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RESEARCH ARTICLE

Comparative genomic analysis of expansin superfamily gene members in zucchini and cucumber and their expression profiles under different abiotic stresses

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Abstract Zucchini and cucumber belong to the Cucurbitaceae family, a group of economical and nutritious food plants that is consumed worldwide. Expansin superfamily proteins are generally localized in the cell wall of plants and are known to possess an effect on cell wall modification by causing the expansion of this region. Although the whole genome sequences of cucumber and zucchini plants have been resolved, the determination and characterization of expansin superfamily members in these plants using whole genomic data have not been implemented yet. In the current study, a genome-wide analysis of zucchini (*Cucurbita pepo*) and cucumber (*Cucumis sativus*) genomes was performed to determine the expansin superfamily genes. In total, 49 and 41 expansin genes were identified in zucchini and cucumber genomes, respectively. All expansin superfamily members were subjected to further bioinformatics analysis including gene and protein structure, ontology of the proteins, phylogenetic relations and conserved motifs, orthologous relations with other plants, targeting miRNAs of those genes and in silico gene expression profiles. In addition, various abiotic stress responses of zucchini and cucumber expansin genes were examined to determine their roles in stress tolerance. *CsEXPB-04* and *CsEXPA-11* from cucumber and *CpEXPA-20 and CpEXPLA-14* from zucchini can be candidate genes for abiotic stress response and tolerance in addition to their roles in the normal developmental processes, which are supported by the gene expression analysis. This work can provide new perspectives for the roles of expansin superfamily genes and offers comprehensive knowledge for future studies investigating the modes of action of expansin proteins.

Keywords *Cucurbita pepo* · *Cucumis sativus* · Expansin · Genome-wide identification · Abiotic stress

Introduction

Zucchini and cucumber belong to the Cucurbitaceae family, which includes both economical and nutritious food plants, consumed worldwide (Cavagnaro et al. 2010). While 75 million tons of cucumber were produced in approximately 2 million hectares of land worldwide in 2018, a total of 28 million tons of zucchini were planted in 2 million hectares in the same year. Additionally, Turkey ranks third worldwide in cucumber production after China and Iran (FAO 2020).

Expansin superfamily proteins are generally localized in the cell wall of plants and are known to have an effect on cell wall modification by causing the expansion of this region (Cosgrove 1998, 2000). This group of proteins was first isolated from cucumber coleoptiles in 1992 (McQueen-Mason et al. 1992) So far, expansin proteins have been divided into 4 subgroups: α-expansin or expansin A (EXPA), β-expansin or expansin B (EXPB), expansin-like A (EXPLA) and expansin-like B (EXPLB) (Kende et al. 2004). The expansin-like X group added by Choi et al. (2008) also represents another expansin group associated with the expansin genes. Numerous studies support the fact that expansin proteins play roles in various biological

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processes in plants such as having a functional effect on leaf development (Pien et al. 2001; Yuan et al. 2015), in stoma opening and closing (Lü et al. 2013), seed germination (Chen et al. 2001), fruit development and ripening (Brummell et al. 1999; Nardi et al. 2015), root hairs development (Lin et al. 2011), and in biotic and abiotic stress tolerance (Sasidharan et al. 2011; Abuqamar et al. 2013).

Abiotic stresses including drought, cold and salinity result the production and accumulation of extremely toxic compounds in plant cells. Previous studies have revealed important clues concerning the roles of expansin proteins in abiotic stress tolerance. In one of those studies, it was determined that *TaEXPB23* obtained from wheat influenced the peroxidase enzyme activity that binds to the cell wall, and consequently, oxidative stress tolerance increases in transgenic tobacco plants with high *TaEXPB23* gene expression rates (Han et al. 2015). In addition, transgenic Arabidopsis plants with high *RhEXPA4* gene expression had an increased potential to adapt to drought and salt stress by developing various modifications (Lü et al. 2013). It was shown that the increase in the expression level of the *GmEXPB2* gene in soybeans plays a role in tolerating abiotic stresses including iron, phosphate and water deficiency (Guo et al. 2011). The effect of heat stress was determined to be less damaging in transgenic tobacco plants with increased expression of the *PpEXP1* gene isolated from *Poa pratensis* (Xu et al. 2014). In addition to these studies, others have examined the results of high expression of the expansin gene in stress responses and concluded that it augments resistance to abiotic stresses (Cosgrove 2015; Zörb et al. 2015; Marowa et al. 2016). Based on these studies, it is important to characterize the expansin superfamily considering the role this gene family plays in the developmental processes of plants, its working mechanisms and its potential to increase stress tolerance.

Expansin genes have been identified in many organisms including grape, sugar cane, potato, soybean and tomato plants to date (Dal Santo et al. 2013; Zhu et al. 2014; Lu et al. 2016; Santiago et al. 2018; Chen et al. 2019). Cucumber was the seventh plant whose genome sequence was published in 2009 (Huang et al. 2009). The zucchini genome sequence was published in 2018 (Montero-Pau et al. 2018). To our knowledge, determination and characterization of expansin superfamily members in these plants using whole genomic data have not been implemented yet. In the current study, a genome-wide analysis of zucchini (*Cucurbita pepo*) and cucumber (*Cucumis sativus*) genomes was performed to determine and characterize the expansin family using bioinformatics tools. Moreover, the gene expression levels of expansin in leaf and root tissues under salt, drought, heat and cold stress factors and ABA application were evaluated to gain insight into the roles of these genes in the stress tolerance of these plants.

Materials and methods

Determination of the expansin genes in zucchini and cucumber genomes

Comprehensive research was conducted to identify expansin genes in the genomes of zucchini and cucumber with the experiance gained during our previous studies (Baloglu et al. 2014; Altunoglu et al. 2016, 2017, 2019; Altunoğlu 2016). First, the expansin amino acid sequences were retrieved from the Expansin Central database (http://personal.psu.edu/fsl/ExpCentral/) and compared using BLASTP (Protein Blast Sequence Comparison) search in the Cucurbit Genomics and NCBI (National Biotechnology Information Center) databases. The matches that met the expectation value of $\geq e^{-50}$ were retained as meaningful matches. Thereafter, Pfam (https://pfam.xfam.org/) domain analysis was performed and conserved regions associated with expansin protein sequences were identified through Hidden Markov Models (HMM) (Zhang et al. 2014; Finn et al. 2016). Any repetitive sequences were removed, and possible expansin protein sequences were determined. Finally, the physical and chemical parameters of the determined proteins were calculated using the Expasy PROTPARAM tool (https://web.expasy.org/protparam/) (Gasteiger et al. 2005).

Chromosomal localization distribution and prediction of gene structure

Exon–intron regions were identified by comparing the genomic sequences with the predicted coding sequences (CDS) in the Gene Structure Display Server (GSDS, http://gsds.gao-lab.org/Gsds_about.php) (Hu et al. 2015). The genomic sequences of the related proteins were examined in the Cucurbit Genomics Database (http://cucurbitgenomics.org/) to find the chromosomal localizations of the genes. Mapchart software was used to visualize chromosomal localities (Voorrips 2002).

Determination of phylogenetic relationship between expansin proteins and analysis of conserved motifs

Multiple sequence alignment was performed using the ClustalW algorithm (Larkin et al. 2007) in the MEGAX program (Kumar et al. 2018). After alignment, a phylogenetic tree was constructed by using the Neighbor Joining (NJ) method with bootstrap analysis for 1000 repetitions to obtain high bootstrap values (Saitou and Nei 1987). The constructed phylogenetic tree visualization was improved

by utilizing the interactive tree of life tool (iTOL; http://itol.embl.de/index.shtml) (Letunic and Bork 2011).

The protected motif structures of the zucchini and the cucumber expansin proteins were analyzed using a motif-based search tool (MEME Suite) (https://meme-suite.org/meme/tools/meme) (Bailey and Elkan 1994). In the analysis, using classical mode, the maximum number of motifs was determined as 20 and the optimum width of the motifs was tested between ≥ 2 and ≤ 300.

Gene ontology analysis

Functional analyses of zucchini and cucumber plant expansin proteins were performed using the Blast2GO program (Conesa and Götz 2008). Amino acid sequences of the expansin proteins were used into the Blast2GO program and functional analysis was performed in three-steps (BlastP, Mapping and Annotation). As a result, classification of cellular locations and biological functions of the expansin proteins were obtained.

Determination of gene orthologous of zucchini and cucumber expansin genes and predicted divergence times

Tandem and segmental duplications were detected for expansin genes and a comparison was made between the duplicating expansin genes and orthologous genes in the genomes of Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*), poplar (*Populus trichocarpa*) and soybean (*Glycine max*). By performing a BLASTP scan in NCBI, values with an expectation value of $\leq 1e^{-50}$ and matching the 50% similarity parameters were accepted among the sequences showing similarity. Tandem and segmentally duplicated expansin genes and their orthologs in four other plants were aligned using the Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo) online tool (Sievers et al. 2011). The calculation of synonymous (Ks) and non-synonymous (Ka) change rates with protein sequences and coding sequences (cDNA) of expansin genes was performed using the program PAL2NAL (http://www.bork.embl.de/pal2nal) (Suyama et al. 2006). Thus, using the equation T (MYA, Million years ago) = Ks / 2λ ($\lambda = 6.5 \times 10^{-9}$), the divergence times of orthologous gene pairs in the evolutionary process were estimated (Lynch and Conery 2000; Yang et al. 2008).

