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RESEARCH

Genome-Wide Analysis of *Glutathione S-Transferase* Gene Family in *P. vulgaris* Under Drought and Salinity Stress

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Abstract

The enzymes known as glutathione S-transferases (GST), which are present in many evolved organisms, are essential for the defense against reactive oxygen species. GSTs have a role in the development of defenses against biotic and abiotic challenges, especially defending plants from various stresses such as drought, salinity, and heavy metal. This study uses a genome-wide investigation of the *GST* gene family in *Phaseolus vulgaris* to pinpoint several distinctive traits. 55 Pv-GST proteins have been identified in *P. vulgaris*. The molecular weights of these proteins range from 15.02 kDa to 47.99 kDa; the range of amino acid numbers 132 to 420 and the range of theoretical isoelectric points 5.03 to 9.61 were identified. *Pv-GST* genes are estimated to have at least 2 and a maximum of 10 exons, with an average of 4 exons. Phylogenetic analysis was performed with GST proteins from *Arabidopsis thaliana*, *Glycine max*, and *Phaseolus vulgaris* species, and subfamilies of these GSTs were identified. Using RNAseq data, the expression profiles of *Pv-GST* genes in leaf tissue of common bean during drought and salinity stress were identified. Using the obtained sequence data, primers for qRT-PCR were designed. Changes in the expression profiles of *GST* genes caused by salt and drought stress and melatonin treatments in two different common bean cultivars were determined by qRT-PCR experiments. Under drought and salt stress, the expression levels of GSTs decreased, while melatonin treatment with few exceptions increased these expression levels. As a result of this study, it was observed that *Pv-GST* genes may play a role in the growth and development of bean and may be involved in the response to abiotic stresses. Moreover, the results of this study will provide a basis for functional gene research and the expression profiles and qRT-PCR results under different stress conditions in common bean will provide a valuable contribution to the understanding of the functions of the *GST* gene family.

Keywords *Cis-acting* elements · GST · In silico analysis · Phylogenetic analysis · qRT-PCR · RNAseq

Key Message
Our main discovery is the identification of *GST* family genes in common bean and their expression levels under salt and drought stress. This study is our results, and this will provide benefits, such as understanding GST's roles in defense against drought and salt stress conditions and melatonin's healing effects, in this area. We think that these findings can be a basis and support for future studies especially transgenic studies in plants.

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Introduction

Cellular detoxification processes are vital for plants to continue their development and growth. Toxic effects of different substances such as reactive oxygen species (ROS) that occur during normal energy release processes need to be neutralized. Glutathione S-transferases (GST; E.C. 2.5.1.18) are a heterogeneous group of cellular detoxification enzymes that catalyze the binding of the tripeptide glutathione (GSH) to electrophilic sites on phytotoxic substrates and are widely found in plants (Mohsenzadeh et al. 2011; Ahmad et al. 2020). GSTs play an important role in neutralizing xenobiotics and eliminating the toxic effects of stress mechanisms in plants. GSTs are collection of many phase II energy-releasing isozymes (Hayes et al. 2005). They increase the affinity of the GSH to various electrophiles. If there is a toxic

compound containing sulfur, nitrogen, or carbon atoms in apolar structure, the reduced glutathione attacks this compound and catalyzes the formation of the thioether bond, resulting in increased solubility of the apolar compound and increased metabolic efficiency. GSTs have a wide range of functions, including the formation of oxylipins as a precursor to jasmonic acid. GSTs eliminate cytotoxic or genotoxic components in the cell that can damage DNA, RNA, or proteins (Noctor et al. 2002). GSTs act as a stress signal in a non-catalytic manner, acting as a flavonoid-binding protein, controlling, and reducing apoptosis, and protecting against ozone action (Kampranis et al. 2000; Dixon et al. 2003, 2010; Mohsenzadeh et al. 2011; Ahmad et al. 2020).

