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ORIGINAL ARTICLE

# Comparative bioinformatics analysis and abiotic stress responses of expansin proteins in Cucurbitaceae members: watermelon and melon

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## Abstract

Watermelon and melon are members of the Cucurbitaceae family including economically significant crops in the world. The expansin protein family, which is one of the members of the cell wall, breaks down the non-covalent bonds between cell wall polysaccharides, causing pressure-dependent cell expansion. Comparative bioinformatics and molecular characterization analysis of the expansin protein family were carried out in the watermelon (*Citrullus lanatus*) and melon (*Cucumis melo*) plants in the study. Gene expression levels of expansin family members were analyzed in leaf and root tissues of watermelon and melon under ABA, drought, heat, cold, and salt stress conditions by quantitative real-time PCR analysis. After comprehensive searches, 40 expansin proteins (22 ClaEXPA, 14 ClaEXPLA, and 4 ClaEXPB) in watermelon and 43 expansin proteins (19 CmEXPA, 15 CmEXPLA, 3 CmEXPB, and 6 CmEXPLB) in melon were identified. The greatest orthologous genes were identified with soybean expansin genes for watermelon and melon. However, the latest divergence time between orthologous genes was determined with poplar expansin genes for watermelon and melon expansin genes. *ClaEXPA-04*, *ClaEXPA-09*, *ClaEXPB-01*, *ClaEXPB-03*, and *ClaEXPLA-13* genes in watermelon and *CmEXPA-12*, *CmEXPA-10*, and *CmEXPLA-01* genes in melon can be involved in tissue development and abiotic stress response of the plant. The current study combining bioinformatics and experimental analysis can provide a detailed characterization of the expansin superfamily which has roles in growth and reaction to the stress of the plant. The study ensures detailed data for future studies examining gene functions including the roles in plant growth and stress conditions.

**Keywords** Expansin genes · Characterization · *Citrullus lanatus* · *Cucumis melo* · Abiotic stresses

## Introduction

Watermelon and melon are members of the Cucurbitaceae family including economically significant crops in the world. There are 119 genera and 825 species belonging to the Cucurbitaceae family in tropical and subtropical regions (Yeşil 2019). Plants such as dicotyledonous watermelon, melon, zucchini, and cucumber are in that reptile, sticky, and broadleaf plant family. Total production of watermelon and melon reached 100 and 27 million tons in the world, respectively. Turkey is the second most producer country after China for both of those crops (FAOSTAT 2019).

Cell walls include cellulose and many other polysaccharides that vary in structure, function, and multiplicity and can be modified by the activities of enzymes and other proteins synthesized by the cell. The expansin protein family, which is one of the members of the cell wall, breaks down the non-covalent bonds between cell wall polysaccharides such as cellulose and hemicellulose especially xyloglucan, causing pressure-dependent cell expansion (Fukuda 2014). It is known that the expansin protein family has roles in developmental stages including meristems and developmental

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zones of root and stem (Cho and Cosgrove 2002) and root hairs (Reinhardt et al. 1998) and has function in fruit softening (Brummell et al. 1999) and in seed germination (Chen and Bradford 2000). Moreover, previous studies reported that expansins have roles in the abiotic stress tolerance of plants (Dai et al. 2012; Han et al. 2012; Lu et al. 2013). Expansins contain four gene subfamilies according to the current information: α-expansin or expansin A (EXPA), β-expansin or expansin B (EXPB), expansin alpha-like (EXPLA), and expansin beta-like (EXPLB) (Choi et al. 2006). The function of α-expansin proteins is to bind closely to cellulose and hemicellulose. However, they do not have hydrolytic features against large polysaccharide compounds of the cell wall (McQueen-Mason and Cosgrove 1995). Although a study has not yet been published to compare the binding and hydrolytic activity of EXPB proteins, their effect for relaxation is almost identical to that of α-expansin proteins (Cosgrove et al. 1997a). Moreover, EXPB proteins are known to contain a subset of proteins called group-I grass pollen allergens and some other proteins that are not well-defined (Cosgrove 2015; Sampedro et al. 2015). Ectopic expression of *AtEXPLA2* has been determined to increase the root and hypocotyl lengths of *Arabidopsis* plants (Boron et al. 2015).

When plants are subjected to unfavorable environmental situations, their growth is adversely affected. A plant's ability to adapt and resist stress is essential for its survival in stressful conditions. Struggle for the plant to survive despite adverse environmental conditions is defined as stress resistance (Levitt 1980). Abiotic stress factors consist of many adverse environmental conditions including lack of nutrients or water, low and high temperature, UV, and salinity. Plants that live in adverse environmental conditions for a long time trying to adapt in such a way that these environmental factors are minimally impacted (Dolferus 2014; Öztürk 2015). Abiotic stresses cause serious product losses worldwide. For this reason, to uncover information about the stress tolerance mechanisms of plants, it is necessary to conduct genome-wide studies of plants of agricultural importance.

Although the full genome sequence data of watermelon and melon plants were available in 2013 and 2012, respectively, gene identification and characterization studies using these genomes were limited (Garcia-Mas et al. 2012; Guo et al. 2013). Gao et al. (2020) has determined 30 expansin genes in watermelon. They used Blast search in relevant databases using only *Arabidopsis* expansin genes. However, in the current study, watermelon expansin genes were identified via CLC Genomic Workbench v.11.1 package program using all identified expansin genes before in other plants. This package program preserved detailed and comprehensive information about expansin genes in watermelon. Therefore, detailed and comparative bioinformatics and molecular characterization analysis of the expansin protein family were carried out in the watermelon (*Citrullus lanatus*) and melon (*Cucumis melo*) genomes in the current study. Moreover, gene expression rates of expansin genes were analyzed in leaf and root tissues of watermelon and melon plants under ABA, drought, heat, cold, and salt stress conditions using available RNA sequencing data and a quantitative real-time PCR system.

## Materials and methods

### Determination of expansin family members in watermelon and melon

To identify the expansin genes in watermelon and melon genomes, the expansin genes (EXPA, EXPB, EXPLA, EXPLB) in previously determined plants were scanned in the Expansin Central Database (http://personal.psu.edu/fsl/ExpCentral/) and the relevant sequences were found. These sequences were compared with all proteins in watermelon and melon using BLASTP (Protein Blast Sequence Comparison) in the Cucurbit Genomics Database (CuGenDB, http://cucurbitgenomics.org/) and the repeated sequences were eliminated. According to the algorithm used in the comparison, sequences meeting the expectation value of $\geq e^{-50}$ and $\geq 50\%$ similarity were selected. To compare the sequences related to the whole genome sequences of watermelon and melon, a BLAST search was performed by the CLC Genomics Workbench 11.1 program. Domain analysis in the PFAM Database was performed to determine the connection of the protein sequences with expansin. Pfam Sequences with DPBB_1 and/or Pollen_allerg_1 domains were included in the study by comparing conserved regions with Hidden Markov Models (HMM) using PF01357 (Pollen_allerg_1 domain) when applying this connection (http://pfam.xfam.org/) (Zhang et al. 2014; Finn et al. 2016). Then, determined expansin gene sequences were retrieved for the study. Information about the properties of expansin proteins (physical position on the chromosomes, amino acid length, molecular weight, isoelectric points (pI), and instability index) was collected using the Expasy ProtParam Tool server (https://web.expasy.org/protparam/) (Gasteiger et al. 2005).

### Determination of the chromosomal position of expansin genes and prediction of gene structure

Through scanning of the Cucurbit Genomics Database server, BLASTP was employed to define the chromosomal locations of watermelon and melon genes encoding expansin proteins (CuGenDB, http://cucurbitgenomics.org/). Then, they were mapped using the MapChart program (Voorrips 2002). To determine the gene structure of the expansins, complementary DNAs (cDNA) or predictive coding sequences and genomic sequences were compared

by the Gene Structure Display Server (GSDS, http://gsds.gao-lab.org/) tool and exon–intron organizations were determined (Guo et al. 2007).

## Sequence alignment, phylogenetic analysis, and determination of preserved motifs

Amino acid sequences of watermelon and melon expansin proteins were loaded into the MEGA X program (Kumar et al. 2018). Then, multiple sequence alignment was performed using ClustalW (Larkin et al. 2007). A phylogenetic tree was created using the Neighbor-Joining (NJ) method with aligned sequences (Saitou and Nei 1987) using bootstrap analysis for 1000 iterations. The created tree was visualized with the Interactive tree of life tool (iTOL, https://itol.embl.de/) (Letunic and Bork 2011).

For the identification of conserved motifs, protein sequences were uploaded to the Multiple Em for Motif Elicitation (MEME, https://meme-suite.org/meme/tools/meme) motif search tool (Bailey and Elkan 1994). Using the classical mode, a total of 20 motifs were identified. Obtained MEME motifs were scanned with InterProScan, which ensures functional investigation of proteins by scanning their significant domains and areas (Quevillon et al. 2005).

## Gene ontology (Go) analysis

Blast2Go server (https://www.blast2go.com/) was used for functional analysis of the expansin proteins of watermelon and melon (Conesa and Gotz 2008). Functional analysis was performed in 3 stages with amino acid sequences of the expansin proteins. (i) A match was made with arrays installed on the server (BLASTP). (ii) Mapping was carried out for the data coming from BLAST analysis (MAPPING). (iii) Documentation of the sequence information has been obtained (ANNOTATION). As a result of the analysis, Gene Ontology (Go) classification was made, which was divided into 3 categories as biological process, cellular components, and molecular function.