Identification of miRNAs targeted zucchini and cucumber expansin genes

Sequences of known plant miRNAs from miRBase v21 (http://www.mirbase.org/) were used in order to find miRNAs that have an important function in regulating gene expression (Kozomara and Griffiths-Jones 2014). The transcripts of the targeted expansin genes were identified using the Plant Small RNA Target Analysis Server program (psRNATarget, https://www.zhaolab.org/psRNATarget/) (Dai and Zhao 2011). Using the Cytoscape software, miRNAs targeting expansin gene transcripts were visualized (Shannon et al. 2003).

Predicted three dimensional (3D) protein structures

A BLASTP scan was performed in the Protein Data Bank (PDB) to determine the predicted three-dimensional structure of the expansin proteins (Berman et al. 2000). Next, homology modeling for cucumber and zucchini expansin proteins was performed using intensive mode in Phyre2 program (ProteinHomology/AnalogY Recognition Engine; http://www.sbg.bio.ic.ac.uk/phyre2), and the predictive structure of the proteins was obtained (Kelley et al. 2015).

Expression analysis of expansin genes by in silico method

In order to evaluate expansin gene expression profiles in zucchini and cucumber, a search was conducted for transcriptome data from the Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra). The accession numbers used for zucchini and cucumber in transcriptome analysis are listed as follows: SRP082555 [fruit samples from variable days (fruit 5, 10, 15, 20 and 40 days) after fertilization], SRP136062 [zucchini leaf samples infected by *Aphis gossypi* for 24, 48 and 96 h (A24, A48, A96) and controls (C24, C48, C96)], SRP134937 [samples taken from both female and male nectars at four stages of zucchini flower maturation (24 h prior to anthesis, 15 h prior to anthesis, 0 h full anthesis, 12 h post anthesis)], SRP174527 (salt-stressed cucumber root tissues), SRP137949 (salt-stressed cucumber root and leaf tissues), and SRP071224 (samples taken from 23 different normal cucumber tissues). Illumina HiSeq reads were downloaded in the 'fastq" format for RNA-seq analysis and the quality of the obtained read was checked using FASTQC software. Using CLC Genomics Workbench 11 software, normalization and transformation processes were performed and gene expression ratios were calculated. Then, heatmaps were created with Permut Matrix software by using gene expression levels obtained by converting RPKM values to log2 (Caraux and Pinloche 2005).

Expression analysis of expansin genes under drought, salt, heat, cold and ABA applications by real time PCR method (qRT-PCR)

Plant materials, growth parameters and stress treatment

Zucchini and cucumber seeds (Seyden F1 and Cemre F1 cultivars, respectively) were procured from the Monsanto Food and Agriculture Trade Limited Company (Antalya, Turkey). The seeds were washed and then kept in distilled water for two hours for imbibition. After this process, the seeds were planted in containers with vermiculite. Subsequently, plants were grown in a culture medium containing Hoagland's solution (Caisson Labs, USA) at 24 ± 2 °C for 21 days and a photoperiod of 16 h' light and 8 h' dark at 400 µmol m^{-2} s^{-1} light intensity in a plant growing cabinet (Hoagland and Arnon 1950). Stress-treated plants and untreated (control) plants were grown under the same conditions. When plants reached to the level of at least three leaves of the normal growing period, stress applications were initiated (Altunoglu et al. 2016) (Supplementary Fig. 1). To mimic drought stress, 20% PEG was added to the Hoagland solution (Baloglu et al. 2014). For ABA application, a 100 µM ABA solution prepared with the Hoagland solution was sprayed on the leaves of zucchini and cucumber plants. For the salt stress application, 200 mM sodium chloride (NaCl) was added to the Hoagland solution and applied to the plants. Plants were exposed to 10 °C and 50 °C for cold and heat stresses, respectively (Unel 2018). Leaf and root samples were collected from the stress-treated plants at 0, 1st, 3rd, 6th, 12th and 24th hours. The beginning of the stress was taken as the zero point and the plant samples taken at this stage were evaluated as control. The collected root and leaf samples were kept at − 80 °C to measure tissue specific expression profiles of expansin genes.

RNA isolation and qRT-PCR analysis

Trizol reagent (ABP Biosciences, USA) was used to isolate RNA. The purity and concentration of RNA were measured by using agarose gel electrophoresis and MultiscanGO nano-spectrophotometer (ThermoFisher Scientific, USA), respectively. DNA contamination was removed from RNA samples using the *DNase* I enzyme (Thermo Scientific, USA). The iScript cDNA synthesis kit (Biorad, USA) was used for cDNA preparation according to the manufacturer's instructions. The expansin genes with increased gene expression in different tissues of zucchini and cucumber plants were detected through transcriptome analysis. Primers for these selected expansin genes were designed through the NCBI Primer 3 software. In qRT-PCR analysis, *18S rRNA* (GenBank ID: X51542.1) (Baloglu et al. 2014) and *TUA* (Tubulin alpha chain) (GeneID: XM_004149597) (Kong et al. 2014) were used as housekeeping genes in zucchini, while *18S rRNA* and *β-actin* (ID: Csa017310) (Ling et al. 2011) were used for cucumber (Supplementary Table 1).

Gene expression analysis was performed on the CFX96 Touch Real-Time PCR cycler (BIO-RAD, USA). qRT-PCR conditions using SYBR Green Supermix (BIO-RAD, USA) were as follows: initial denaturation was conducted for 3 min at 95 °C, then 5 s of denaturation at 95 °C, 10 s of binding at 52 °C, and 10 s of elongation at 60 °C were conducted for 35 cycles. Melting curve analysis was used at the end of the qRT-PCR reaction to ensure that the desired DNA region was replicated. After the Ct values were determined, the results were normalized considering the control status and housekeeping gene. ΔCT and ΔΔCT values were calculated using the formula: $\Delta CT = CT_{sample} - CT_{housekeeping\ gene}$ and $\Delta\Delta CT = \Delta CT_{stressed\ sample} - \Delta CT_{control\ (0\ h)}$. The difference between expression levels was determined as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001). Heat maps were drawn for expression profiles of zucchini and cucumber expansin genes under variable abiotic stresses.

Statistical analysis

Three biological replicates were used for every tissue sample and three technical replicates were analyzed in qRT-PCR for every sample in the study. Statistical values of the difference between stressed samples and control samples were evaluated by One-way Anova in the Minitab 18 package program. Differences were considered significant when p-values were less than 0.05.

Results and discussion

Determination of the expansin genes in zucchini and cucumber genomes

After multiple searches, 49 expansin genes (29 *CpEXPA*, 14 *CpEXPLA*, 6 *CpEXPB*) for zucchini and 41 expansin genes (12 *CsEXPA*, 18 *CsEXPLA*, 4 *CsEXPB*, 7 *CsEXPLB*) for cucumber were identified. Nomenclature for the expansin genes was classified according to chromosomal localization and expansin subgroup. Gene names included the first letters of the plant's Latin name followed by expansin subgroup and number. When molecular weights and amino acid lengths of the determined expansin genes were analyzed, the molecular weights of the zucchini expansin genes were between 13,670.58 Da and 53,001.62 Da, and their protein lengths varied between 132 and 484 aa (Supplementary Table 2). For cucumbers, it was

determined that molecular weights of the expansin genes varied between 6654.67 Da and 31,038.50 Da and their protein lengths were between 64 and 269 aa (Supplementary Table 3).

There are numerous studies in the literature related to the identification and characterization of expansin genes in plant genomes. Accordingly, in studies conducted on other plants, 38 expansin gene numbers were identified in Arabidopsis (Li et al. 2002), 29 in grape (Dal Santo et al. 2013), 92 in sugarcane (Santiago et al. 2018), 36 in potato (Chen et al. 2019), 75 in soybean (Zhu et al. 2014), 38 in tomato (Lu et al. 2016), 30 in jujube (Hou et al. 2019), 88 in maize (Zhang et al. 2014), 52 in tobacco (Ding et al. 2016), and 241 in common wheat (Han et al. 2019). Considering the number of expansin subgroups in these plants, *EXPA* genes were the most common in the zucchini genome followed by EXPLA proteins, whereas the most abundant in cucumber was *EXPLA*. These differences in gene numbers can be explained by detailed searches and the use of many different plants. In addition, the differences in the number of genes of different plants may be related to the functions and needs of these genes in those plants.

Chromosomal localization distribution and prediction of the gene structure

The chromosomal distributions of the expansin genes visualized using the Mapchart program were found scattered on the 16 chromosomes of the zucchini genome (not observed on chromosomes 6, 12, 14, 17), and 7 chromosomes of the cucumber genome. It was observed that most of the expansin genes were located on chromosome 1 (8 genes), followed by chromosome 15 with 6 expansin genes. The chromosomes carrying the least expansin genes in the zucchini genome were the 19th and 20th chromosomes with 1 gene each (Fig. 1). In the cucumber genome, chromosome 7 carried the most number of expansin genes (11 genes), while the 4th chromosome had the least count of expansin genes with 3 (Fig. 2). The structure analysis of the zucchini and cucumber expansin genes revealed that all genes contained introns (Supplementary Fig. 2–3).

