Afyon Kocatepe Üniversitesi Fen ve Mühendislik Bilimleri Dergisi

Afyon Kocatepe University Journal of Science and Engineering

AKÜ FEMÜBİD **23** (2023) 021001 (292-299) AKU J. Sci. Eng. **23** (2023) 021001 (292-299)

DOI: 10.35414/akufemubid.1205241

Araştırma Makalesi / Research Article

Cloning of full-length gene encoding homologue of CBF1 transcription factor from *Olea europaea L.* leaves

Munise YURTSEVER[1], Maide ŞEKER[2], Şenay VURAL KORKUT[1*]

[1]*Department of Molecular Biology and Genetics, Faculty of Arts and Science, Yildiz Technical University, İstanbul, Turkey*

[2]*Department of Molecular Biology and Genetics, Faculty of Science, Gebze Technical University, Kocaeli, Turkey*

e-mail: munise@yildiz.edu.tr *ORCID ID: https://orcid.org/0000-0003-2446-9914*

e-mail: maideseker@gmail.com *ORCID ID: https://orcid.org/0000-0003-0013-2516*

Corresponding author e-mail: skorkut@yildiz.edu.tr *ORCID ID: https://orcid.org/0000-0002-6260-0357*

Geliş Tarihi: 15.11. 2022 Kabul Tarihi: 12.03.2023

Keywords
CBF/DREB; *Olea europaea L.*; Gene Cloning ; DNA Sequencing, Phylogenetic analysis

Abstract

One of the most prevalent environmental stresses that affects plants physically and physiologically is the low temperature. Since low temperature adversely affects plant productivity Researchers investigated the molecular processes that regulate the effects of cold stress on plants and discovered many cold responsive genes as well as the control mechanisms that regulate them. CBF/DREB (C repeat binding factor/dehydration response element binding factor) transcription factors induces the expression of corresponding genes in plants in response to abiotic stress conditions such as cold, drought and salinity. These transcription factors contribute to plant resistance to stress by activating genes in various pathways. Genes encoding CBF transcription factors were first discovered in *Arabidopsis thaliana* (L.). Later, Gene homologs were isolated and cloned from a variety of plants. They belong to the AP2/EREBP protein family. In the present study, the full-length cDNA encoding homologue of the transcription factor CBF1 was cloned from *Olea europaea L*. cv. Gemlik leaves. A 748 bp long cDNA encodes a hypothetical protein of 224 amino acids. BLAST analysis revealed that the CBF1 sequences of Gemlik cultivar and wild olive were nearly identical. A phylogenetic tree was built using *Olea europea L*. cv. Gemlik CBF1 sequence.

Olea europaea L. yaprağından CBF1 transkripsiyon faktörü homoloğunu kodlayan tam uzunluktaki genin klonlanması

Anahtar kelimeler
CBF/DREB; *Olea europaea L.*; Gen Klonlama; DNA dizileme; Filogenetik analiz

Öz

Bitkileri fiziksel ve fizyolojik olarak etkileyen en yaygın çevresel streslerden biri düşük sıcaklıktır. Düşük sıcaklık bitki verimliliğini olumsuz etkilediğinden, araştırmacılar soğuk stresinin bitkiler üzerindeki etkilerini düzenleyen moleküler süreçleri araştırmışlar ve soğuğa duyarlı genlerle birlikte bunların kontrol mekanizmalarını da ortaya çıkarmışlardır. *CBF/DREB* (C tekrar bağlama faktörü/dehidrasyon yanıt elemanı bağlama faktörü) transkripsiyon faktörleri, bitkilerde soğuk, kuraklık ve tuzluluk gibi abiyotik stres koşullarına yanıt olarak ifade edilen genlerin ekspresyonunu indüklemektedir. Bu transkripsiyon faktörleri, çeşitli yolaklardaki genleri aktive ederek strese karşı bitki direncine katkıda bulunurlar. CBF transkripsiyon faktörlerini kodlayan genler ilk olarak *Arabidopsis thaliana*'da (L.) keşfedildi. Daha sonra çeşitli bitkilerden gen homologları klonlandı. CBF/DREB transkripsiyon faktörleri AP2/EREBP protein ailesi içinde yer alırlar. Bu çalışmada, transkripsiyon faktörü CBF1'in homologunu kodlayan tam uzunluktaki cDNA, *Olea europaea* L. cv. Gemlik yapraklarından klonlanmıştır. 748 bp uzunluğunda cDNA, 224 amino asitlik varsayımsal bir proteini kodlar. BLAST analizi, Gemlik çeşidinin ve yabani zeytinin *CBF1* dizilerinin neredeyse aynı olduğunu ortaya çıkardı. *Olea europea L.* cv Gemlik *CBF1* dizisi kullanılarak bir filogenetik ağaç oluşturuldu.