GSTs are divided into three main classes: cytosolic, mitochondrial (kappa), and microsomal. Dissolvable GSTs can be found mostly in the cytosol and mitochondria, while "membrane-associated eicosanoid and glutathione" (MAPEG) enzymes involved in energy-release mechanisms are generally associated with microsomal GSTs (Hayes et al. 2005; Pearson 2005; Ahmad et al. 2020). GST classes such as zeta (Z), tau (U), lambda (L), phi (F), dehydroascorbate reductase (DHAR), theta (T), tetrachlorohydroquinone dehalogenase (TCHQD), microsomal prostaglandin E-synthase type 2 (mPGES-2), γ-lower eukaryotic elongation factor 1B (EF1Bγ), and glutathionyl-hydroquinone reductase (GHR) were differentiated by factors such as kinetic properties and functions, immunological cross-reactivity, and whole genome organization (Lallement et al. 2014). While dimeric GSTs such as phi, tau, zeta, theta, and TCHQD contain serine residues in their active sites, monomeric DHAR and lambda contain cysteine residues in their active sites (McGonigle et al. 2000). However, F-, U-, T-, and Z-GSTs are produced very specifically, while F and U are more present. The GST structure is bifolded, the first of which is the N-terminal domain containing the glutathione binding site and thioredoxin folds, and the second is the C-terminal domain, which has a quadruple helix structure confirming the substrate specificity (Frova 2003). It is known that GSTs are found in bacteria, fungi, plants, and animals, and they are well-known proteins in stress adaptation pathways (Sheehan et al. 2001; Pearson 2005; Ahmad et al. 2020). In mammalian cells, GST overexpression has resulted in resistance to various anticancer and carcinogenic agents. However, increased expression of GSTs in bacteria, plants, fish, and mammals has been reported under exposure to various prooxidants (Hayes et al. 2005). *NbGSTU1* and *NbGSTU3* expression resistance to *Colletotrichum destructivum* or *Colletotrichum orbiculare* infections has been developed in *Nicotiana benthamiana* (Dean et al. 2005). In addition, the increased GST activities have been shown to be associated with tolerance of insecticide and herbicide in cereal crops (Edwards and Dixon 2004). An increase in GST activity was observed in *P. sativum* (pea) plant whose leaves and roots were exposed to cadmium (Dixit et al. 2001). Overexpression of *Nt*107 GST connect with cold and salt stress in tobacco and *LeGSTU2* associated with drought and salt stress in tomato improved resistance to abiotic stress in *Arabidopsis* (Roxas et al. 2000; Xu et al. 2015). Oxidative stress scavenging is strongly associated with GSTs (Ahmad et al. 2020), while some factors such as herbicide, plant hormones, heavy metals, GSH, and hydrogen peroxide have been shown to increase GSTs' catalytic activity and substrate affinity (Marrs 1996; Ahmad et al. 2020).

Genome-wide analysis of GSTs has been performed in many plants and the results have been shown. *GST* genes have been reported in various plant species such as *Oryza sativa*, *Arabidopsis*, maize, sugar orange, Japanese black pine, *Capsella rubella*, potato, *Gossypium raimondii*, *Gossypium arboreum*, *Glycine max*, *Malus domestica*, and *Medicago ruthenica* (McGonigle et al. 2000; Sappl et al. 2009; Jain et al. 2010; Licciardello et al. 2014; Yang et al. 2014; Hu et al. 2015; He et al. 2016; Islam et al. 2018; Ahmad et al. 2020; Zhao et al. 2021; Wang et al. 2022).

As a bean plant, *Phaseolus vulgaris* is the only annual herbaceous plant that has edible seeds or whose immature fruits are harvested and used as food, which can be grown in most climate zones worldwide (Gentry 1969). Botanically, it is in the same family as other legumes. As the main category, it can be supplied as dry beans, green beans, and shell beans based on usage. Except for the fruit, the remaining parts are generally used as straw in animal husbandry, and like most members of the *Fabaceae* family, they have a mutualistic relationship with nitrogen-fixing bacteria in their roots and supply the nitrogen they need in this way. Common bean, which is cultivated all over the world, is also valuable in terms of breeding (Luna-Vital et al. 2015). Stress is characterized as any change that affects or interrupts the physiological and metabolic balance of plants in their habitat. *P. vulgaris* crop loss is typically attributed to unfavorable environmental factors like drought and salinity in the soil. One of the most significant abiotic stresses that has a negative impact on plant development, growth, and worldwide agricultural productivity is drought. Drought and salinity are considered as the second most important factors limiting bean yield and quality after diseases (Maas and Hoffman 1977; Shulaeva et al. 2008; Villordo-Pineda et al. 2015). 30 years ago, melatonin was first reported to be able to act as an antioxidant by Tan et al. (1993) and it was also reported that melatonin enhances the effectiveness of other antioxidants in plants (Arnao and Hernández-Ruiz 2006). Under conditions of stress, the amount of endogenous melatonin, a plant's natural antioxidant, increases, which is connected to an increased amount of ROS (Arnao and Hernández-Ruiz 2013; Aygören et al. 2022). Melatonin is known to play an important role in how plants react to stress, according to numerous studies (Arnao and Hernández-Ruiz 2009; Li