Phylogenetic relations between expansin genes and analysis of conserved motifs

The phylogenetic tree drawn for the zucchini expansin proteins revealed 3 distinct phylogenetic clusters (Fig. 3A). Among those clusters, Cluster I contained 1 expansin protein, Cluster II had 21 expansin proteins, and Cluster III had 27 expansin proteins. According to this distribution, Cluster I included CpEXPA and, except for one CpEXPLA protein, Cluster II also included the CpEXPA subgroup of the proteins. The CpEXPA, CpEXPLA and CpEXPB subgroups of proteins comprised Cluster III. In addition, 20 different motifs were determined using the MEME database. According to the motif analyses of the zucchini expansin proteins, it was observed that motifs 1, 3, 4, 5, 6, 7 and 12 were usually found in the CpEXPA subgroup of the proteins. However, only motif 4 was in the CpEXPA-19 protein. The same motifs, in addition to 16 and 19, were found in the subgroup of CpEXPLA proteins, and in the subgroup of CpEXPB proteins, motifs 4, 5, and 7 were common (Supplementary Fig. 4).

In the phylogenetic tree of the cucumber expansin proteins, 4 separate clusters were observed (Fig. 3B). Accordingly, there was 1 protein in Cluster I, 4 in Cluster II, 15 in Cluster III, while in Cluster IV there were 8 proteins in the "a" arm and 13 in the "b" arm. When the distribution of the expansin subgroups in those clusters was examined, the CsEXPA protein was predominant in Cluster I, the CsEXPLB subgroup of proteins in Cluster II, and the CsEXPLB and CsEXPLA subgroup of proteins in Cluster III. In the "a" arm of Cluster IV, the CsEXPA and CsEXPLA subgroups of proteins were common. In the "b" arm of Cluster IV, mainly the CsEXPA and CsEXPLA subgroups of proteins and 1 CsEXPB subgroup were collected. Considering the motif analyses, while motifs 1a, 7a and 10a were usually included in the CsEXPA subgroup of proteins, the motif content in the CsEXPB subgroup of proteins varied considerably. The CsEXPLA subgroup of proteins commonly contained motifs 1a, 5a and 6a, while motifs 1a and 9a were typically found in the CsEXPLB subgroup of proteins (Supplementary Fig. 5).

According to the phylogenetic analysis, it was determined that proteins belonging to the same expansin subgroup were also found in the same clusters of the tree and distributed in very close branches. In order to examine these distributions in more detail, the conserved motif structures of the expansin proteins were also analyzed. According to the determined motif structures, it was observed that members of the expansin subgroups generally have the same motif structures with a few exceptions. Common motifs observed among the same protein subgroups were thought to have been conserved during evolution and function as determinants in the subgroups. In this respect, it is thought that the conserved motif analysis in proteins also supports the phylogenetic tree analysis for these proteins and proves the high accuracy of the cluster distributions. In addition, the reason some proteins are members of the same expansin subgroup and are close but in different clusters in the phylogenetic tree may be that the motif contents of these expansin proteins have different additions from one other.

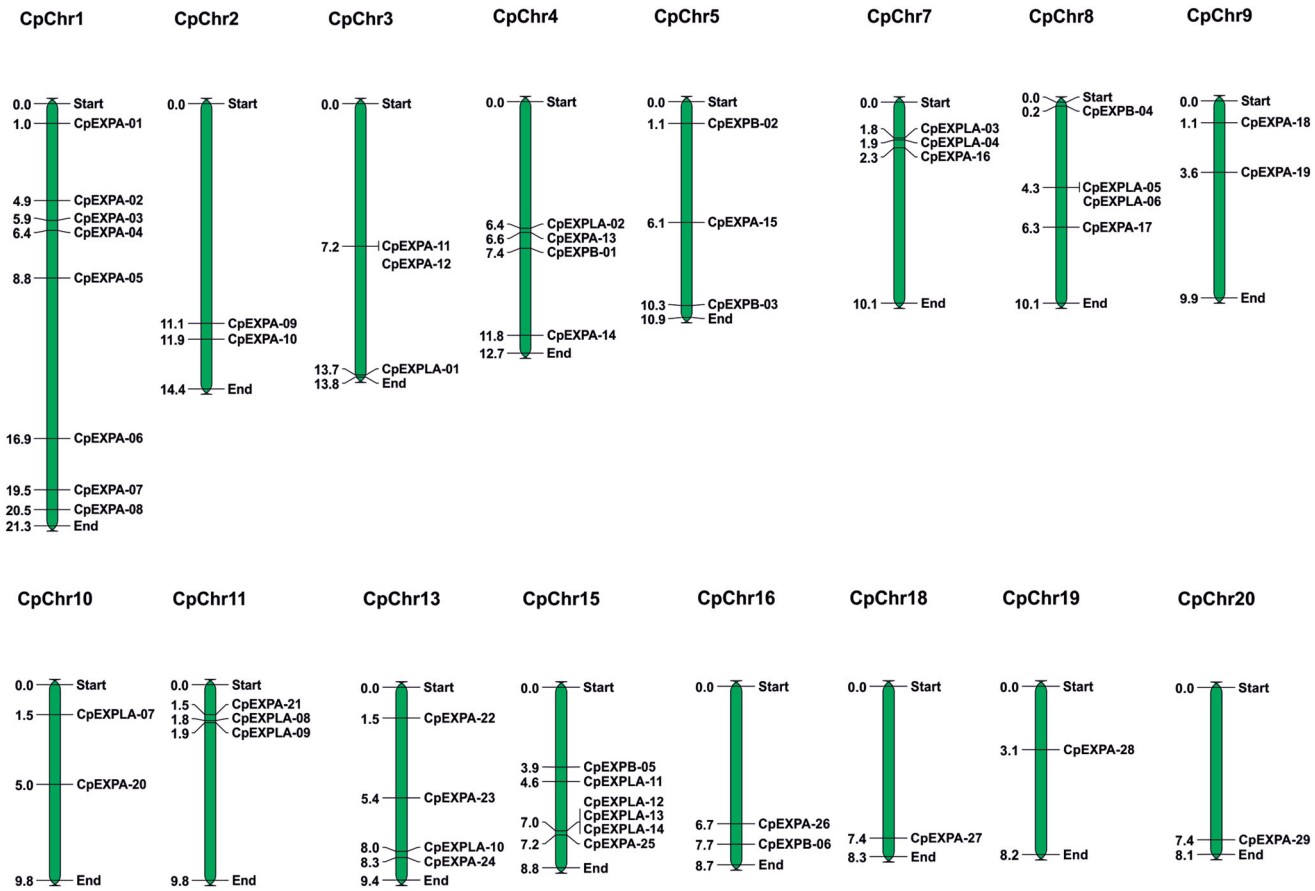

Fig. 1 Chromosomal distribution of 49 expansin genes on zucchini chromosomes. Physical locations of zucchini expansin genes are shown on 16 zucchini chromosomes and chromosomal distances are given as Mbp

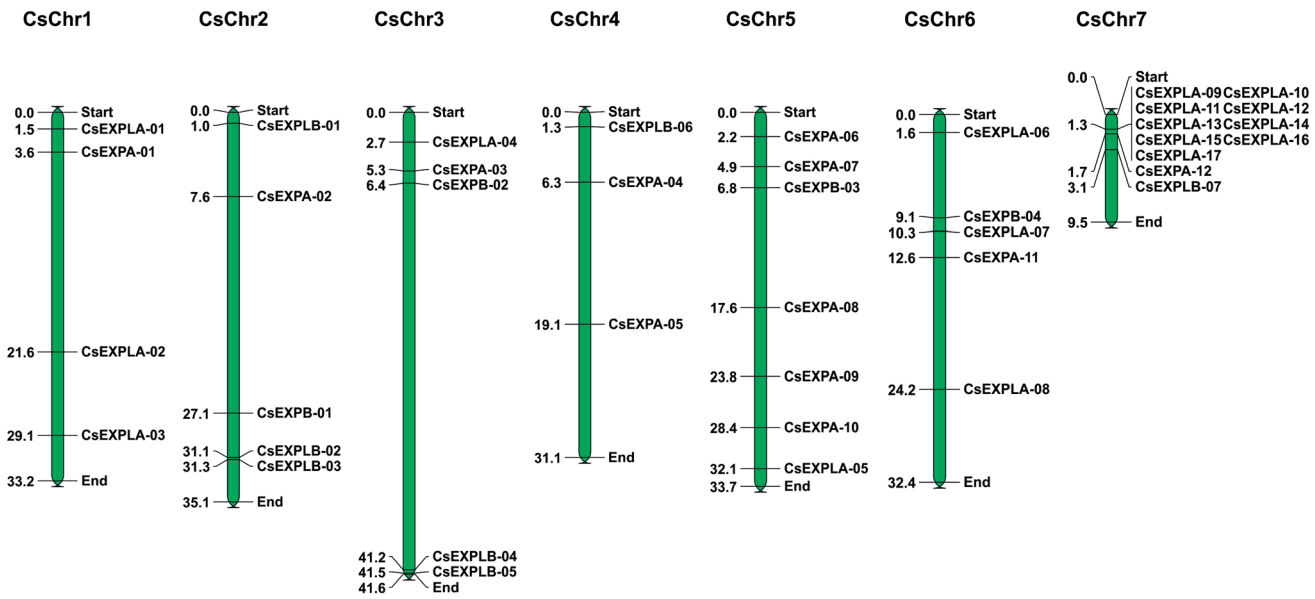

Fig. 2 Chromosomal distribution of 41 expansin genes on cucumber chromosomes. Physical locations of cucumber expansin genes are shown on 7 cucumber chromosomes and chromosomal distances are given as Mbp