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1. Introduction

Low temperature causes several mechanical and physical damage to the plants and is one of the major stresses that limit plant growth, productivity, and dispersal (Boyer 1982, Mosa *et al.* 2017). Cold acclimation is a process by which plants increase their tolerance to low, non-freezing temperatures (Palva and Heino 1997). It causes some changes in gene expression levels. The best understood mechanism of cold acclimation to date is *CBF* (C-repeat binding factor) cold response pathway (Lissarre *et al*. 2010; Thomashow 2001). CBFs are transcription factors belonging to the AP2/ERF domain of the DNA-binding protein family (Riechmann and Meyerowitz 1998). These transcription factors bind cis-acting regulatory elements termed C repeats/dehydration response elements (CRT/DRE) (Baker *et al*. 1994; Stockinger *et al*. 1997). Many cold-responsive genes, such as COR (cold-regulated) (Zarka *et al*. 2003) and LTI (low temperature-induced) genes (Yamaguchi-Shinozaki and Shinozaki 1994) contain CRT/DRE elements in their promoter regions. *CBF* expression increases cold tolerance by inducing these genes (Gilmour *et al.* 1998, Jaglo-Ottosen *et al*. 1998, Liu *et al.* 1998).

The first *CBF1* transcription factor was cloned from Arabidopsis thaliana with yeast hybrid method (Stockinger *et al.* 1997). In the following years *DREB1A, DREB1B, and* DREB1C genes were isolated from *Arabidopsis thaliana* which was grown under cold and drought stress. In this study, it was discovered that *DREB1B* clone identical to CBF1 (Liu *et al.* 1998). The *CBF1, CBF2, CBF3* proteins are also named as DREB1C, DREB1B, and DREB1A, respectively (Stockinger *et al*. 2001).

CBF/DREB1 transcription factor homologues have also been identified in various plants such as wheat, tomato, maize, cotton, and tobacco. (Chen *et al*. 2008, Guo *et al*. 2011, Huang *et al*. 2007, Qin *et al.* 2004, Zhang *et al.* 2004). Although CBF sequences from different plants are similar in conserved regions, their full-length sequences differ significantly. (Agarwal *et. al.* 2006, Mizoi *et. al.* 2012, Shi *et. al.* 2018). This feature of CBF encoding sequences makes their cloning challenging.

Cloning *CBF* genes, particularly in stress-tolerant crop species, will allow researchers to gain a better understanding of the molecular mechanisms behind abiotic stresses like cold, drought, and salinity. Additionally, increasing the expression of the stress-related genes in transgenic plants created utilizing CBF genes would aid in the development of plants that are resistant to stress in a variety of environmental conditions. In the present study, we cloned the CBF1 coding sequence of *Olea europaea L. var. europaea cv.* Gemlik which has been exposed to cold stress.

2. 2. Materials and methods

2.1 Plant material and cold treatment

For adaptation, *Olea europaea cv.* Gemlik (olive) seedling was kept at 24 °C under 14-hours photoperiod for one week. For cold treatment, the plants were kept at 4 °C with a 12-hour photoperiod for 24 hours. Under cold stress, olive leaves were harvested at specific intervals of time. Control plants were maintained at 24°C under the 14 hours photoperiod.