## Determination of expansin gene orthologs and prediction of divergence times

Tandem and segmental duplication events of expansin genes were found in watermelon and melon genomes. In addition, the amino acid sequences of the expansins found in the watermelon and melon and their orthologs in *Arabidopsis* (*Arabidopsis thaliana*), rice (*Oryza sativa*), soybean (*Glycine max*), and poplar (*Populus trichocarpa*) were aligned using the ClustalW multi-sequence alignment program (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al. 2011). This analysis was performed using the BLASTP scan in the NCBI database (National Biotechnology Information Center), concerning the expected rate of $\leq 1e^{-50}$ and 50% similarity parameters between sequences showing similarity. The PAL2NAL (http://www.bork.embl.de/pal2nal/) (Suyama et al. 2006) program was used to calculate the homologous (Ks) and non-homologous (Ka) variation rates of the expansin genes using the multiple sequence alignment of amino acid sequences and the corresponding original complementary DNA (cDNA) sequences. As a result, using the formula $T = Ks / 2\lambda$ ($\lambda = 6.5 \times 10^{-9}$), the duration of replication and separation of each expansin gene in the evolutionary process was determined as million years ago (Mya) (Lynch and Conery 2000; Yang et al. 2008).

## Computer identification of miRNAs targeting expansin genes

Identification of miRNA-controlled gene targets is essential for evaluating miRNA functions. The miRBase server v.2.1 (http://www.mirbase.org/) (Kozomara and Griffiths-Jones 2014) was used to obtain previously determined plant miRNA sequences for the discovery of miRNAs targeting transcripts of watermelon and melon expansin genes. All known plant miRNA sequences and watermelon or melon expansin gene transcripts were aligned. Using the Plant Small RNA Target Analysis Server tool (psRNATarget, https://www.zhaolab.org/psRNATarget/), identification of miRNA sequences targeting watermelon and melon expansin transcripts was provided (Dai and Zhao 2011). All known plant miRNA sequences and potential targets of these sequences were determined concerning the parameters previously described by Zhang (2005). Expansin genes and target miRNA matches were visualized with the Cytoscape program (Shannon et al. 2003).

## Homology modeling of expansin proteins

Protein Data Bank (PDB) (Berman et al. 2000) provides information about the three-dimensional structures of proteins. Expansin proteins of watermelon and melon were searched in this database. The best three-dimensional architecture of the proteins was determined by BLASTP analyses. The obtained information was used to predict protein structure through homology modeling using the PHYRE2 program (Protein Homology/Analog/YRecognitionEngine; http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index), which acts as a tool for three-dimensional structure prediction of proteins (Kelley et al. 2015).

## Determination of expression profiles of watermelon and melon expansin genes using transcriptome data

Transcriptome data belonging to watermelon and melon was obtained by scanning the Sequence Read Archive

database (SRA, https://www.ncbi.nlm.nih.gov/sra), which contains raw sequencing data and their access codes. The access codes for the raw sequencing data for watermelon are the following: SRP168317 (Zhu et al. 2020), SRP051354 (Guo et al. 2015), SRP012853, SRP078211 (Li et al. 2016), and SRP143549 (Mo et al. 2016), and for melon are the following: SRP162091 (Sanz-Carbonell et al. 2019) and SRP066337 (Shin et al. 2017). The Illumina HiSeq readings deemed suitable for the study were downloaded in ".fastq" format for RNA-Seq analysis by NCBI SRA Run Browse. Sequence quality, quality scores, nucleotide content, and sequence replication levels for each reading base were checked by FastQC analysis. An experimental setup was created using the CLC Genomics Workbench 11.1 for RNA-Seq analysis. Then, normalization and transformation processes were applied using the same program. As a result of these processes, gene expression levels were determined and heat maps of hierarchical clusters (heatmap) were drawn with Permut Matrix software (Caraux and Pinloche 2005).

## Expression analysis of expansin genes under abiotic stress application with quantitative real-time PCR method (qRT-PCR)

### Growth of watermelon and melon plants and stress applications

Watermelon (Dizayn F1 cultivar) and melon (Sigal cultivar) seeds used in this study were kindly supplied by Monsanto Food and Agriculture Trade Limited Company (Antalya, Turkey). Firstly, the washing process was applied with distilled water ($dH_2O$) three times and kept in distilled water for 2 h. Then, the seeds were grown in pots containing vermiculite irrigated with Hoagland solution (Caisson Labs, USA) (Hoagland and Arnon 1950) for 21 days at $24 \pm 2$ °C and 400 µmol $m^{-2}$ $s^{-1}$ light intensity in a plant growth cabinet with a photoperiod of 16 h of light and 8 h of darkness (Supplementary Fig. 1). The development of the plants continued until they reached the level of three leaves; then, stress conditions were applied (Altunoglu et al. 2016, 2017). In the stress application of watermelon and melon plants with sufficient growth, drought stress was applied by irrigation with a Hoagland solution containing 20% polyethylene glycol 6000 (PEG-6000) (Baloglu et al. 2014). ABA application was carried out by spraying 100 µM ABA solution to the leaves. In the salt stress application, the plants were irrigated by dissolving 200 mM sodium chloride (NaCl) in the Hoagland solution. The application of heat stress was carried out at 50 °C, and the application of cold stress at 10 °C by ensuring the growth of the plants (Ünel 2018; Arslan et al. 2021). Irrigation was not done 1 day before giving stress to the plants. The 0th hour was accepted as the control group of plants. Root and leaf samples were collected at the 1st, 3rd, 6th, 12th, and 24th hours of stress applications from the plants. Both three biological and technical replicates were used for each sample in the experiments. Plant samples frozen by liquid nitrogen treatment were stored at − 80 °C for later molecular genetic analysis.

### RNA isolation, quantitative real-time PCR analysis (qRT-PCR), and statistical calculations

RNA isolation was performed using Trizol reagent (ABP Biosciences, USA). The quality and reliability of the RNA were calculated by agarose gel electrophoresis and MultiscanGO nano-spectrophotometer (ThermoFisher Scientific, USA), respectively. DNA fragments were eliminated from all RNA samples by using *DNase* I enzyme (Thermo Scientific, USA). The cDNA synthesis was done using the iScript cDNA synthesis kit (Biorad, USA) according to the manufacturer's directives. For qRT-PCR, genes with upregulated expression levels in the tissues of watermelon and melon plants were selected according to the transcriptome data. Primers specific to the expansin genes were synthesized using the NCBI Primer Blast program. *18S rRNA* gene (GenBank ID: X51542.1) (Baloglu et al. 2014), *TUA* gene (tubulin alpha chain) (Gene ID: XM_004149597) (Kong et al. 2014), and *β-actin* gene (ID: Csa017310) (Ling et al. 2011) were used as reference genes in qRT-PCR analysis (Supplementary Table 1). Of these reference genes, *TUA* and *β-actin* genes were used for watermelon; *18S rRNA* and *β-actin* genes were used for melon.

SYBR Green Supermix (Biorad, USA) was used in the study. CFX96 Touch Real-Time PCR device (Biorad, USA) was utilized for gene expression analysis; suitable operating temperatures for the melting temperature (Tm) values of the primers were determined and optimization of the primers was performed. The qRT-PCR conditions were as follows: after 3 min of the first denaturation at 95 °C, 40 cycles of 5 s of denaturation at 95 °C, 10 s of binding at 52 °C, and 10 s of elongation at 60 °C were carried out. At the end of the qRT-PCR reaction, it was checked that the related DNA region was copied by melting curve analysis. Melting curve analysis was performed by denaturing the reaction mixture at 95 °C, then incubating at 65 °C and reading the fluorescent signals at every 0.5 °C increment from 65 to 95 °C. Using cycle threshold (Ct) values from qRT-PCR, ΔCT and ΔΔCT values were calculated by the formula: $\Delta CT = CT_{sample} - CT_{reference}$ and $\Delta\Delta CT = \Delta CT_{stressed\ sample} - \Delta CT_{control\ (0\ h)}$, and the difference between expression levels was estimated as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001). Heat maps were constructed for the expression profiles of the expansin genes using the data.

Statistical analysis was conducted using the One-way ANOVA method in Minitab 18 package program. The difference between control and stress-applied samples was considered meaningful when the $p$-value was $< 0.05$.

## Results and discussion

### Determination of expansin family members and their structure analysis

After comprehensive searches, 40 expansin proteins (22 ClaEXPA, 14 ClaEXPLA, and 4 ClaEXPB) in watermelon and 43 expansin proteins (19 CmEXPA, 15 CmEXPLA, 3 CmEXPB, and 6 CmEXPLB) in melon were identified. While the molecular weights of watermelon expansin proteins were between 8438.70 and 66,871.89 Da, the amino acid contents of proteins varied between 71 and 607 aa. The molecular weights of the melon expansin proteins were between 6145.04 and 31,098.62 Da and the protein lengths were between 54 and 284 aa. According to the physicochemical analysis, all of ClaEXPA proteins and most of ClaEXPLA proteins were in basic character (pI > 7) whereas ClaEXPB proteins were basic or acidic. In melon, a major part of CmEXPA proteins was in basic character. Basic and acidic proteins were defined in the CmEXPB subgroup while most of the CmEXPLA proteins were basic (pI > 7) and most of the CmEXPLB proteins were acidic (pI < 7) (Supplementary Table 2–3). When considering expansin protein subgroups in the plants, the EXPA subgroup was the dominant group among expansins both in watermelon and melon and this was followed by the EXPLA subgroup. EXPLB subgroup was not found in watermelon. Results of the study were consistent with previous studies that identified 49 expansins in zucchini, 41 expansins in cucumber (Arslan et al. 2021), 38 expansins in tomato (Lu et al. 2016), 38 proteins in *Arabidopsis* (Li et al. 2002), 52 proteins in tobacco (Ding et al. 2016) etc. It can be concluded that the number of expansin genes in different organisms can reflect the roles and importance of expansin proteins in those organisms. Although the identification of expansin genes in watermelon was done in a recent study (Gao et al. 2020), they found 30 expansin genes in the watermelon genome. However, 10 more expansin genes were detected in the watermelon genome in the study. Those differences can be explained by using of CLC Genomics Workbench 11.1 program in Pfam database domain searches, genome-wide examination study, and comparative genomics searches of the genes in the current study. Besides, detailed and multiple searches made in the current study might be the reason that it identified more expansin genes in the study.