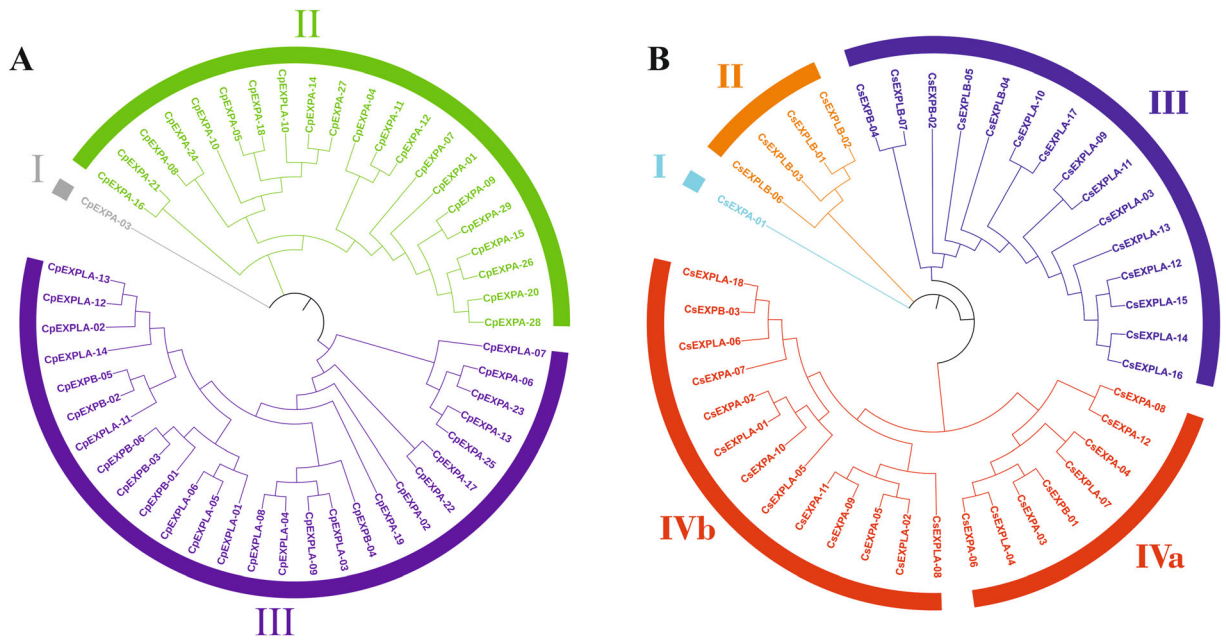

Fig. 3 Phylogenetic comparison of zucchini and cucumber expansin proteins. **A** Phylogenetic tree of zucchini expansin proteins with three clusters. **B** Phylogenetic tree of cucumber expansin proteins with four clusters

Gene ontology analysis

Cellular localization, biological role, and molecular function analyses of the zucchini and the cucumber expansin genes were predicted by using GO analysis through the Blast2GO program. The predicted cellular localization of the expansin proteins in zucchini was observed in the extracellular region, cell part, cell and membrane categories, respectively. When examining the biological role played in cells, expansin proteins had roles in cellular processes, cellular component organization or biogenesis, reproductive processes, multi-organism processes, reproduction and developmental processes, respectively (Fig. 4A).

It was observed that the predicted cellular localization of cucumber expansin proteins was located in particular the extracellular region, cell part, cell and membrane categories, respectively. Predictive roles in biological processes were observed to be cellular processes, cellular component organization or biogenesis, reproductive processes, multi-organism processes, reproduction, and developmental processes (Fig. 4B). Classification by the program for molecular functions of the cucumber or the zucchini expansin genes could not be obtained.

It can be said that the most common biological roles of the expansin proteins in zucchini and cucumber were in cellular processes, cellular component organization, or biogenesis and reproductive processes. These emerging predictive biological roles appear to be compatible with the roles of expansin proteins in the cell. Comprehensive analyses provided by the program related to predicted biological roles of the proteins determined that the plant type cell wall organization was dominant. The predicted cellular localization of the expansin proteins in zucchini and cucumber were generally in the extracellular region, cell part, and cell membrane categories. Localization regions of the proteins are compatible with the functions of the expansin proteins that act on the cell wall and thus expand the cell.

Gene orthologs of the zucchini and cucumber expansin genes and predicted divergence times

Gene duplications have an important function in the spread of gene families (Mehan et al. 2004). In order to understand the expansion of the expansin genes in plants that are members of the Cucurbitaceae family, the relationships of Darwin's positive selection in duplication and separation of the identified expansin genes of these plants were examined. When detailed examinations and calculations were made, non-synonymous (Ka) versus synonymous (Ks) substitution rates (Ka/Ks) were determined for the expansin genes.

When orthologous relations among Cucurbitaceae family members were evaluated, 206 pairs of expansin genes were orthologous between zucchini and cucumber. This was followed by melon (*Cucumis melo*) and watermelon (*Citrullus lanatus*) expansin genes with orthologous gene pair numbers of 187 and 168, respectively. For cucumber expansin genes, 186 orthologous genes between melon

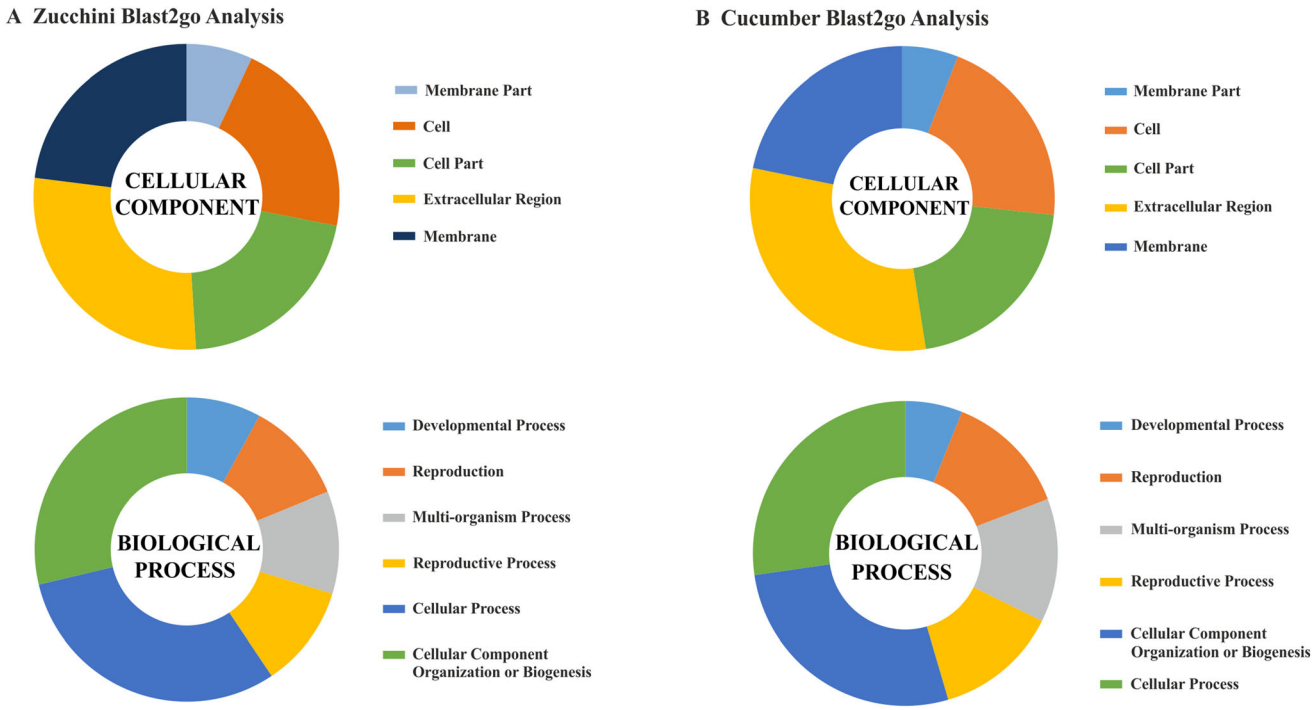

Fig. 4 Gene ontology analysis of **A** zucchini and **B** cucumber expansin genes by Blast2Go program with two categories named as biological process and cellular component

expansin genes and 147 orthologous genes between watermelon expansin genes were identified. The earliest separation that occurred between zucchini and cucumber expansin genes was approximately 106 million years ago (Mya), and the latest separation determined between cucumber and watermelon expansin genes was approximately 83 Mya (Fig. 5; Supplementary Table 4).

Tandem and segmental duplications of the zucchini and cucumber expansin genes were determined. Accordingly, it was observed that 70 pairs of the zucchini expansin genes showed tandem duplication and 747 pairs displayed segmental duplication. The genes with the highest duplication were located on the chromosome 1 of the zucchini genome, followed by the expansin genes located on the chromosomes 15 and 13 (average of ka/ks = 0.12). When duplication events were analyzed in the cucumber expansin genes, 45 expansin gene pairs showed tandem and 88 expansin gene pairs showed segmental duplication. Genes showing tandem duplication were mostly found on the 7th cucumber chromosome, followed by those located on the 5th chromosome (average of ka/ks = 0.11) (Supplementary Table 5).

In addition to these, the orthologous relationships of the expansin genes in the genomes of zucchini and cucumber were determined with the genomes of Arabidopsis (*Arabidopsis thaliana*), soybean (*Glycine max*), rice (*Oryza sativa*) and poplar (*Populus triocarpa*) separately and divergence times for gene orthology were also estimated.