2.2 RNA isolation, cDNA synthesis, and genomic DNA isolation

Total RNA was extracted from the leaves of control and cold-treated *Olea europaea L*. cv. Gemlik seedlings using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. Genomic DNA and chemical residues were removed using mini-columns supplied in RNeasy Mini Kit (Qiagen, The Netherlands). First-strand cDNA synthesis was carried out according to iScript cDNA Synthesis Kit (Bio-Rad, USA) instructions using 3 µg of total RNA.

Genomic DNA was extracted from olive leaves using Plant DNA Preparation Kit (Jena Bioscience, Germany) according to the manufacturer's instructions.

2.3 Polymerase chain reactions (PCR)

Olea europaea L. *europaea* var. sylvestris (wild olive) CBF1 sequence (XM_023032549.1) was used for the design of gene-specific forward primer (CBF.F) 5'-TCAAGATTAATGGATATTTTC-3' and reverse primer (CBF.R) 5'-AGCACGGCTAAGAAGGAACCT-3'. PCR reaction was performed with Advantage 2

Polymerase Mix (Clontech, USA). PCR conditions were 2 min of initial denaturation at 95 °C followed by 35 cycles of 95 °C for 30 sec, 50 °C for 40 sec, 68 °C for 1 min, and a final extension at 68 °C for 5 min. The same conditions were applied for both cDNA and genomic DNA.

2.4 Cloning into E. coli and sequencing

PCR fragments were purified from agarose gel using the MEGAquick-spin™ Plus Total Fragment DNA Purification Kit (Intron Biotechnology, South Korea). T/A cloning of purified products was carried out with pGEM-T Easy Vector System (Promega, USA). Plasmids were transformed into the competent *E.coli* DH5α cells prepared according to Inoue ultra-competent cell protocol (Inoue *et al*. 1990). Colony PCR was used for selecting positive clones. To analyze PCR products, 1% agarose gel was prepared with 1xTBE Buffer and EtBr. λ PstI DNA ladder was loaded to first well as a molecular weight marker and samples were run at 70 V for 40 minutes. Plasmid DNA isolations were made from positive clones using High Pure Plasmid Isolation Kit from Roche. Since PGEM-T Easy Vector has EcoRI restriction sites surrounding the insert site, EcoRI restriction digestion was performed for insert control. Plasmid DNAs were sent for sequencing (Triogen Biotechnology, Türkiye).

2.5 Phylogenetic analysis

Homology analyses were performed with nBLAST and pBLAST tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The Protein sequences with high similarity were selected. MEGA 11 program was used to create a phylogenetic tree using the neighbor-joining (NJ) method with a bootstrap analysis of 1000. The amino acid sequence was translated from the nucleotide sequence with EMBOSS Transeq Tool and the BLAST analysis was performed. Homolog amino acid sequences were selected and aligned with the Multiple alignment tool.

3. Results

3.1 Cloning and sequence analysis

cDNA of *CBF* was cloned both using total RNA and genomic DNA isolated from leaves of olive seedlings. Total RNA isolated from samples exposed to 4 hours of cold was used to synthesize first-strand cDNA. Gene-specific primers were designed using wild olive nucleotide sequence. RT-PCR and TA cloning method used for cloning. Vector sequences in raw sequence data was extracted using Chromas DNA sequencing software and 748 base pairs long cDNA nucleotide sequence was obtained by RT-PCR (Figure 1). For genomic DNA PCR, the same primers were used, and the amplified DNA fragment was 748 base pairs long (Figure 2). In the agarose gel images shown in Figures 1 and 2, the size of the digested fragment is approximately 800 base pairs. The fragment is cut from the PGEMT eaay vector with the EcoRI enzyme, which explains why it is longer than the cDNA size in the results of the sequencing analysis. The vector sequences are introduced during this process.