Chromosome 2 in the watermelon genome contained the most expansin genes with 11 genes, while chromosomes 6 and 11 carried the least expansin genes (1 gene) (Fig. 1). There were 11 expansin genes on the 1st chromosome of the melon, while only 2 expansin genes were found on the 4th, 5th, and 7th chromosomes (Fig. 2).

Interestingly, the watermelon genes *ClaEXPA-20*, *ClaEXPB-02*, and *ClaEXPB-03* do not contain introns (Supplementary Fig. 2). All expansin genes, except *CmEXPA-07* and *CmEXPA-18*, have intron regions, according to the analysis of the melon expansin gene structures (Supplementary Fig. 3). The melon *EXPA* subgroup contains one to three exons, whereas the watermelon *EXPA* subgroup contains two to five exons. In general, the *EXPB* subgroup consists of four or five exons. Furthermore, *EXPLA* and *EXPLB* subgroups had variable numbers of exons in watermelon and melon.

### Phylogenetic analysis and conserved motifs of expansin proteins

According to the phylogenetic tree analysis based on the Neighbor Joining method with bootstrap analysis for 1000 iterations, four distinct clusters were observed for watermelon expansin proteins. ClaEXPA group proteins were mostly clustered in I and II clusters when the distribution profile of these proteins was analyzed based on their subgroups. Cluster III was dominated by ClaEXPA proteins except for one protein. ClaEXPLA group proteins (13) were predominantly found in the cluster IV (Fig. 3A). In the analysis of the motif distributions of watermelon expansin proteins, it was found that mostly motifs 2A, 3A, 4A, 6A,

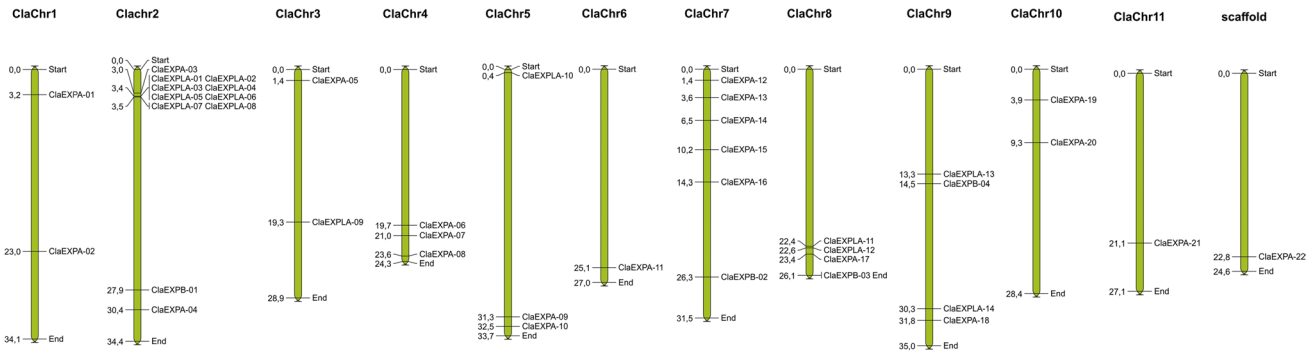



**Fig. 1** Chromosomal localizations of expansin genes on watermelon chromosomes. Chromosomal intervals are determined as Mbp

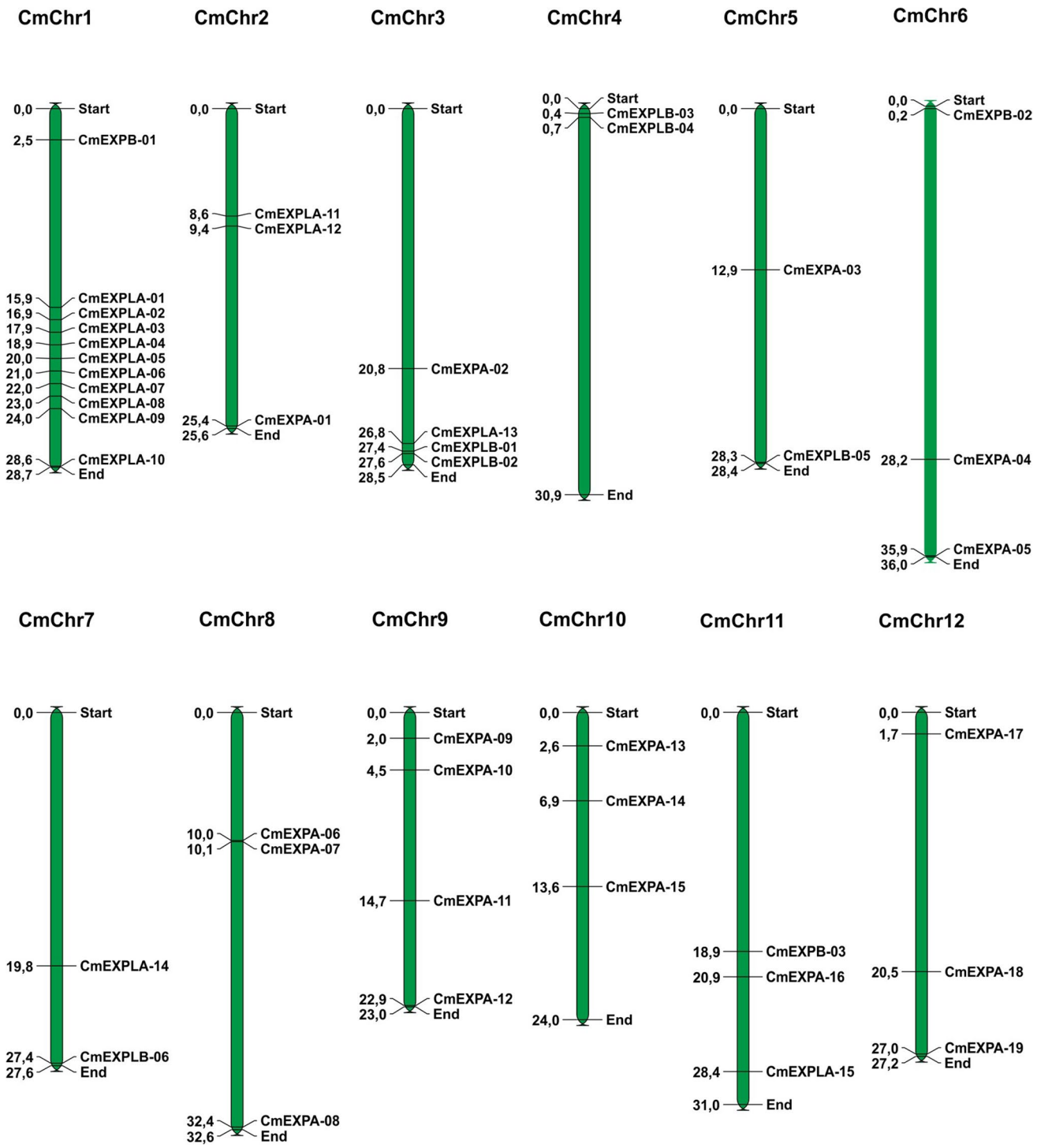



**Fig. 2** Chromosomal localizations of expansin genes on melon chromosomes. Chromosomal intervals are determined as Mbp

and 11A appeared in ClaEXPA proteins. While motifs 1A, 5A, and 6A were common in ClaEXPLA proteins, ClaEXPB proteins had variable motifs and contained motif 1A as a common motif (Supplementary Fig. 4).

In the phylogenetic tree of the expansin proteins in melon, 4 distinct clusters were formed. Upon further examination of the distribution groups of these proteins according to the classes, Cluster I consisted largely of CmEXPLB and CmEXPB proteins, while Cluster II had predominantly CmEXPLB proteins. Cluster III mainly contained CmEXPLA proteins. CmEXPA proteins were found mostly in Cluster IVa and IVb (Fig. 3B). Considering the motif structures

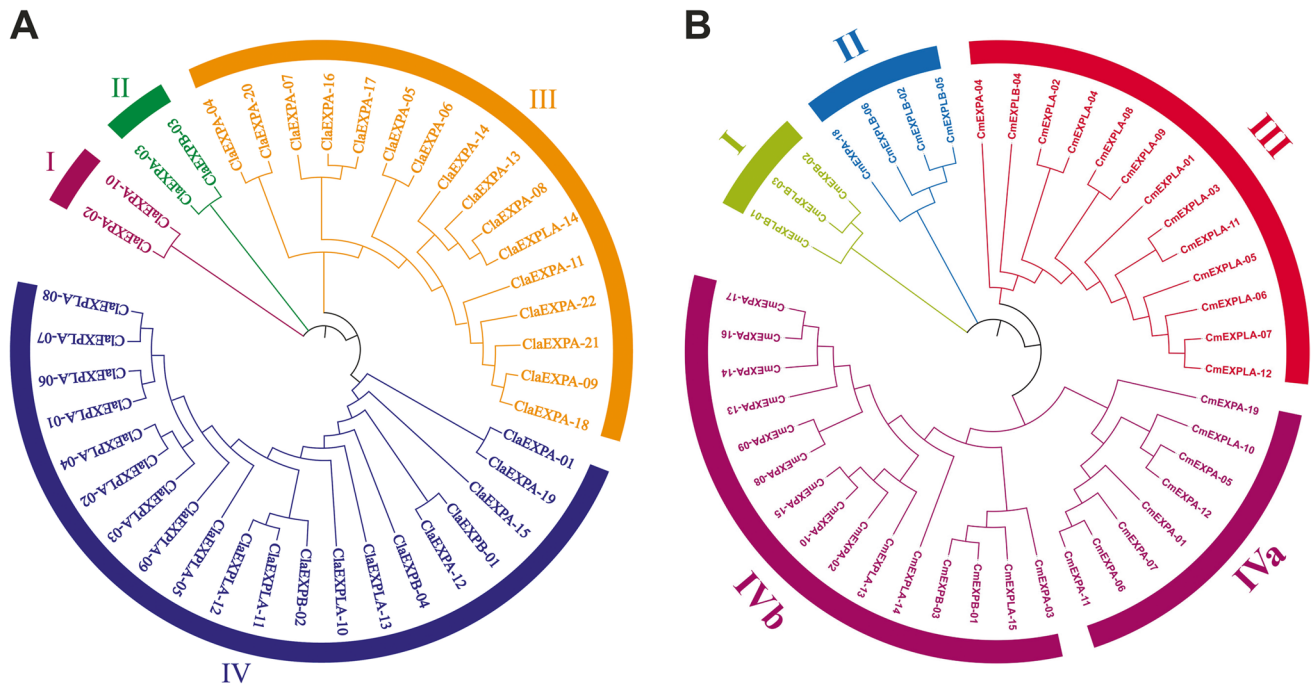



**Fig. 3** Phylogenetic relationships of watermelon and melon expansin proteins. **A** Phylogenetic tree diagram of watermelon expansin proteins. **B** Phylogenetic tree diagram of melon expansin proteins