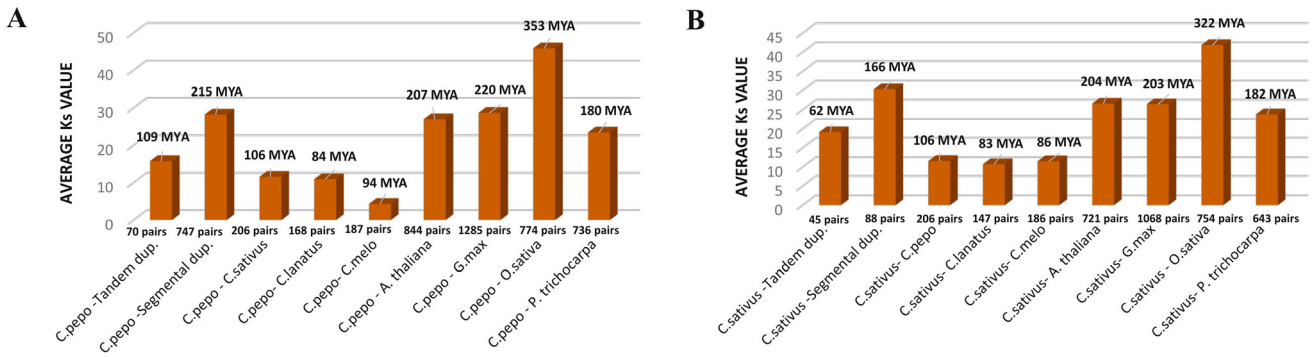

Fig. 5 **A** Gene orthologous of zucchini expansin genes in other organisms and predicted divergence times. **B** Gene orthologous of cucumber expansin genes in other organisms and predicted divergence times

Accordingly, 1285 pairs of orthologous genes were identified between zucchini expansin genes and soybean expansin genes. This was followed by Arabidopsis with 844 pair of genes, rice with 774 pairs of genes, and poplar with 736 pairs of genes, respectively. Looking at the estimated separation times between orthologous genes, it was estimated that these separations occurred between 180 and 353 Mya. It was calculated that the earliest separation of the zucchini expansin genes was between rice expansin genes with an average of 353 Mya. This was followed by soybean expansin genes with an average of 220 Mya, Arabidopsis expansin genes with 207 Mya, and poplar expansin genes with 180 Mya, respectively. When orthologous relationships of *CpEXP* genes in zucchini genome were determined, the average Ka/Ks ratio was calculated as 0.16, 0.26, 1 and 0.03 in poplar, soybean, Arabidopsis, and rice, respectively (Fig. 5A; Supplementary Table 6).

Looking at the orthologous relationships of the cucumber expansin genes, it was observed that the greatest number of orthologous expansin gene pairs were with soybean genes with 1068 pairs, followed by rice with 754 pairs, Arabidopsis with 721 pairs, and poplar with 643 pairs, respectively. The estimated separation times of these orthologous genes were calculated to be: rice with an average of 322 Mya, Arabidopsis with an average of 204 Mya, soybean with an average of 203 Mya, and poplar with an average of 182 Mya, respectively. The calculated average Ka/Ks ratios were 0.04 with poplar, 0.13 with soybean, 0.15 with rice, and 0.36 with Arabidopsis (Fig. 5B; Supplementary Table 7).

When the results are examined, the number of gene pairs showing tandem and segmental duplication was highest in zucchini. This may be related to the number of expansin genes in zucchini. One of the reasons for the spread of expansin genes in different plant species may be tandem and segmental duplication events that affect the distribution of gene families. The latest predicted divergence was observed with the poplar expansin genes for both the expansin genes in zucchini and cucumber. Here, it can be said that there is a selection pressure that is stronger in terms of these genes in zucchini, cucumber and poplar compared to others. The most orthologous genes were observed with soybean expansin genes. Other orthologous expansin genes determined for zucchini were with Arabidopsis, rice, and poplar expansin genes, respectively and for cucumber with rice, Arabidopsis, and poplar expansin genes, respectively. The results were consistent with the previous research conducted by our team, in which the heat shock protein (*Hsp*) genes in watermelon were analyzed and the most orthologous *Hsp* genes (*sHsp, Hsp40, Hsp60, Hsp70* and *Hsp100*) were found with soybean (Altunoğlu et al. 2019). It can be said that zucchini and cucumber expansin genes are more similar to soybean expansin genes than other expansin genes in Arabidopsis, rice, and poplar.

Analysis of miRNAs targeted zucchini and cucumber expansin genes

miRNAs are known as small non-coding regulatory RNA sequences and affect gene expression in many organisms. miRNAs are been used frequently to suppress the expression of the target gene in plants (Bartel 2004; Ambros and Chen 2007). A total of 100 different miRNAs for zucchini expansin genes were determined through psRNATarget: A Plant Small RNA Target Analysis Server. The most targeted zucchini expansin genes were *CpEXPA-27* (targeted by 46 miRNAs), *CpEXPA-05* and *CpEXPA-26* (targeted by 27 miRNAs), respectively. The miRNA known as osa-miR2919 was a common miRNA targeting both *CpEXPA-05* and *CpEXPA-26*. Among the miRNAs targeting *CpEXPA-27*, the most frequently observed ones were miR164 and miR395 (Fig. 6A).

In the analysis for miRNAs targeting cucumber expansin genes, the most targeted genes were *CsEXPLA-11* targeted by 27 miRNAs, *CsEXPLA-02* targeted by 22 miRNAs and *CsEXPA-06* targeted by 21 miRNAs, respectively. A shared miRNA among those genes was not observed in the analyses. In addition, miRNAs from different plants that mostly target the *CsEXPLA-11* gene were miR156 and miR164. The miRNA from different plants that mostly target *CsEXPLA-02* was miR395 while *CsEXPA-06* was mostly targeted by miR159 and miR168 (Fig. 6B) (Supplementary Table 8).

It has been reported that a loss in miR164 activity caused a serious deterioration in shoot development (Sieber et al. 2007). In another study, it was shown that miR156 in the Arabidopsis was necessary for the expression of the young plant stage and regulated the timing of the transition from the young to adult plant by coordinating the expression of various developmental pathways. It has been shown that miR156 acts by suppressing the expression of functionally different SPL transcription factors (Wu et al. 2009). In addition, it was determined that three of the four isoforms of ATP sulfurylase, which is the first enzyme of sulfate assimilation, are targeted by miR395 in Arabidopsis. It has been stated that miR395 is strongly stimulated by sulfate deficiency and this miRNA is an integral part of the regulatory mechanism that controls plant sulfate assimilation with a complex mechanism of action (Kawashima et al. 2011). The results obtained from previous studies investigating the roles of miRNAs that target different expansin genes in the Cucurbitaceae family suggest that expansin proteins play significant role in the stress tolerance of the plant both in normal tissue development and in abiotic stress conditions.

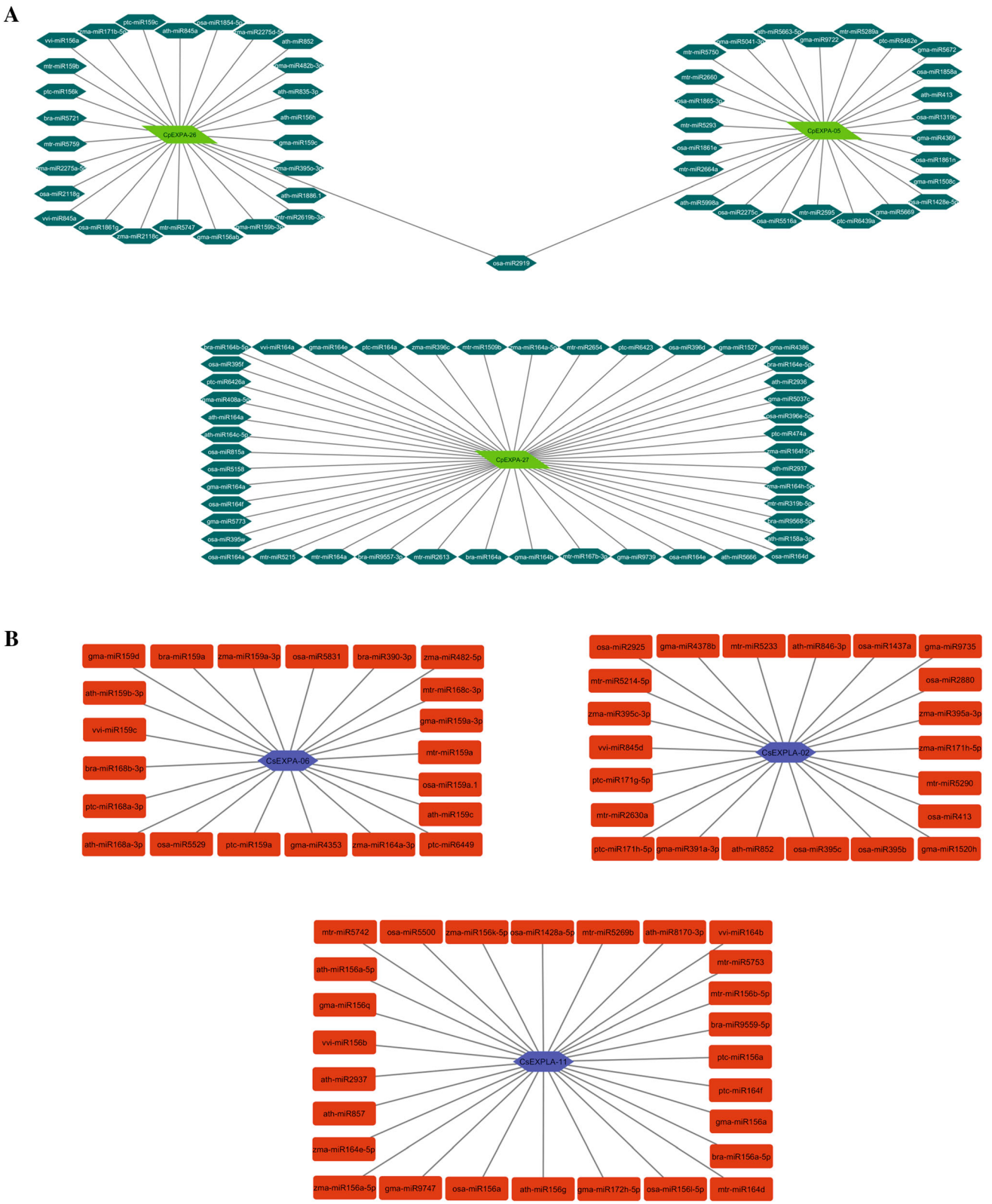

Fig. 6 **A** miRNAs targeting zucchini expansin genes **B** miRNAs targeting cucumber expansin genes