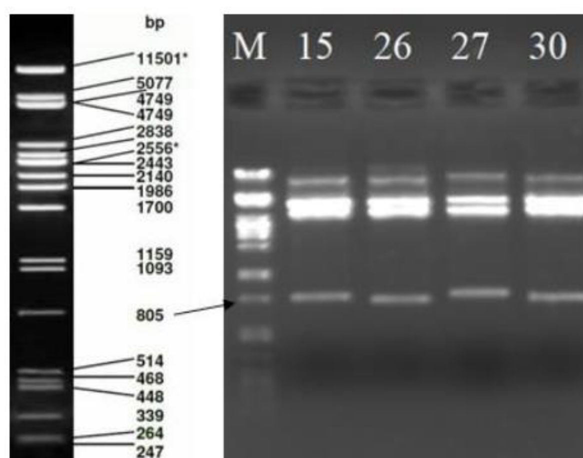

Figure 1. Plasmid insert control from cDNA clone by EcoRI digestion (M: Marker, 15-26-27-30 are different clones, we obtained our gene from 15. clone).

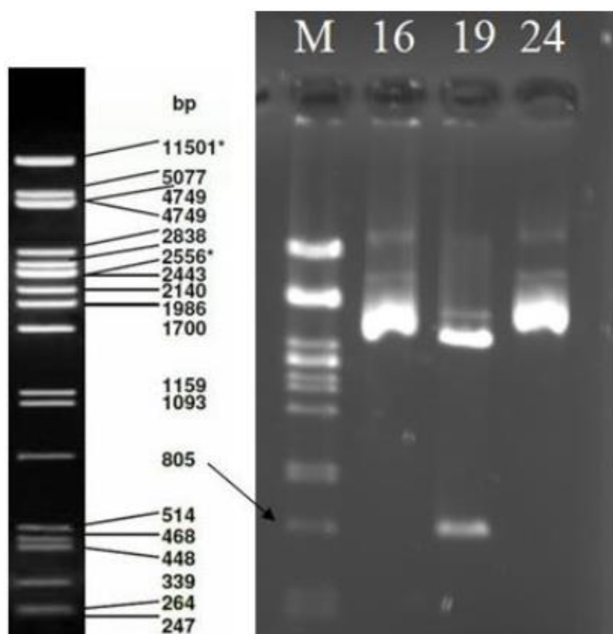

Figure 2. Plasmid insert control from genomic DNA clone by EcoRI digestion (M: Marker, 16-19-24 are different clones, we obtained our gene from 19. clone).

