤0.05 using REST programme: These findings show that after applying the stress, gene expression is significantly (roughly 52.619 fold) higher than it was in the control group. Std. Error: Standard Error, C.I.: Confidence interval, P(H1): P- Value. The number of iterations of Bootstrap in this software is considered to be 2000 by default.

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