of melon expansins, CmEXPA proteins mostly contained 1B, 2B, 3B, 4B, 5B, 7B, 9B, and 10B motifs. All of the CmEXPB proteins included 1B, 3B, 4B, 7B, 9B, 11B, 12B, and 16B motifs. All of the CmEXPLA proteins contained 4B and 7B motifs, while all of the CmEXPLB proteins shared 1B motif. In addition, it was observed that some of the CmEXPLB proteins shared 12B motif and some of them shared 9B motif (Supplementary Fig. 5).

Typically, expansin proteins are comprised of two domains (Domain I and II) and a signal peptide (Sampedro and Cosgrove 2005). Watermelon motif 2A and melon motif 5B contain conserved His-Phe-Asp (HFD, histidine-phenylalanine-aspartate) structures that are part of Domain I of expansin proteins. The majority of ClaEXPA and CmEXPA proteins had HFD structure which is a part of the catalytic site of glycoside hydrolase family-45 (GH45) proteins (Cosgrove 1997b; Cosgrove 2000). HFD motif was not observed in ClaEXPLA (except ClaEXPLA-10–14), CmEXPLA (except CmEXPLA-10–13-14–15), and CmEXPLB subgroups, which correlated well with the previous studies (Ding et al. 2016; Hou et al. 2019; Lv et al. 2020). Among expansin proteins in watermelon and melon, members of the same subgroup often tended to congregate on the same branch of the phylogenetic tree. It was observed that the motif structures within the same expansin subgroup were also preserved among both watermelon and melon expansin proteins. These findings demonstrate consistency between the phylogenetic tree and the conserved motif structures in expansin proteins.

## Gene ontology of expansin proteins

Based on the gene ontology analysis, expansin proteins are predicted to play roles primarily in cell processes, cellular component organization, or reproductive processes in watermelon. Localization of watermelon expansin proteins was defined as a cellular anatomical entity (Fig. 4A). According to the gene ontology classification of expansin proteins in melon, their cellular location was in the category of cellular anatomical entity. Their predictive roles in biological processes were mostly in cellular processes, cellular component organization or biogenesis, and reproductive processes. Besides, the molecular function of the expansin proteins in melon was catalytic activity (Fig. 4B). According to the detailed analysis of watermelon and melon expansin proteins, GO categories including plant-type cell wall organization, extracellular region, cell wall, and integral component of the membrane were determined predominantly as biological roles. In addition to these roles, sexual reproduction was determined in some expansin proteins. The roles of expansin proteins in jute (*Corchorus olitorius*) were determined as predominantly involved in plant-type cell wall organization, cell wall loosening, and syncytium formation. Also, root development and sexual reproduction were the other functions according to GO analysis (Hossain et al. 2021). According to GO analysis of maize expansin proteins using ProtFun server, it was observed that 86% of the expansins were categorized in the cell envelope functional category,

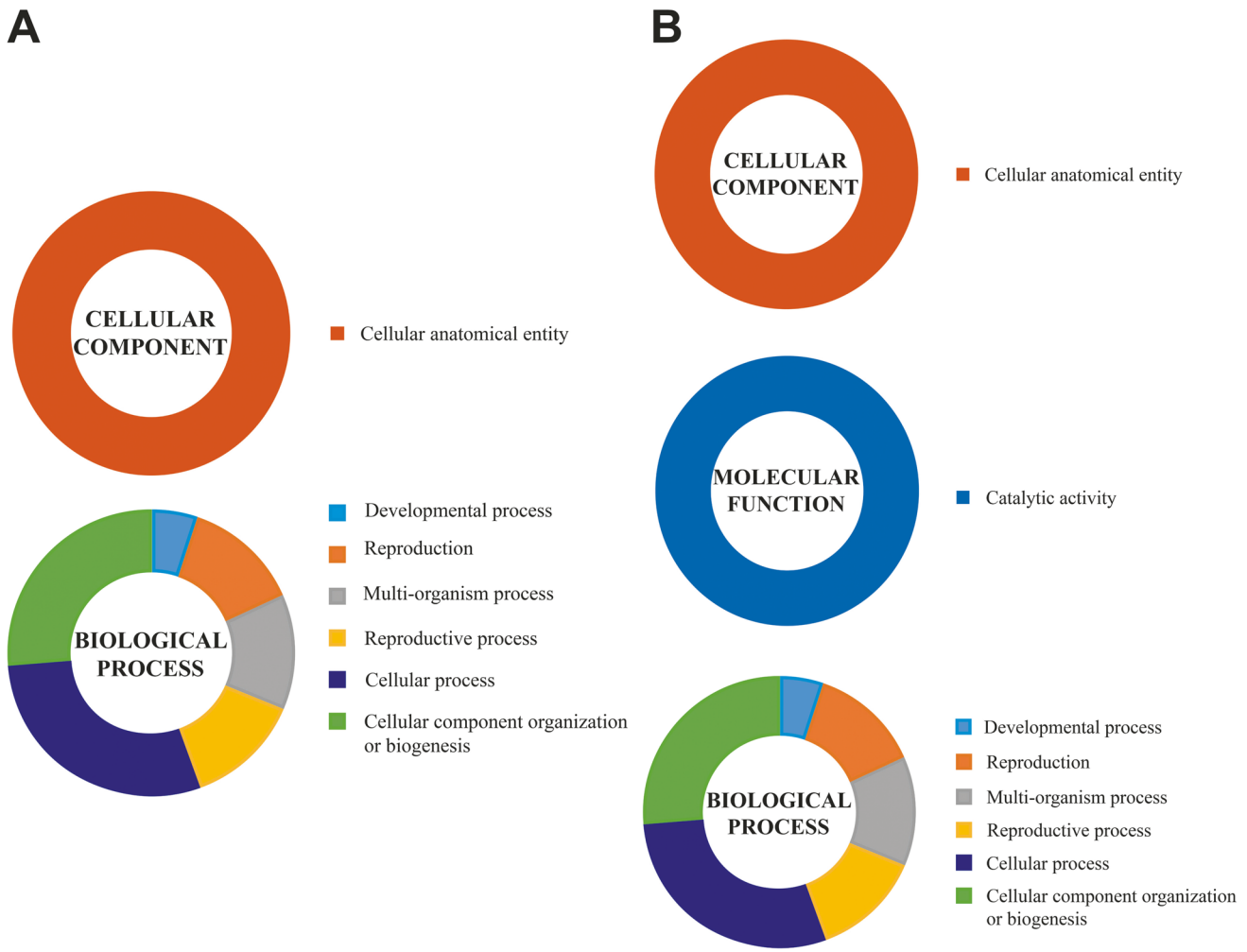



**Fig. 4** Gene ontology results of expansin proteins by Blast2Go program. **A** Watermelon and **B** Melon

and more than 43% of expansins belonged to the stress response group (Zhang et al. 2014). Moreover, *Arabidopsis* and tobacco GO analysis revealed that annotated biological processes of GO categories were including plant-type cell wall organization and sexual reproduction (Ding et al. 2016). According to sugarcane GO analysis using ProtFun server, the proteins were classified into the cell envelope functional category and the most determined GO categories were stress response and immune responses in sugarcane expansin proteins (Santiago et al. 2018). The results of our study are consistent with those of previous studies despite using different GO analysis programs. It can be concluded that the roles of expansin proteins mainly in plant-type cell wall organization and in reproductive processes were shared among expansins in different organisms. These analyses can present preliminary knowledge about the roles and the localization of expansin proteins, which can be attributed to the evolutionarily conserved nature of those proteins.

## Orthologous genes and their predicated divergence times

The expansion of gene families is influenced by gene duplication and transposition (Cao and Shi 2012). A comparison was made between the expansion modes of expansin genes in watermelon and melon. For this purpose, nonsynonymous (Ka) versus synonymous (Ks) substitution rates (Ka/Ks) were calculated for expansin genes. This approach was also used to calculate the substitution rates between *Arabidopsis* (*Arabidopsis thaliana*), soybean (*Glycine max*), rice (*Oryza sativa*), poplar (*Populus triocarpa*), and watermelon and melon expansin genes, too. Among watermelon genes, 37 pairs indicated tandem duplication while 85 pairs displayed segmental duplication. A majority of tandem duplications occurred on the watermelon's 2nd chromosome (average Ka/Ks = 0.17). When the melon genes were considered, 32 pairs of tandem duplications and 77 pairs of segmental

duplication events were determined. Besides, expansin genes showing tandem duplication were mostly observed on the 1st chromosome of melon (average Ka/Ks = 0.14) (Supplementary Table 4).