Alignment of these nucleotide sequences showed that there were no introns in the gene. The open reading frame encodes a 224-amino acid-long hypothetical protein (Figure 3). The molecular weight of the hypothetical protein was predicted as 24.7 kDa and its isoelectric point was predicted as 5.22 using EXPASY online tool. Conserved amino acid sequence of AP2 domain ranging from 70-127 had been shown in the alignment of plant CBF sequences (Figure 4). A 3D model of the domain was constructed with SWISS-MODEL online tool (Figure 5).

```
  1 ATGGATATTTTCAACAAGTTCAACTCGGACCCAGTTTCTTGTGTG
     M  D  I  F  N  K  F  N  S  D  P  V  S  C  V
 46 CAAGATTTCTCGTCCTTGACTGAAGCACCAGAGTCTTCATCTTTA
     Q  D  F  S  S  L  T  E  A  P  E  S  S  S  L
 91 TCTGATAATAGCAATAGCGTTAAAAGAGCCACTTTTTCTGATGAT
     S  D  N  S  N  S  V  K  R  A  T  F  S  D  D
136 GAAGTTTTGCTAGCTTCAAACAACCCGAAAAAGCGTGCCGGGAGG
     E  V  L  L  A  S  N  N  P  K  K  R  A  G  R
181 AAGAAATTCCGCGAGACGAGGCACCCTGTTTATCGGGGAGTGAGG
     K  K  F  R  E  T  R  H  P  V  Y  R  G  V  R
226 CGGAGGAACTCTGGTAAGTGGGTGTGTGAAGTCAGAGAACCCAAC
     R  R  N  S  G  K  W  V  C  E  V  R  E  P  N
271 AAGAAGTCAAGAATCTGGCTGGGAACTTTCGCCACAGCGGAAATG
     K  K  S  R  I  W  L  G  T  F  A  T  A  E  M
316 GCAGCGAGAGCTCACGACGTGGCGGCAATAGCGCTTCGCGGTAGG
     A  A  R  A  H  D  V  A  A  I  A  L  R  G  R
361 TCAGCGTGTTTAAACTTTGCTGATTCAGCTTGGAAGCTACCGGTT
     S  A  C  L  N  F  A  D  S  A  W  K  L  P  V
406 CCGACCTCCACTGATGCTAAGGACATTCAGAAAGCGGCAGCAGAA
     P  T  S  T  D  A  K  D  I  Q  K  A  A  A  E
451 GCGGCCAAGGCCTTTGGGCAACCAAATTCCGAGTCGGATGCGAGG
     A  A  K  A  F  G  Q  P  N  S  E  S  D  A  R
496 GAGGAAGCTACCGCTATATCGCAGGAAAATGTGTCCTTTTCCGAT
     E  E  A  T  A  I  S  Q  E  N  V  S  F  S  D
541 GAGGAGGATCTTTTCGGAATGCCTGGATTGATTGACAATATGGCT
     E  E  D  L  F  G  M  P  G  L  I  D  N  M  A
586 GAAGGGTTGATGCTACCTCCACCTTACTGCATGGACACTGATGTC
     E  G  L  M  L  P  P  P  Y  C  M  D  T  D  V
631 GTGGATGCATATGCTGACATGTCTTTATGGAGTTATTCCATTTAA
     V  D  A  Y  A  D  M  S  L  W  S  Y  S  I  *
```

Figure 3. Full length CBF1 open reading frame.