Predicted three-dimensional (3D) protein structures

According to the estimated 3D structure analysis for the zucchini expansins, the structure of 27 zucchini expansin proteins displayed high homology. Residues were modeled between 90–100% homology and with > 90% confidence intervals. These proteins were CpEXPA-01-04-05-07-08-10-11-15-16-20-21-22-24-25-26-27-28-29 (PDB ID: 2HCZ), CpEXPA-03 (PDB ID: 5FUD), CpEXPA-02-13-17 (PDB ID: 1N10), CpEXPB-02-05 (PDB ID: 2HCZ) and CpEXPLA-06-10-11 (PDB ID: 2HCZ). When the structures were examined, generally two groups of β sheets and one or two small groups of α helix structures in all subgroups were abundant (Fig. 7).

Twenty cucumber expansin proteins were modeled between 90–94% homology and with > 90% confidence intervals. These proteins were CsEXPA-01-02-07-10-11-12 (PDB ID: 2HCZ), CsEXPA-03-04-06-08 (PDB ID: 1N10), CsEXPB-01 (PDB ID: 2HCZ), CsEXPLA-01-02-05-07-15 (PDB ID: 2HCZ), CsEXPLA-06 (PDB ID: 1N10) and CsEXPLB-04-05-07 (PDB ID: 2HCZ). When the structure of these proteins was examined, it was seen that generally two groups of β sheets and one or two small groups of α helix structures were common in all subgroups modeled (Fig. 8).

Identification of similar three-dimensional structures suggests that there are structures preserved for the function of these proteins. Among the proteins whose three-dimensional structure was determined, there were the most EXPA proteins in zucchini and cucumber. This may be because these subgroups of proteins are usually the most abundant groups. According to a molecular modeling study on an expansin protein called FaEXPA1 in strawberry, it was determined that the structure of the protein contained two domains: Domain 1 with 6 β-sheets and 1 α-helix constructing a β-barrel with the helices to the outside, while Domain 2 has 8 β-strands, adhered in two anti-parallel β-sheets. Moreover, in the same study, the structure of the 2HCZ protein from PDB was used as a template to construct a 3D-structure, which displayed high homology for most of the zucchini and cucumber expansin proteins in PBD (Valenzuela-Riffo et al. 2018). From this view, results of the current study related to predictive 3D structures of EXPA proteins and the predominantly found β-sheet structures in these proteins are consistent with previous studies (Sampedro and Cosgrove 2005; Valenzuela-Riffo et al. 2018). Moreover, these similar and conserved sites may denote the conserved functions of the expansin superfamily members in various plants.

Expression analysis of zucchini expansin genes by in silico method and their expression under abiotic stress applications by qRT-PCR

In order to determine in silico expression levels of zucchini expansin genes, samples taken from zucchini leaves infected by *Aphis gossypi* for 24, 48 and 96 h (A24, A48, A96) and controls (C24, C48, C96) (Vitiello et al. 2018), samples taken from both female and male nectars at four stages of zucchini flower maturation (Solhaug et al. 2019), and fruit samples from variable days after fertilization data (Wyatt et al. 2016) were used. When the heat map of *Aphis gossypi* infection data was analyzed, no expression was observed for *CpEXPA-01* and *CpEXPB-06* genes in the control samples, but an increase was observed only at the 48th hour of treatment. In *CpEXPA-20*, while no expression was observed in the control group, it was determined that its expression increased at the 24th, 48th and 96th hours of the treatment. It has been observed that the expression levels of the *CpEXPA-03, CpEXPA-20*, *CpEXPA-26* and *CpEXPLA-14* genes were elevated in all four maturation stages of both female and male nectars. It has also been determined that the expressions of *CpEXPA-01* and *CpEXPB-01* genes increased in all maturation stages of male nectars. When the transcriptome data of the fruit samples on different days after fertilization (DAP) were examined, the *CpEXPA-01-03-05-06-09-18-19-20-23-24-26-28*, and CpEXPLA-*02-14* genes were expressed in all the fruit developmental stages (Supplementary Fig. 6). The qRT-PCR results show, a significant increase in the *CpEXPA-20* gene expression level at different time point used in the study for ABA application compared to controls. Similarly, an increase in the expression of this gene in root tissues was observed as compared to controls. An increase in CpEXPLA-14 gene expression was observed in the root tissues, as well. A decrease was observed at different time points after a rapid increase in all genes within the 1st hour of drought stress induction in the root tissues. In the leaf tissues, a decrease in the expressions of *CpEXPA-01*, *CpEXPA-18*, *CpEXPA-20,* and *CpEXPLA-14* genes after a rapid increase in the 1st and 3rd hours of stress was observed. In all genes examined in zucchini leaf tissues exposed to heat stress, the increase at the 1st hour of stress was determined compared to the control group. For the expression levels of the *CpEXPA-01*, *CpEXPA-18*, *CpEXPB-01*, *CpEXPB-06,* and *CpEXPLA-14* genes in zucchini root tissues under cold stress, the first significant trend towards increase raise was detected in the 3rd hour of the stress. A significant trend of higher expression was observed in all genes examined in the zucchini root tissues under salt stress starting from the 1st hour (Fig. 9; Supplementary Fig. 7). The Arabidopsis expansin-like A2, *EXLA2* (NP_195553.1) which is

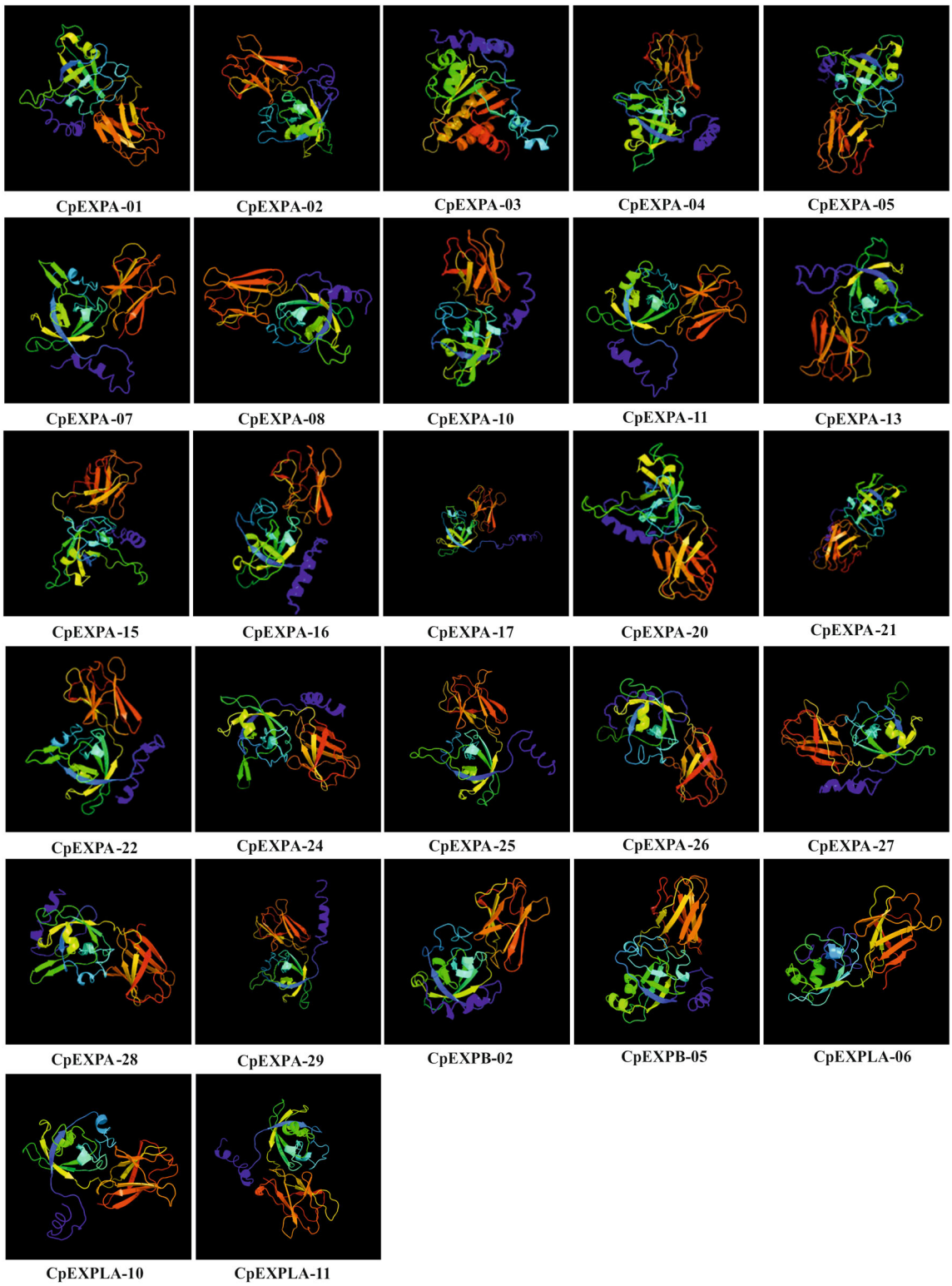

Fig. 7 Predicted structures of zucchini expansin proteins. The structures of 27 expansin proteins with > 90% confidence level are shown