et al. 2012; Wang et al. 2012; Shi et al. 2015; Huangfu et al. 2020). Under various abiotic conditions, plants treated with exogenous melatonin have greater plant heights, biomass, and organic matter contents than untreated plants. In plants treated with melatonin, ROS, electrolyte leakage, and cell damage were all decreased (Aygören et al. 2022).

This study aimed to characterize the *GST* genes in common bean and to determine the protective effect of melatonin against stresses such as salt and drought and evaluate the expression levels of *GST* genes in these stress conditions. In this study, a genome-wide comprehensive analysis of GSTs in *P. vulgaris* was performed and a total of 55 candidate members were identified. These genes were found to be distributed within 10 defined subclasses of GSTs and members from various subclasses were found to be abundant in *P. vulgaris*. Additional analyses such as gene positions, biochemical characteristics, conserved motifs, and cellular localization were performed, and *Pv-GST*s were identified. Then, the expression profiles of *Pv-GST*s under salt and drought stress were analyzed and the expression levels of some *Pv-GST* genes were determined by qRT-PCR experiments. Considering the economic and agricultural importance of common bean, the discovery of salt and drought stress-related genes has a serious potential for the future to improve the response to drought and salt stress in common bean, to develop resistant varieties to these abiotic stress factors, and to make advanced molecular biology and genetic studies more applicable.

Material and Method

Detection of *GST* Genes in the *P. vulgaris* Genome

The protein sequences of the *P. vulgaris GST* genes were obtained through the Phytozome v12.1 database (Goodstein et al. 2012). PANTHER Accession Number (PTHR11260) was obtained from both Phytozome and PANTHER database (http://www.pantherdb.org/). To identify all possible GSTs in the bean genome, both the blastp tool in Phytozome v12.1 and the hidden Markov model (HMM) screening have been performed using HMM's default parameters. The molecular weight (mW) and theoretical isoelectric point (pI) of the obtained GST proteins were estimated using the "ProtParam tool" (Wilkins et al. 1999), and this information of the proteins was recorded.

Clarifying the Structure, Physical Location, Gene Duplications, and Conserved Motifs of *GST* Gene Family

Gene Structure Display Server v2.0 was used to obtain information about intron–exon regions of *GST* genes (Guo et al. 2007). Genome sequence information and encoded DNA (CDS) data were used to predict the positions of *GST* genes. Then, using the Phytozome v12.1 database, the positions and sizes of the *GST* genes in the chromosomes were determined.

GST genes were marked on *P. vulgaris* chromosomes and plotted using TBtools and the same program was also used to identify gene duplications (Chen et al. 2020). Pair-to-pair non-synonymous ratios (Ka), synonymous ratios (Ks), and evolutionary strain (Ka/Ks) values of *GST* genes were calculated in the PAL2NAL (Suyama et al. 2006) PAML web interface (Yang 2007).

The "Multiple EM for Motif Elimination (MEME Suit)" tool was used to perform additional conserved motif analysis of GST proteins (Bailey et al. 2006). The limits in the MEME Suit tool for the maximum/minimum width and the maximum number of Motifs are set to 6, 50, and 10, respectively. The region distribution is set to any number of repeats. Identified motifs were scanned in the InterProScan database using default settings (Quevillon et al. 2005).