According to orthologous gene analysis between watermelon and model organisms, 1129 pairs of orthologous genes were detected between watermelon expansin genes and soybean expansin genes. There were 759 pairs of orthologous genes between watermelon and rice genes, 735 pairs between watermelon and *Arabidopsis* genes, and 690 pairs between watermelon and poplar genes. The estimated separation times of those genes were 315 Mya for rice, 191 Mya for soybean, 176 Mya for *Arabidopsis*, and 160 Mya for poplar. The average Ka/Ks ratio was calculated as 0.22 for soybean, 0.05 for poplar, 0.01 for rice, and 0.3 for *Arabidopsis* (Fig. 5A) (Supplementary Table 5). Among the orthologous genes detected for melon expansin in model organisms, 852 orthologous pairs of genes were found in soybean genes. This was followed by *Arabidopsis* (633 gene pairs), rice (549 gene pairs), and poplar (544 gene pairs). Among the identified orthologous genes, the average divergence time was estimated as 198 Mya with *Arabidopsis*, 334 Mya with rice, 205 Mya with soybean, and 187 Mya with poplar expansin genes. The average of Ka/Ks ratios were also calculated as 0.01 for rice, 0.03 for *Arabidopsis*, 0.15 for soybean, and 0.22 for poplar (Fig. 5B) (Supplementary Table 6).

In a study on the duplication of expansin genes in other plants, segmental and tandem duplication events were found to play a critical role in the expansion of the tomato expansin family (Lu et al. 2016). During a maize segmental duplication event, five gene pairs were involved in both tandem and segmental duplication events (Zhang et al. 2014). A study was conducted on the tobacco plant, which determined the number of genes with tandem duplication corresponding to 11.5% of total expansin genes. (Ding et al. 2016). In the jute (*Corchorus olitorius*) genome, 11.54% of expansin genes were formed by segmental duplication and only one pair of genes displayed tandem duplication, which was located on the same linkage group (Hossain et al. 2021). In soybean expansin genes, 68% were part of segmental duplication events, whereas 14.7% were tandem duplications (Zhu et al. 2014). In watermelon and melon genomes, segmental duplication seemed to be the mechanism for expansion of expansin genes, which was by expansin genes in tobacco, soybean, and jute. According to the calculated Ka/Ks substitution rates, watermelon and melon expansin genes undergo a strong purifying pressure. Both watermelon and melon were found to have the most orthologous genes with soybean expansins. Zucchini and cucumber expansin genes displayed the most orthologous relations with soybean expansin genes in our group's previous study, too (Arslan et al. 2021). However, the latest divergence time between orthologous genes was determined with poplar expansin genes for both watermelon and melon, which was similar for expansin genes in zucchini and cucumber (Arslan et al. 2021). Moreover, concerning the other studies conducted by our research group, the most current divergence times were obtained with poplar genes for *LEA* (Altunoglu et al. 2016, 2017) *bZIP* (Baloglu et al. 2014; Unel et al. 2019), and *Hsp* (Altunoğlu et al. 2019) genes in cucumber, watermelon, or melon genomes. The data presented here suggest that poplars and Cucurbitaceae family members are more closely related from an evolutionary perspective than other model plants.

## Computer identification of miRNA target genes for expansins

A total of 89 miRNAs were identified for watermelon expansin genes by psRNATarget: a Plant Small RNA Target

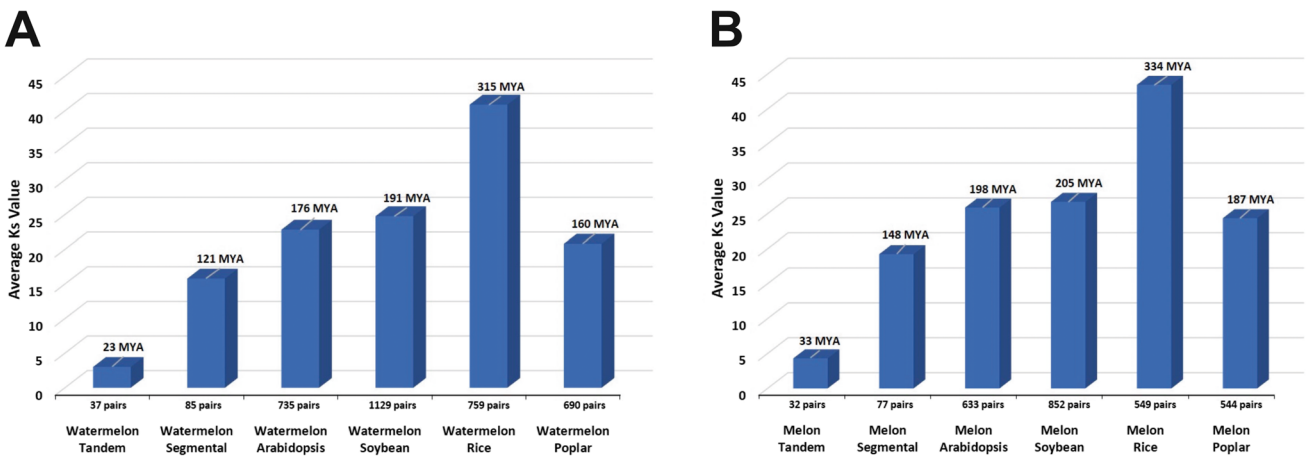



**Fig. 5** **A** Orthologous genes of watermelon expansin genes in model organisms and approximative differentiation times. **B** Orthologous genes of melon expansin genes in model organisms and approximative differentiation times

Analysis Server. The most targeted watermelon expansin genes were *ClaEXPB-04* targeted by 32 miRNAs, *ClaEXPA-17* targeted by 30 miRNAs, and *ClaEXPA-08* targeted by 27 miRNAs, respectively. Nine miR156 from different plants targeting *ClaEXPA-17* and *ClaEXPA-08* were shared. Moreover, miR164 from different plants mostly targeted *ClaEXPB-04*. Many of miR156 and miR395 from different plants targeted *ClaEXPA-17*. *ClaEXPA-08* was predominantly targeted by miR156 and miR399 (Fig. 6A). In terms of miRNAs that target melon expansin genes, 57 miRNAs have been identified. The most targeted melon expansin genes were *CmEXPA-12* and *CmEXPA-17* by 20 miRNAs and *CmEXPA-16* by 17 miRNAs, respectively. miR395 and miR171 mostly targeted *CmEXPA-17*. *CmEXPA-16* was targeted mainly by miR528 and miR845. miR168 was determined as miRNA which predominantly targets *CmEXPA-12* (Fig. 6B) (Supplementary Table 7).

The miRNAs that most target watermelon expansin genes were miR156, miR164, miR395, and miR399. The most frequently observed miRNAs targeting melon expansin genes were miR168, miR171, miR395, miR528, and miR845. According to previous studies about the roles of miRNAs which target watermelon or melon expansin genes, it was observed that the miR164 family members participated in the drought and salt stress responses of *Populus euphratica* and the expression of peu-miR164a-e, one of the members of this family, was increased in plants that were rapidly dehydrated by stress (Li et al. 2009). Moreover, it was determined that the expression level of peu-miR164a decreased after ABA administration (Duan et al. 2016). In a study with *Arabidopsis*, it was determined that overexpression of miR395c or miR395e, with minor sequence differences between them, retarded and accelerated seed germination in *Arabidopsis* under high salt or dehydration stresses, respectively (Kim et al. 2010). In a study conducted in the salt marsh plant *Spartina alterniflora*, it was observed that some miRNAs, including miR168, miR399, miR395, miR171, and miR164, were downregulated in leaf tissues under salt stress (Qin et al. 2015). Downregulation of some miRNAs targeting expansin genes in watermelon and melon may indicate the mode of action and importance of expansins under different abiotic stresses. Therefore, the determination of miRNAs can contribute to resolving the roles of expansin proteins.

## Homology modeling of expansin proteins

By considering the homology modeling of the watermelon expansin proteins, the three-dimensional structure of 23 of them was calculated with a modeling > 90% confidence level. Estimated three dimensional structures belong to ClaEXPA-01–03-04–05-08–09-11–13-14–15-16–18-21–22 (PDB ID: 2hcz), ClaEXPA-07 (PDB ID: 2ahn), ClaEXPB-01–04 (PDB ID: 2hcz), ClaEXPB-02 (PDB ID: 1n10), ClaEXPB-03 (PDB ID: 2z6g), ClaEXPLA-01 (PDB ID: 1n10), and ClaEXPLA-10–13-14 (PDB ID: 2hcz) (Fig. 7). The β layer structure was dominant in most of the examined expansin proteins in watermelon and there were one or two small α helixes. On the other hand, it has been observed that the three-dimensional structure of ClaEXPB-03 protein consisted of only a large number of α helix structures. When the three-dimensional structure of expansin protein in melon was estimated, the structure of 23 melon expansin proteins was predicted at a per cent modeling > 90% confidence level. Modeled proteins were CmEXPA-01–02-03–04-05–09-12–13-14–15-16–17-19 (PDB ID: 2hcz), CmEXPA-07 (PDB ID: 5xbu), CmEXPA-18 (PDB ID: 1n10), CmEXPB-01–03 (PDB ID: 2hcz), CmEXPLA-10–13-14–15 (PDB ID: 2hcz), and CmEXPLB-03–04 (PDB ID: 2hcz) (Fig. 8). The dominant structure was generally 2 groups of β sheets and a small 2 α helix structure in modeled proteins. Besides, only α helix structure was observed in CmEXPA-07, while only β layer structure was observed in CmEXPA-18.