orthologous to the *CpEXPLA-14* gene in zucchini, the results show that *EXLA2* expression was induced significantly by abiotic stresses particularly salinity, cold, and ABA (Abuqamar et al. 2013). The *CpEXPA-20* orthologous gene in Arabidopsis known as *AtEXP2* (NP_196148.1) was defined as being involved in

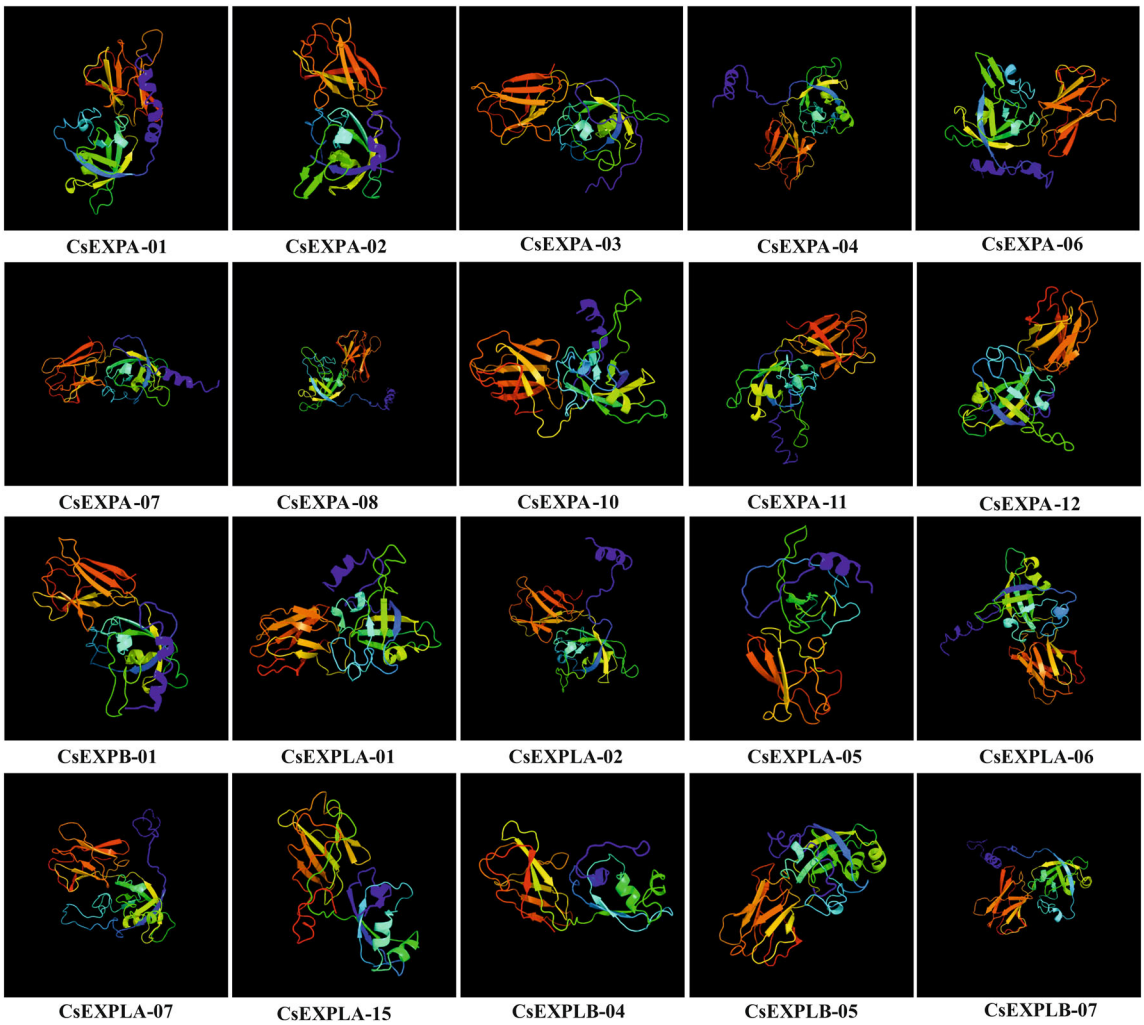

Fig. 8 Predicted structures of cucumber expansin proteins. The structures of 20 expansin proteins with > 90% confidence level are shown

gibberellic acid-mediated seed germination and providing salt and osmotic stress tolerance in Arabidopsis (Yan et al. 2014). The *AtEXPA1* (NP_177112.1) an orthologous gene of CpEXPA-20 present in Arabidopsis. It has been shown that upregulation of *AtEXPA1* accelerated stomatal opening by decreasing the volumetric elastic modulus (Zhang et al. 2011). It can be concluded that the *CpEXPA-20* and *CpEXPLA-14* genes can have roles in abiotic stress response and tolerance in addition to their roles in the normal developmental processes of the zucchini, which are promoted by the analysis of their counterparts orthologous in other plants.

Expression analysis of cucumber expansin genes by in silico method and their expression analysis under abiotic stress applications by qRT-PCR

To determine the expression of the cucumber expansin genes, transcriptome data from salt-stressed cucumber root tissues (Zhu et al. 2019a), salt-stressed cucumber root and leaf tissues (Zhu et al. 2019b) and samples taken from 23 different cucumber tissues (Chen et al. 2020) were examined. It was observed that the expression of *CsEXPB-02, CsEXPLA-04-15* and *CsEXPLB-04* genes increased in the samples under salt stress (Zhu et al. 2019a). However, when the other set of data including salt-stressed cucumber root and leaf tissues were examined, the *CsEXPB-04*, *CsEXPLB-01* and *CsEXPLB-07* gene expressions were observed to be upregulated in stressed cucumber root and leaf samples (Zhu et al. 2019b). Considering the transcriptome data from 23 different cucumber tissues, the expression of *CsEXPLA-17* gene was observed in all tissues except in the S22, and S23 (S22: peel of 3-week-old fruit; S23: flesh of 3-week-old fruit) (Supplementary Fig. 8). From the qRT-PCR results, it was observed that the expression levels of *CsEXPA-08*, *CsEXPLA-06, CsEXPB-04,* and *CsEXPLB-07* genes in cucumber root tissues tended to decrease after reaching their highest point at the 3rd hour of ABA application. In cucumber leaf