Sequence Alignments and Phylogenetic Analysis

Phylogenetic analysis was performed using the neighbor-joining (NJ) method (Saitou and Nei 1987) with a 1000-repeat bootstrap value. Sequences of GST proteins were aligned using ClustalW (in MEGA v7) (Thompson et al. 1997). Phylogenetic tree was drawn using MEGA v7 software (Tamura et al. 2011). Then, this phylogenetic tree was shaped and clarified using the Interactive Tree of Life (iTOL) interface (Letunic and Bork 2011).

Synteny Analysis

For synteny analysis, orthologs of *P. vulgaris*, *G. max*, and *A. thaliana GST* genes were obtained from the Phytozome v12.1 database and collinear genes were determined and synteny map was created using Tbtools program (Chen et al. 2020).

Intracellular Localization of *Pv-GST* Genes and Promoter Analysis

Intracellular localization of members of the bean *GST* gene family was estimated using the WoLFPSORT web interface (Horton et al. 2007).

The 2000 bp upstream regions of the *Pv-GST* genes were accessed using the Phytozome v12.1 database, and examinations were performed using the PlantCARE interface in these regions (Lescot et al. 2002). However, a large-scale search was made in the literature and the relationship between the *cis-acting* elements and stresses was scanned. Then, the positions of the *cis-acting* elements in the promoter regions of the genes were visualized with the Tbtools program (Chen et al. 2020).

Predictive Three-Dimensional Protein Structure Analysis

After obtaining the sequence information of the proteins, this sequence information was entered into the "Protein Homology/analogY Recognition Engine V 2.0 (Phyre^2)" database and the search was performed by activating the "intense" mode (Kelley et al. 2015). It has been added to the table by considering the "confidence level" specified in the reports of the proteins whose three-dimensional structures were created (> 95%).

Protein–Protein Interactions and miRNA Analysis of Bean GSTs

The STRING (https://string-db.org/) database was used to determine the physical, functional, and experimental interactions of protein–protein interactions. The results obtained here were transferred to the Cytoscape program and visualized using the database's own Cytoscape transfer tool.

In addition to all these, miRNAs associated with the *Pv-GST* genes were screened using "A Plant Small RNA Target Analysis Server (psRNATarget)" database (Dai et al. 2018).

In Silico Gene Expression Analysis

In order to do this analysis, Illumina RNAseq dataset was obtained from NCBI SRA (Sequence Read Archive) database. For this experiment, SRR957668 (saline stress treated leaf), SRR955869 (saline treatment control) (Hiz et al. 2014), SRR8284481 (drought stress treated leaf), and SRR8284480 (drought treatment control) (Gregorio Jorge et al. 2020) data have been used. The expression profiles of *Pv-GST* genes in leaf tissue of *P. vulgaris* were determined. In silico expression profiles were calculated by creating the log2 transform of RPKM (Reads per Kilobase Million: Transcript per kilobase, normalized form of transcript expression) values, and "heatmap" was obtained with the CIMMiner algorithm (Weinstein et al. 1997).

Plant Material, Stress Treatment, RNA Isolation, cDNA Synthesis, and qRT-PCR Analysis

Two common bean varieties, *P. vulgaris* cv. Serra and *P. vulgaris* cv. Elkoca-05, obtained from the Department of Molecular Biology and Genetics at Erzurum Technical University were used in this study. These two varieties were studied for their resistance to salt and drought stress by Aygören et al. (2022). Surface sterilization of the seeds was performed with a solution containing 1% (v/v) NaOCl for 5 min. Then, for germination, seeds were sown in pots filled with perlite moistened with plant nutrient solution (Hoagland's No. 2 Basal Salt solution) and seedlings were kept in pots in a controlled environmental growth chamber (25ºC, 70% relative humidity, 250 mmol m^{-2} s^{-1} photosynthetic photon flux) until the three-leaf stage of the plants. Plants reaching the same size were selected and transferred to hydroponic medium containing modified Hoagland's solution at a ratio of 1/10 (Hoagland and Arnon 1950). *P. vulgaris* seedlings were grown under the same experimental conditions. Then, at the seventh day of transfer, melatonin (Cat. No: M5250, Sigma-Aldrich, Deisenhofen, Germany) was sprayed to the leaves at a concentration of 0 (control; distilled water) and 200 µM M (melatonin) 24 h before salt and drought stress treatments. Salt stress was carried out with Hoagland's solution (for moderate salt stress) containing 150 mM NaCl for 9 days under the same growth conditions. However, for the drought stress experiment, *P. vulgaris* genotypes grown under the same conditions were kept in Hoagland solution containing 0 (control) and 20% PEG6000 for 24 h. After these periods, the leaf tissues of *P. vulgaris* genotypes were placed in liquid nitrogen and stored at – 80°C until the study was performed. The *P. vulgaris* genotypes used in the study were grown as triplicate, and qRT-PCR analyses were performed as triplicate biological repeats.