In comparison with previous studies involving three-dimensional (3D) structure analysis of expansin proteins, investigation of the predicted protein structure of an expansin A protein (ATEXPA23) using PSIPRED server displayed 5 β sheets, 5 hairpins, 3 psi loops, 7 β bulges, 16 strands, 3 helices, 27 β turns, 5 γ turns, and 3 disulfide bonds in the 3D structure of the protein. In addition, amino acid sites of Gly 206, Asp 207, Ile 208, Leu 210, and Met 211 were determined at a stretch in the cellulose-binding domain of the protein (Basu et al. 2019). According to the 3D model of a wheat expansin A protein (TaEXPA1-A) using Swiss-model, β sheets were dominantly found in the structure and the secondary structure image was similar to most of the watermelon and melon EXPA proteins (Han et al. 2019). There was also a high degree of similarity between the crystal structure of watermelon and melon expansin proteins and ZmEXPB1 from maize (PDB ID: 2hcz). Similarity, the predicted 3D structure of watermelon and melon expansin proteins and expansins in other organisms by using different servers supports the reliability of the current study results and the conserved nature of the expansin proteins in different organisms, which may be related to their mode of action.

## Expression analysis of expansin genes in watermelon

To determine the expression profiles of watermelon expansin genes using transcriptome projects from the SRA database, unfertilized ovaries collected before

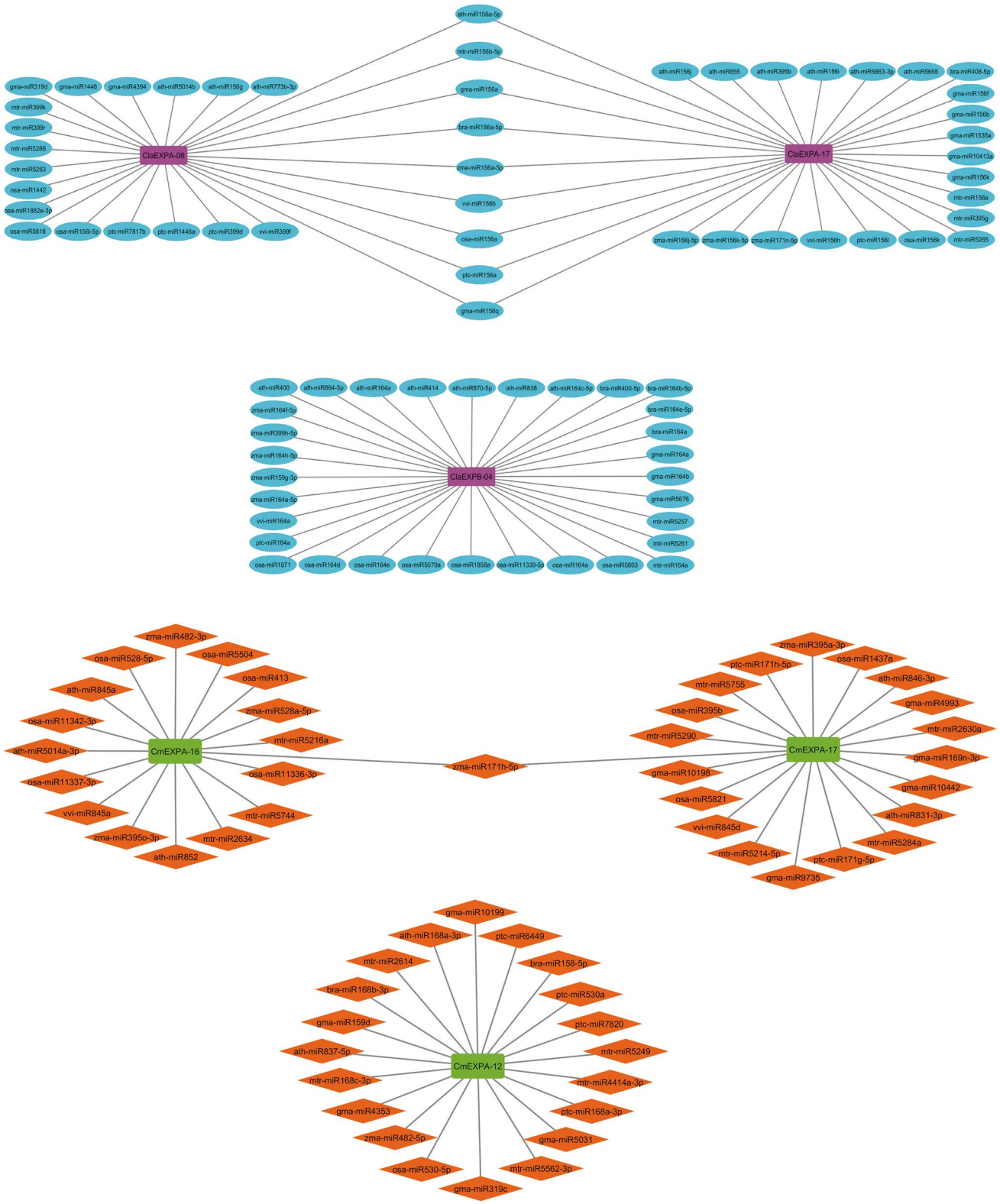
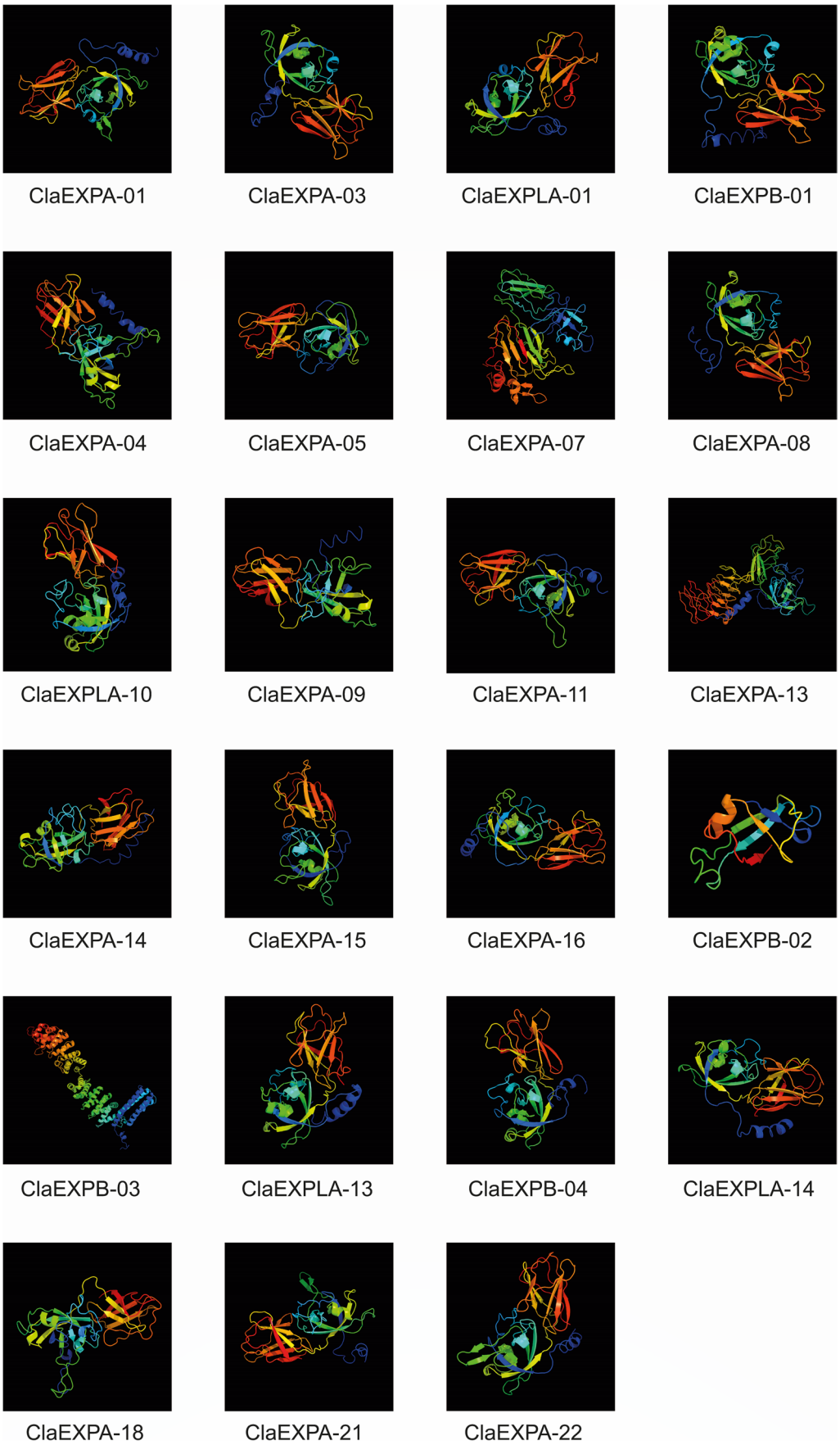