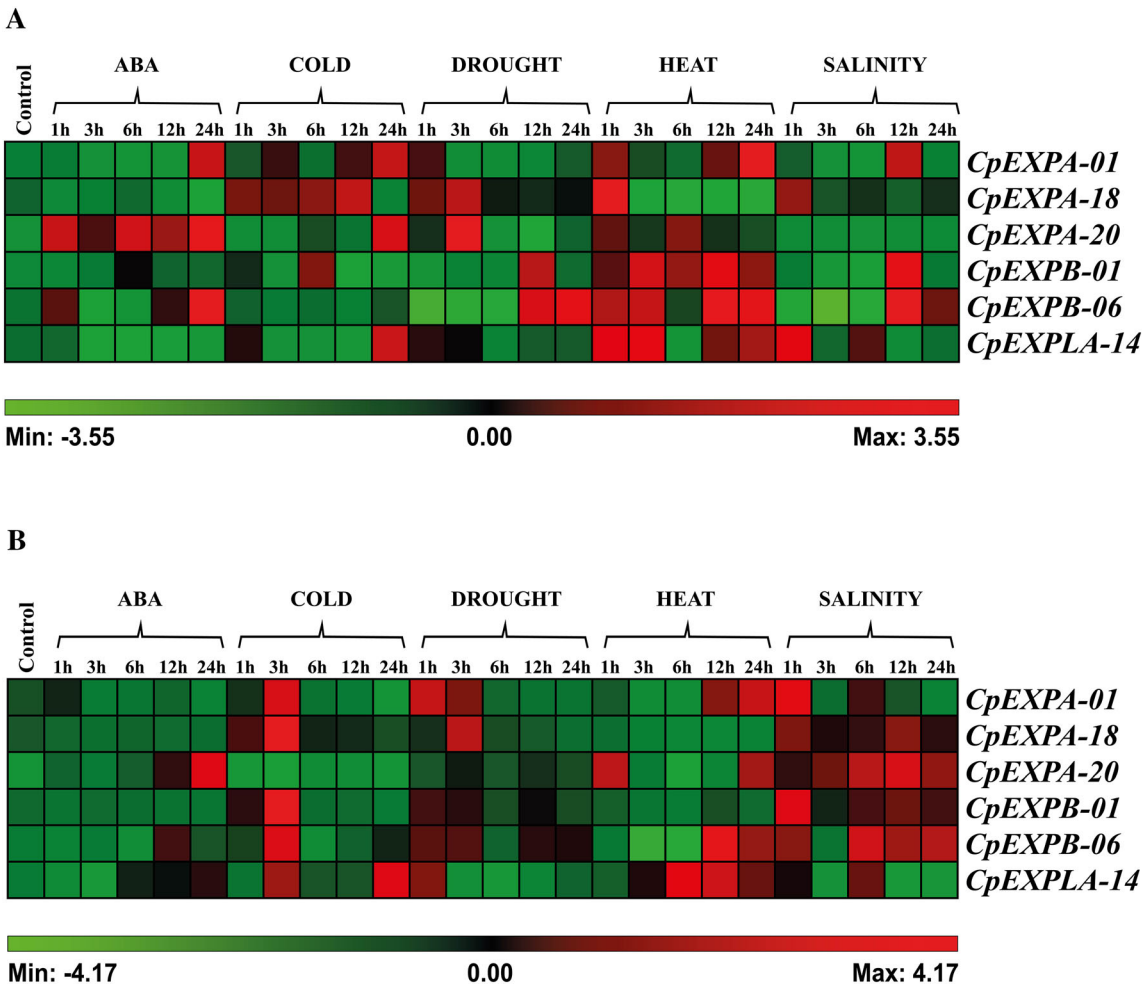

Fig. 9 Expression analysis of selected zucchini expansin genes in 0 (control), 1, 3, 6, 12 ve 24 h in the **A** leaf **B** root tissues under cold, drought and heat stresses and ABA treatment

tissues under drought stress, *CsEXPA-11*, *CsEXPB-04* and *CsEXPLA-06* genes showed the highest tendency to increase at the 6th hour of the stress and decrease at the next hour. *CsEXPA-08, CsEXPA-11, CsEXPB-04,* and *CsEXPLB-01* genes tended to increase the most at the 12th hour in cucumber leaf tissues under heat stress, then after show a sharp decrease in the expression. The expressions of *CsEXPA-11, CsEXPB-04,* and *CsEXPLB-07* genes in leaf samples under salt stress and *CsEXPA-08, CsEXPA-11,* and *CsEXPLA-06* genes in root samples under cold stress were found to increase rapidly after the first hour of stress application (Fig. 10; Supplementary Fig. 9). Based on transcriptomic data, *CsEXPB-04* and *CsEXPLB-07* gene expression levels were upregulated under salt stress in cucumber root and leaf samples which were further confirmed by the qRT-PCR analysis, while *CsEXPA-11* gene expression was observed in only root samples. *CsEXPA-08* gene expression was detected only in control root and leaf samples of salt stress data while *CsEXPLA-06* expression was determined in both normal and salt stressed leaf and root samples. It can be concluded that our qRT-PCR results are in agreement with the transcriptome data obtained for these genes. In addition, the expressions of *CsEXPA-08, CsEXPA-11,* and *CsEXPLA-06* genes were upregulated in variable normal tissues of cucumber including root, stem, leaf, tendril, ovary, and flower. The genes whose expression levels increase after the 1st hour of stress application can be considered to play significant roles against cold stress in these plants. Expression of soybean *GmEXPB3* (NP_001276154.1) and *GmEXPB5* (NP_001248362.1) genes which are the orthologous genes of *CsEXPB-04* gene were determined exclusively in flowers and seeds, respectively, which further attributed to their roles in pollen and young seed development. Other genes orthologous to *CsEXPB-04* are *GmEXPB4* (NP_001244938.1), *GmEXPB6* (NP_001239797.1), *GmEXPB7* (NP_001253998.1), *GmEXPB8* (NP_001248360.1), and *GmEXPB9* (NP_001248361.1) in the soybean genome and it was found that increased expression of these genes among the studied tissues demonstrated that they are expressed in generative and vegetative tissues which may be related with

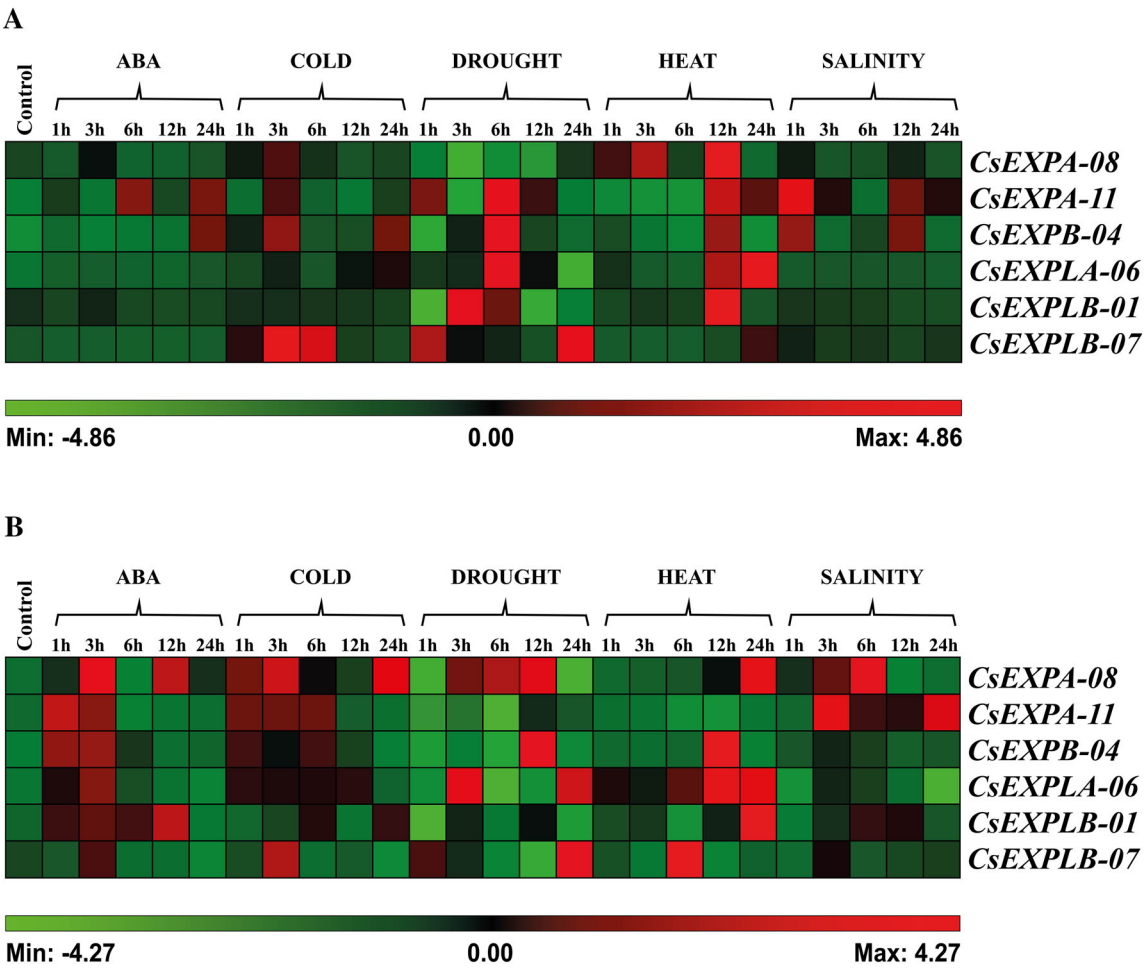

Fig. 10 Expression analysis of selected cucumber expansin genes in 0 (control), 1, 3, 6, 12, and 24 h in the **A** leaf **B** root tissues under cold, drought and heat stress and ABA treatment

their roles in organ development (Li et al. 2014). Consistent with the findings of orthologous genes in soybean, the *CsEXPB-04* gene has roles in the normal growth and development of plant organs in addition to its role in the salt stress response of the plants. Overexpression of the orthologous gene *CsEXPA-11* in rice (*OsEXPA7*-XP_015631937.1) increased tolerance to salt stress and expression of *OsEXPA7* was high in the shoot apical meristem, root, and the leaf sheath (Jadamba et al. 2020). Upregulation of the orthologous gene *CsEXPA-11* in Arabidopsis (AT1G69530), which is orthologous to the *CpEXPA-20* gene, caused accelerated stomata opening (Zhang et al. 2011). In the current study, the results obtained from the expression analysis under the abiotic stress and report from other transcriptomic studies are consistent, which suggests that *CsEXPA-11* has a significant function under salt stress conditions and also in normal plant growth.

Conclusion

The results of the current study have ensured the first comprehensive data to discover zucchini and cucumber expansin gene family based on gene and protein structure, ontology of the proteins, phylogenetic relations and conserved motifs, orthologous relations with other plants, targeting miRNAs of those genes and in silico gene expression profiles. In addition, abiotic stress responses of zucchini and cucumber expansin genes were examined to opine their roles in stress tolerance. This work can provide to look from new perspectives for the roles of expansin gene superfamily and it supplies a comprehensive knowledge for future studies discovering the mode of action of expansin proteins.

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Authors' contributions Planned and designed the research: AYC, BMC, performed experiments: AB, İCY, UF, HE, ÖM, KE, analysed data: BAU, AYC, UF, wrote and edited the manuscript: AYC, AB, BMC.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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