Total RNA was isolated using the TRIzol® reagent (Invitrogen, Life Technologies, California) in accordance with the manufacturer's instructions. RNA concentrations and qualities were controlled by Multiskan™ Go Microplate Spectrophotometer (Thermo Scientific, Massachusetts, USA) and run on a 1% agarose gel as a control.

From the RNAs obtained, cDNA synthesis was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Massachusetts, USA) in accordance with the manufacturer's instructions.

Specific primers for *Pv-GST* genes were designed according to RNAseq data, and qRT-PCR experiments were performed with the real-time qPCR system RotorGene-Q (Qiagen, Hilden, Germany) instrument and using Thermo Fisher Fast SYBR® Green Master Mix (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions, 10 min at 95°C followed by 40 cycles at 95°C for 15 s, at 58–63°C for 30 s. Then, melt curve analysis was performed from 65°C to 94°C for 3 s/temperature to evaluate the specificity and quality of PCR amplification. By monitoring the changes in fluorescence intensity as a function of temperature, the analysis provided information about the melting behavior of the amplified cDNA products. *P. vulgaris β-actin* gene was used as a housekeeping gene. qRT-PCR data were normalized according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Statistical analyses were performed by GraphPad Prism 9 program (http://www.graphpad.com/) with one-way ANOVA method and Dunnett's test was considered at 0.05 significance level.

Results

Identification and Characterization of *Pv-GST* Genes

To understand the presence and potential role of *GST* genes in *P. vulgaris*, chromosome analysis was conducted to identify and characterize these genes. The purpose of this analysis was to elucidate the *GST* gene distribution in *P. vulgaris* and provide a basis for further investigations into their expression patterns and functional significance, particularly in response to various stress conditions. Keysearch was performed on the genome of the *P. vulgaris* in the Phytozome database. As a result of these searches, 55 genes with homology to GSTs were identified. Confirmation was made using the HMMER web interface after both the presence of GST domains and the removal of unrelated sequences from genes with putative GST domains. The chromosomal locations, amino acid numbers, molecular weights, and isoelectric points of 55 candidate *GST* genes found in the *P. vulgaris* genome were listed in Supplementary Table 1.

The distribution of *GST* genes in bean chromosomes was shown according to the families in which GSTs were found. Segmental duplications between some genes were illustrated with red lines in Fig. 1.

All chromosomes in the *P. vulgaris* have *GST* genes. The most genes (9) were found on chromosome 7 and chromosomes 4 and 11 contain only one gene (Fig. 1). Pv-GST proteins range in size from 132 to 420 amino acids, and among these proteins, Pv-EF1BG-1, with a length of 420 amino acids, was found to have the longest amino acid chain. Pv-GSTU-24, with a length of 132 amino acids, has the shortest amino acid chain. It was found that Pv-GSTL-2 had the lowest isoelectric point and Pv-GSTT-2 had the highest isoelectric point out of the various theoretical isoelectric values of Pv-GST proteins, which ranged from 5.03 to 9.61. The molecular weights of Pv-GST proteins were found to range from 15.028 kDa (Pv-EF1BG-2) to 47.990 kDa (Pv-GSTU-24).