**Fig. 6** **A** miRNAs targeting watermelon expansin transcripts. **B** miRNAs targeting melon expansin transcripts

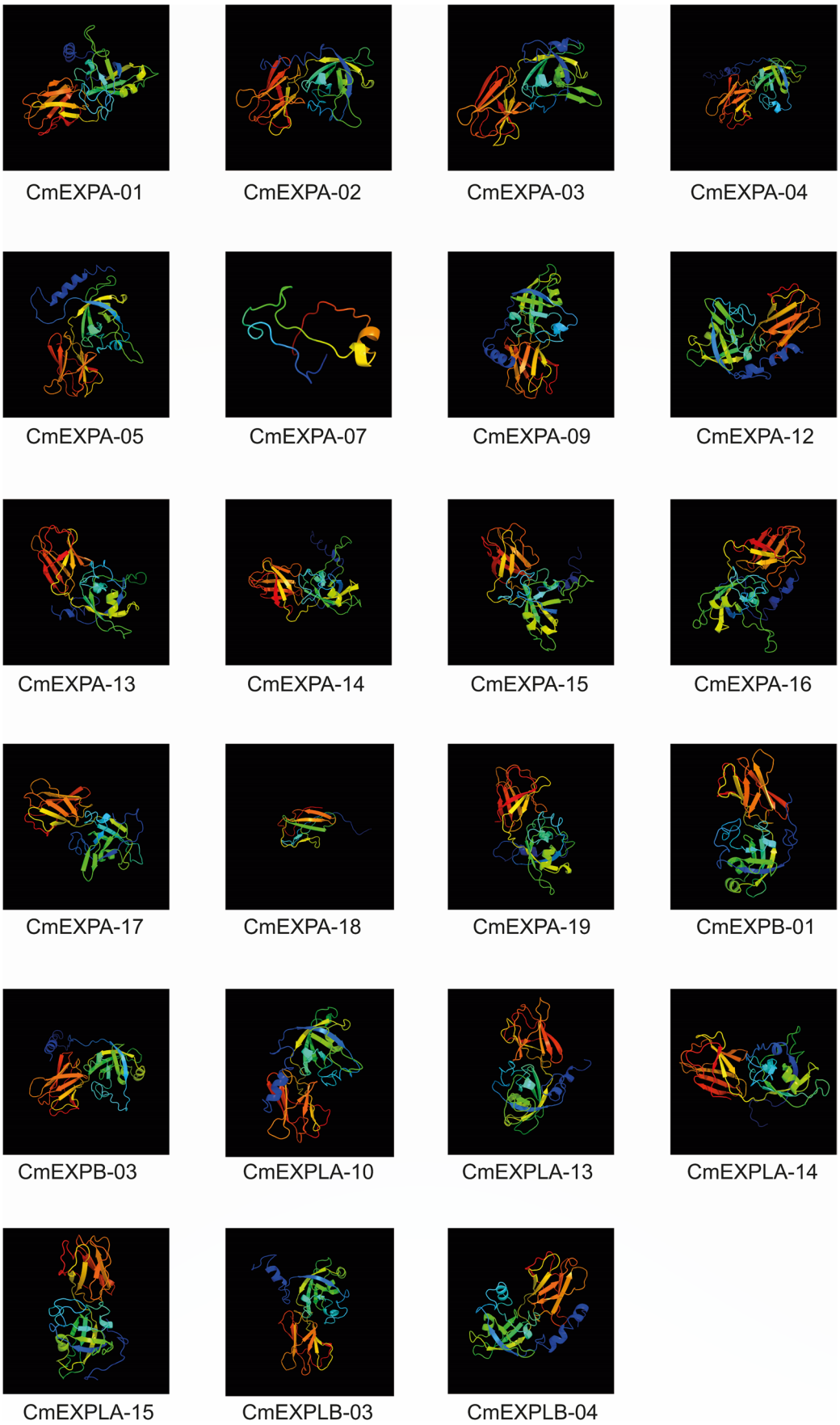

ClaEXPA-01
ClaEXPA-03
ClaEXPLA-01
ClaEXPB-01
ClaEXPA-04
ClaEXPA-05
ClaEXPA-07
ClaEXPA-08
ClaEXPLA-10
ClaEXPA-09
ClaEXPA-11
ClaEXPA-13
ClaEXPA-14
ClaEXPA-15
ClaEXPA-16
ClaEXPB-02
ClaEXPB-03
ClaEXPLA-13
ClaEXPB-04
ClaEXPLA-14
ClaEXPA-18
ClaEXPA-21
ClaEXPA-22

◂**Fig. 7** Estimated three-dimensional structures of watermelon expansin proteins. The structures of expansin proteins with > 90% confidence level are presented

flowering (A0, control); samples in flowering step kept at 37 °C for 4 h (A3), 8 h (A5), 12 h (A7), and 24 h (A8) (SRP168317) (Zhu et al. 2020); medium flesh and mesocarp samples of fruits after pollination (10 days, 18 days, 26 days, 34 days, 42 days, and 50 days after pollination (DAP)) (SRP051354) (Guo et al. 2015); vascular (VT) and phloem (PH) tissue samples (SRP012853); cold stressed samples (SRP078211) (Li et al. 2017); and leaf samples of 4 days and 8 days after drought stress application in M20 and Y34 watermelon genotypes (SRP143549) (Mo et al. 2016) were used. These transcriptome data showed that the expression of the *ClaEXPA-09* gene was upregulated on the 4th and 8th days in the drought-stressed M20 watermelon genotype, while it increased on the 4th day in the drought-stressed Y34 genotype. As part of the same transcriptome data, the expression of *ClaEXPLA-13* and *ClaEXPB-01* genes was observed in both control and drought-stressed samples. According to the transcriptome analysis of cold stress application, the expressions of *ClaEXPA-04*, *ClaEXPB-01*, *ClaEXPB-03*, and *ClaEXPLA-13* genes were determined in both control and stressed tissues (Supplementary Fig. 6). In comparison with those results, qRT-PCR analysis of these genes showed that *ClaEXPA-09* gene expression increased significantly in the root tissues under drought stress during the 3rd hour. It was observed that the expression of *ClaEXPLA-13* and *ClaEXPB-01* genes also tended to increase in the root tissues from the 1st hour, and the expression of the *ClaEXPLA-13* gene also increased in the leaf tissues. qRT-PCR results indicate that in response to cold stress application, *ClaEXPA-04*, *ClaEXPB-03*, and *ClaEXPLA-13* gene expression increased in root tissues, but the greatest increase was observed in leaf tissues. For the *ClaEXPB-01* gene, this was particularly evident in the root tissue at the 24th hour of the cold stress (Fig. 9, Supplementary Fig. 7, Supplementary Fig. 8). According to the findings, transcriptome and qRT-PCR results were compatible in that *ClaEXPA-04*, *ClaEXPA-09*, *ClaEXPB-01*, *ClaEXPB-03*, and *ClaEXPLA-13* genes were frequently upregulated under drought or cold stress in roots or leaves. Moreover, according to transcriptome data of unfertilized ovaries collected before flowering and samples kept at 37 °C in different hours of flowering, mid-flesh and mesocarp samples of fruits after fertilization, and vascular (VT) and phloem (PH) tissue samples, the expression of *ClaEXPA-04*, *ClaEXPB-01*, *ClaEXPB-03*, and *ClaEXPLA-13* genes were usually observed. When the expression of the gene orthologous to watermelon genes in other plants was examined under stress, the plants that overexpressed the *AtEXPA1* (NP_177112.1) gene, which is one of the orthologous of the *ClaEXPA-04* and *ClaEXPA-09* genes in *Arabidopsis*, were less affected by the salt and ABA stress conditions. It has therefore been reported that it helps plants better adapt to stressful conditions (Gao et al. 2010). Moreover, it was determined that the *AtEXP2* gene (NP_178409.2), which is the other orthologous of the same watermelon genes in *Arabidopsis*, was also involved in seed germination and response of the plant to abiotic stresses such as salt and osmotic stress (Yan et al. 2014). It was observed that the *AtEXPB5* (NP_001319806.1) gene, which is the orthologous of the *ClaEXPB-01* gene in *Arabidopsis*, played an important role in pollen tube growth (Liu et al. 2021). According to the obtained data and orthologous gene analysis, it can be concluded that *ClaEXPA-04*, *ClaEXPA-09*, *ClaEXPB-01*, *ClaEXPB-03*, and *ClaEXPLA-13* genes can be involved in tissue development and abiotic stress responses in watermelon.

## Expression analysis of expansin genes in melon

Transcriptome data of melon leaf samples exposed to drought, salt, and cold stress (SRP162091) (Sanz-Carbonell et al. 2019) and samples of melon fruit tissues taken at different stages of fruit development (samples taken 8, 16, 24, and 32 days after pollination (DAP)) (SRP066337) (Shin et al. 2017) were utilized in the cuırrent study. As a result of the transcriptomic analysis, it was found that the expression of the *CmEXPB-05* gene was only detected in the salt-stressed samples, while the expression of the *CmEXPA-10* and *CmEXPLB-02* genes was observed only in the drought-stressed samples. In cold stress application data, the expressions of *CmEXPA-10* and *CmEXPA-13* genes increased only in the stressed sample (Supplementary Fig. 9). Transcriptome data and qRT-PCR data appear to be compatible since the expression of *CmEXPA-10*, *CmEXPLB-05*, and *CmEXPLB-02* genes tends to increase in melon root and leaf tissues under those stresses (Fig. 10, Supplementary Fig. 10, Supplementary Fig. 11). Furthermore, the qRT-PCR analysis showed an increase in the expression of *CmEXPA-10*, *CmEXPA-12*, and *CmEXPLB-02* genes in melon leaf tissue following ABA application from the 1st hour. Using transcriptome analysis, normal tissue samples from melon fruits taken at different stages of fruit development revealed that both *CmEXPLA-01* and *CmEXPLB-05* were expressed. In studies that examined the expression of melon genes in other plants under stress, it was determined that overexpression of the *AtEXPA4* gene (NP_181500.1), which is orthologous of the


CmEXPA-01
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CmEXPB-03
CmEXPLA-10
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CmEXPLA-14
CmEXPLA-15
CmEXPLB-03
CmEXPLB-04

◂**Fig. 8** Estimated three-dimensional structures of melon expansin proteins. The structures of expansin proteins with > 90% confidence level are presented

*CmEXPA-10* and *CmEXPA-12* genes from *Arabidopsis*, resulted in an increase in primary root elongation in the plant (Liu et al. 2021). It has been revealed that the *GmEXP1* (XP_003549683.2) gene, which is orthologous of *CmEXPA-10* and *CmEXPA-12* genes in soybean, played an important role in root development, especially in the elongation and/or the emergence of primary and secondary roots (Lee et al. 2003). Moreover, the expression of *EXLA2* (NP_195553.1), the orthologous of the *CmEXPLA-01* gene in *Arabidopsis*, was significant in the response to many biotic and abiotic stresses, especially in the pathogenesis of necrotrophic pathogens and in tolerance to abiotic stresses including salt, cold, and ABA treatments (Abuqamar et al. 2013). It was observed that the expression of the gene *expansin-A15* (XP_015629199.1), which is orthologous of the *CmEXPA-10* and *CmEXPA-12* genes in rice, was significantly upregulated on the 3rd and 7th days in root tissues exposed to cadmium and arsenic stresses separately and simultaneously (Huang et al. 2019). According to the expression results of gene orthologs in other plants, transcriptome and qRT-PCR analyses, it can be interpreted that prominent *CmEXPA-12*, *CmEXPA-10*, and *CmEXPLA-01* genes can contribute to both normal tissue development and the stress response stages of the plant.