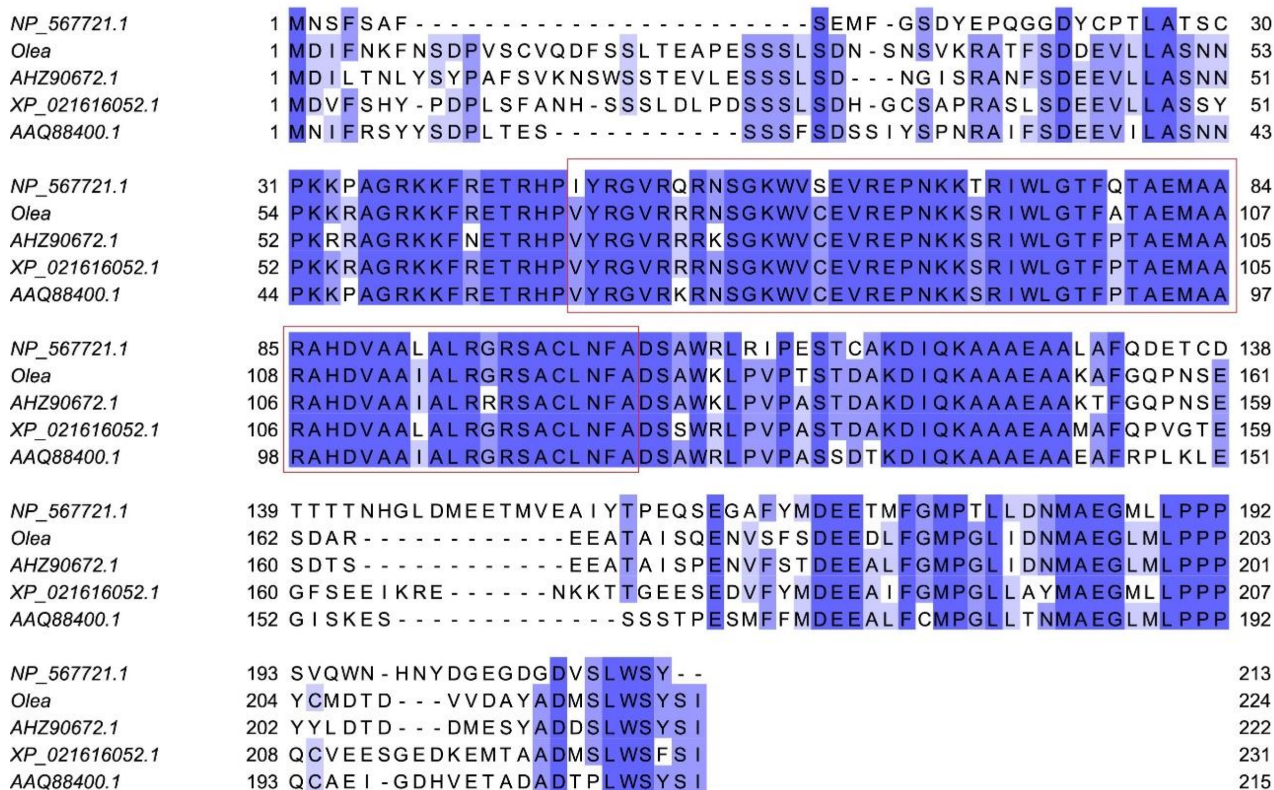

Figure 4. Multiple sequence alignment of *CBF/DREB1* proteins. Conserved sequences are shown in blue, AP2 domians are boxed. NP_567721.1 *Arabidopsis thaliana CBF/DREB1*, *Olea europaea* L. var. *europaea* cv. Gemlik *CBF1*, AHZ90672.1 *Fraxinus mandshurica CBF/DREB1*, XP_021616052.1 *Manihot esculenta DREB1A-like*, AAQ88400.1 *Capsicum annuum CaCBF1B*.

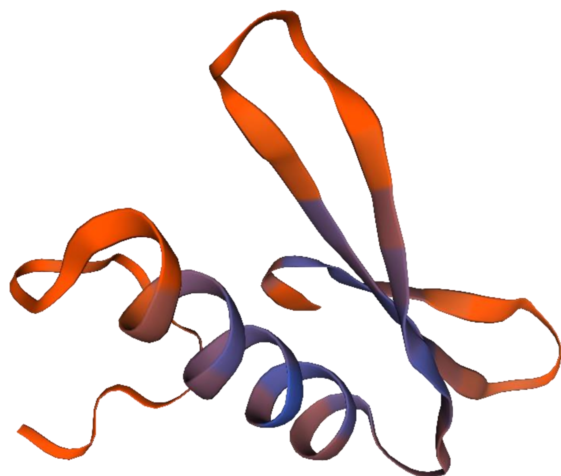

Figure 5. 3D Model of *Olea europaea* cv. Gemlik AP2 domain.

3.2 Phylogenetic analysis

nBLAST analysis revealed that *Olea europaea* cv. Gemlik CBF nucleotide sequence demonstrated 99.87% similarity to *Olea europaea* var. *sylvestris DREB1B*, 86.83% similarity to *Fraxinus mandshurica CBF/DREB1*.