As a result of gene duplication analysis, *Pv-GSTL-1*/*Pv-GSTL3*, *Pv-GSTU-1*/*Pv-GSTU-4*, *Pv-GSTU-6*/*Pv-GSTU26*, and *Pv-TCHQD-1*/*Pv-TCHQD-2* have been determined as segmentally duplicated genes. The Ka and Ks values and Ka/Ks ratio (dN/dS) among these genes and the divergence time of these genes are given in Table 1. The formula used to calculate the divergence time was $T = Ks/2\lambda$ ($\lambda = 6.56 \times 10^{-9}$) and value was given as MYA (million years ago). If the Ka/Ks ratio was greater than 1, it indicates positive selection during the evolution of the gene, if it was less than 1, it was purifying selection, and if it was equal to 1, it indicates natural selection (Juretic et al. 2005; İlhan et al. 2023). The data obtained from the study show that there was a high degree of purifying selection throughout the evolution of the *P. vulgaris* plant, due to the Ka/Ks ratio of the segmental duplicate genes in *P. vulgaris* being less than 1.

Phylogenetic Analysis, Conserved Motifs, and Gene Structures of *Pv-GST*s

Performing a phylogenetic analysis, studying conserved motifs, and examining the gene structures of *Pv-GST*s can provide insight into the evolutionary relationships and potential functional properties of these genes. A phylogenetic tree was created using the GSTs of the plants *P. vulgaris*, *A. thaliana*, and *G. max* to show the interconnections between Pv-GST proteins. Using the MEGA7 (Molecular Evolutionary Genetic Analysis) program and the neighbor-joining method, a phylogenetic tree was constructed using the amino acid sequences of the GST proteins (Fig. 2). Furthermore, the classification of GST proteins into ten groups based on their origin allows the identification of different clusters and subfamilies in *Pv-GST* distributions. Tau and phi were the classes with the most members, respectively. While *A. thaliana* and *G. max* had no members of MGST class proteins, which are evolutionarily different from other GSTs, *G. max* had no members of GHR class and *A. thaliana* had no members of EF1Bγ class. Subclasses containing a serine residue in their active site (tau, phi, theta, and zeta, respectively) were found to contain the most members of GSTs. This analysis enhances our understanding of the evolutionary relationships among GST proteins and offers insights into their potential functional roles and diversification across plant species.

Evolutionarily conserved motif analysis of Pv-GST proteins revealed the presence of 10 highly conserved motifs (Fig. 3). The identified motifs were given in Supplementary Table 2. Motif number 10, which was the longest motif, was only found in *Pv-GSTL* genes, and this motif contains glutathione S-transferase N terminal domain. In addition, motif 1 and motif 3 also contain N terminal domain. Six *Pv-GST* genes were found to not contain motif 1, and eleven *Pv-GST* genes were found to not contain motif 3. All *Pv-GSTU* genes except *Pv-GSTU-24* and all *Pv-GSTL* genes except *Pv-GSTL-4* were found to contain motif 2 which contains the glutathione S-transferase C terminal domain. Surprisingly, motif 4 was not found to contain any domain, but all *Pv-GST* genes contain this motif and even the *Pv-GHR-1* gene contains only this motif. This may indicate that motif 4 may be highly related to the GST superfamily.

Exons and introns were determined using Gene Structure Display Server and *Pv-GST* genes, and the schematic representation of the *Pv-GST* genes was given in Fig. 4. It has been determined that the number of exons in *Pv-GST* genes varies between 2 and 10, and the number of introns varies between 1 and 9. It was determined that the genes containing the most exons were *Pv-GSTL-2*, *Pv-GSTL-3*, *Pv-GSTL-4*, and *Pv-GSTZ-2*. This analysis may offer substantial knowledge into the underlying genomic architecture and the molecular mechanisms which regulate the function of genes.