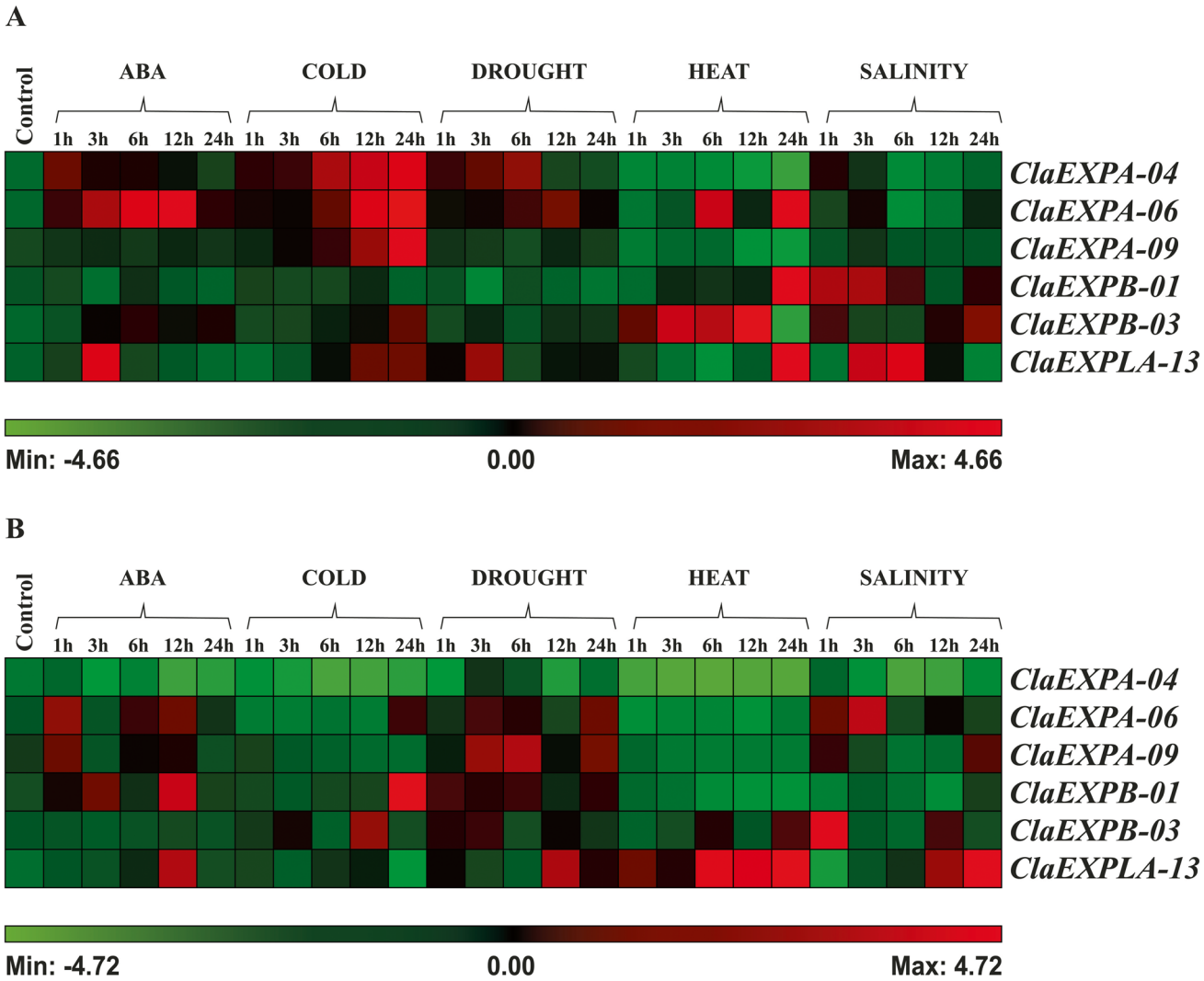


**Fig. 9** Expression patterns of watermelon expansin genes in 0 (control), 1, 3, 6, 12, and 24 h in the **A** leaf and **B** root under ABA treatment and cold, drought, and heat stresses

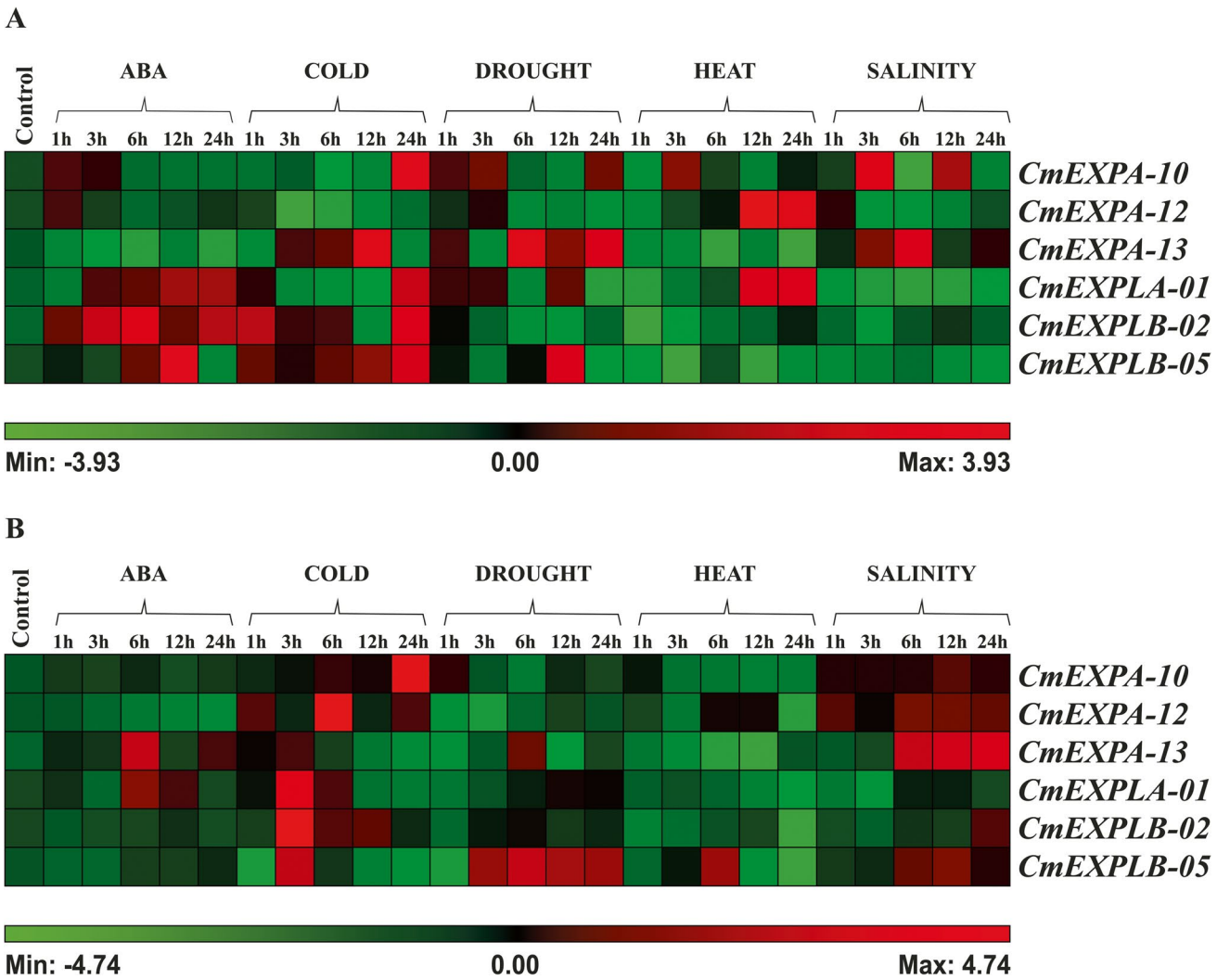


**Fig. 10** Expression patterns of melon expansin genes in 0 (control), 1, 3, 6, 12, and 24 h in the **A** leaf and **B** root under ABA treatment and cold, drought, and heat stresses

## Conclusion

The current study provides detailed and comparative research about features of the expansin gene family in watermelon and melon by using bioinformatics tools. Furthermore, the expression profiles of the expansin genes under variable abiotic stresses were compared using both publicly available transcriptome data and qRT-PCR in tissues from those plants. In the current study, bioinformatics and experimental methods are combined to characterize the expansin superfamily, which plays a role in plant growth and stress response. Consequently, the study provides detailed data that can be used to study gene functions in future studies, including their role in plant growth and under different stress conditions.

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**Author contribution** All authors contributed to the study conception. Planned and designed the research: YCA and MCB; performed experiments: ÇYI, BA, ENYÇ, FU, EH, EÇ, and GB; analyzed data: AUB, YCA, and FU; wrote and edited the manuscript: YCA, ÇYI, and MCB. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Data availability** All data will be available on reasonable request.

## Declarations

**Ethics approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Consent to participate and for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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