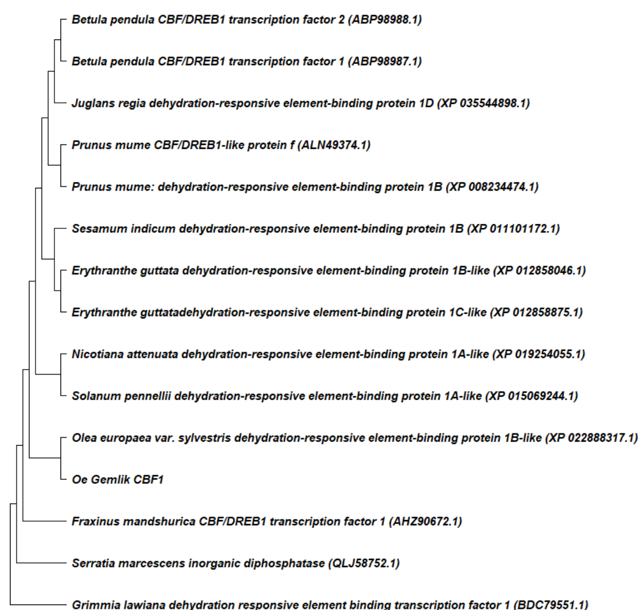

Figure 6. Phylogenetic tree for *Olea europaea* cv. Gemlik CBF1.

A phylogenetic tree was built using MEGA 11 (Figure 6). 14 different *CBF*s were selected according to similarity BLAST analysis and their amino acid sequences retrieved from using the NCBI database. *Grimmia lawiana* moss was used to root the tree. Expectedly *Olea europaea var. sylvestris* (wild olive) *CBF1* as the closest relative, showed 100% identity to the *Olea europaea cv.* Gemlik *CBF1* amino acid sequence. Olive *CBF1*s were clustered with *Fraxinus mandshurica* (Manchurian ash) *CBF/DREB1* which had 80.36% identity. Manchurian ash grows in cool-temperate forest ecosystems in East Asia and has excellent cold tolerance (Liang et al. 2019). *Nicotiana attenuata* (coyote tobacco), *Solanum habrochaites* (wild tomato) and *Solanum commersonii* (wild potato) *DREB* genes are clustered together with olive CBF with an identity of 73.26%, 72.40%, and 70.20%, respectively (Caffagni *et al*. 2014, Pino *et al*. 2013).

4. Discussion

CBF/DREB proteins are the most extensively studied transcription factors involved in the cold response pathway in plants. In this study, we aimed to clone the full-length *CBF1* gene from Gemlik cultivar of olive. Olive seedlings were exposed to cold stress for this purpose, and RNA was extracted from samples collected at various time points. When reverse transcription PCR products of the control RNAs were run on a gel, no band was visible. However, when RNA from plants exposed to 4-h cold stress was used in reverse transcriptase PCR, a band around 750 bp was observed. This demonstrates that cold stress induces CBF expression in *Olea europaea* leaves. Genomic DNA was also used to clone CBF for analyzing the existence of introns. Comparing two sequences with Clustal omega revealed there were no introns. This is the case for the vast majority of CBF genes that are currently known.

Base substitution, indels, and repeats were found to be abundant in the olive gene fragments *OeACP1*, *OeACP2*, *OeLUS*, and *OeSUT1*. These variations are important considering the roles of these genes in primary metabolic pathways (Cultrera *et al.* 2019). *Olea europaea* Gemlik *CBF* nucleotide sequence demonstrated 99.87% similarity to wild olive while the amino acid sequence demonstrates 100% identity which indicates *CBF1* gene was conserved in wild and cultivated forms.

According to phylogenetic analysis Manchurian ash is the closest relative of olive *CBF1*. Manchurian ash is a native species in Northern Asia and can endure -40 °C. Other close relatives include coyote tobacco, wild tomato, and wild potato species. These wild species are thought of as gene sources for resistance phenotype.

Because *CBF* genes are involved in abiotic stress responses, the expression profile of the olive CBF1 homologue in stress conditions can be investigated further using real-time PCR. Transient expression analysis can be performed in a model plant to investigate the effects of the gene in cold acclimation

Despite the fact that olive is not a cold-resistant species, study into the CBF transcription factors in this plant is crucial for comprehending how cold adaptation works and developing stress-resistant cultivars.

Acknowledgement

This work was supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK), project no 1919B011703461.

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