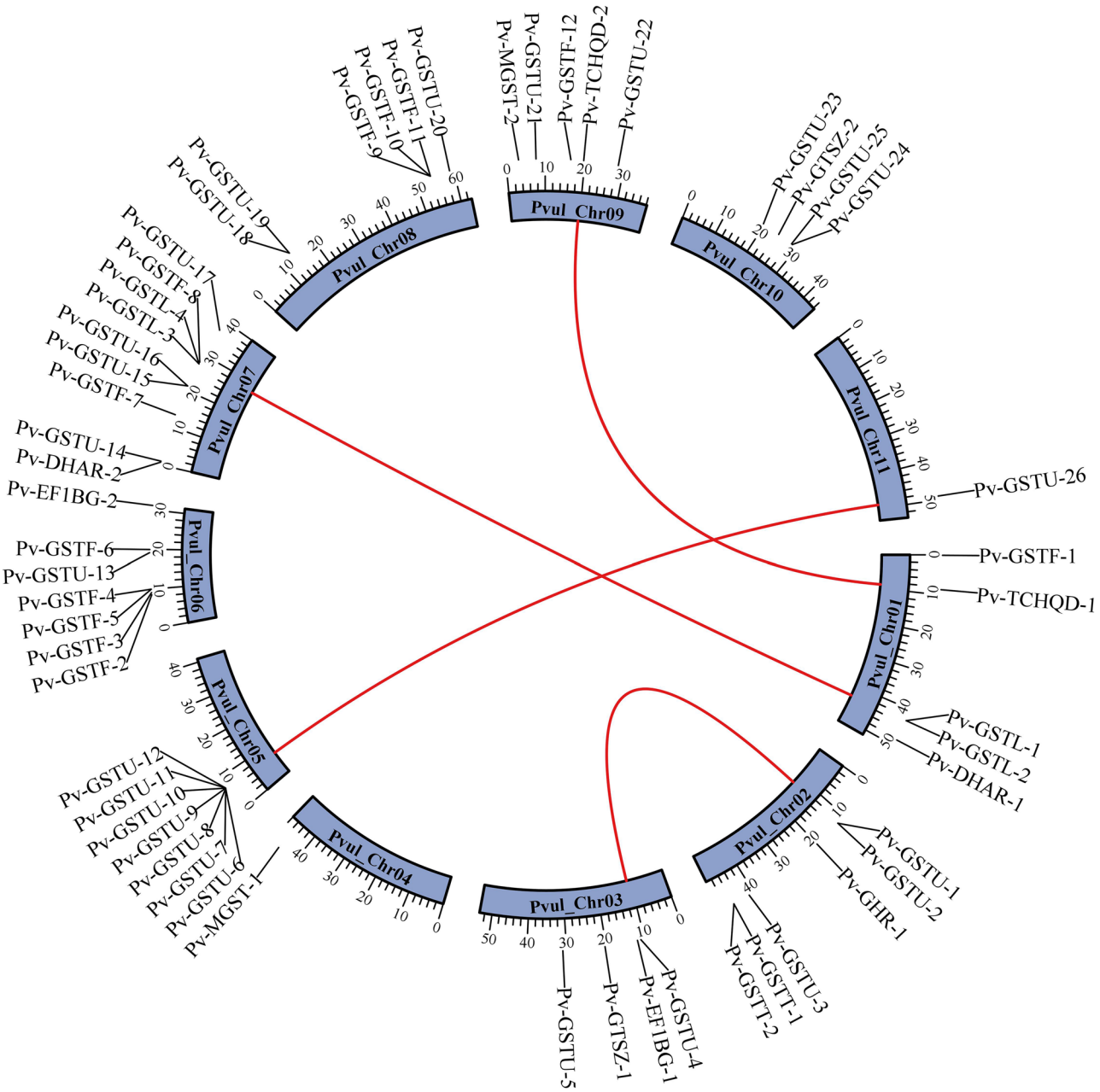

Fig. 1 Putative *Pv-GST* genes' distribution on *P. vulgaris* chromosomes. Chromosomes (named: Pvul_Chr) and positions of genes were represented. Red lines indicate duplicated genes

Table 1 Ka, Ks, and Ka/Ks values and divergence times of segmental duplicate genes of *Pv-GST*s

Gene 1	Gene 2	Ka	Ks	Ka/Ks	MYA
Pv-GSTL-1	Pv-GSTL-3	0.1837	0.6613	0.2499	50.404
Pv-GSTU-1	Pv-GSTU-4	0.366	1.4581	0.1804	111.136
Pv-GSTU-6	Pv-GSTU-26	0.2963	1.2019	0.1993	91.6082
Pv-TCHQD-1	Pv-TCHQD-2	0.0993	0.5325	0.1621	45.5869

Comparative Mapping of the Pv-GST Gene Family with *G. max* and *A. thaliana*

To identify orthologous genes and investigate the conservation and divergence of *GST* gene families across many plant species, comparative mapping of the *Pv-GST* gene family with *G. max* and *A. thaliana* was carried out. The correlation makes it possible to compare and contrast how the three

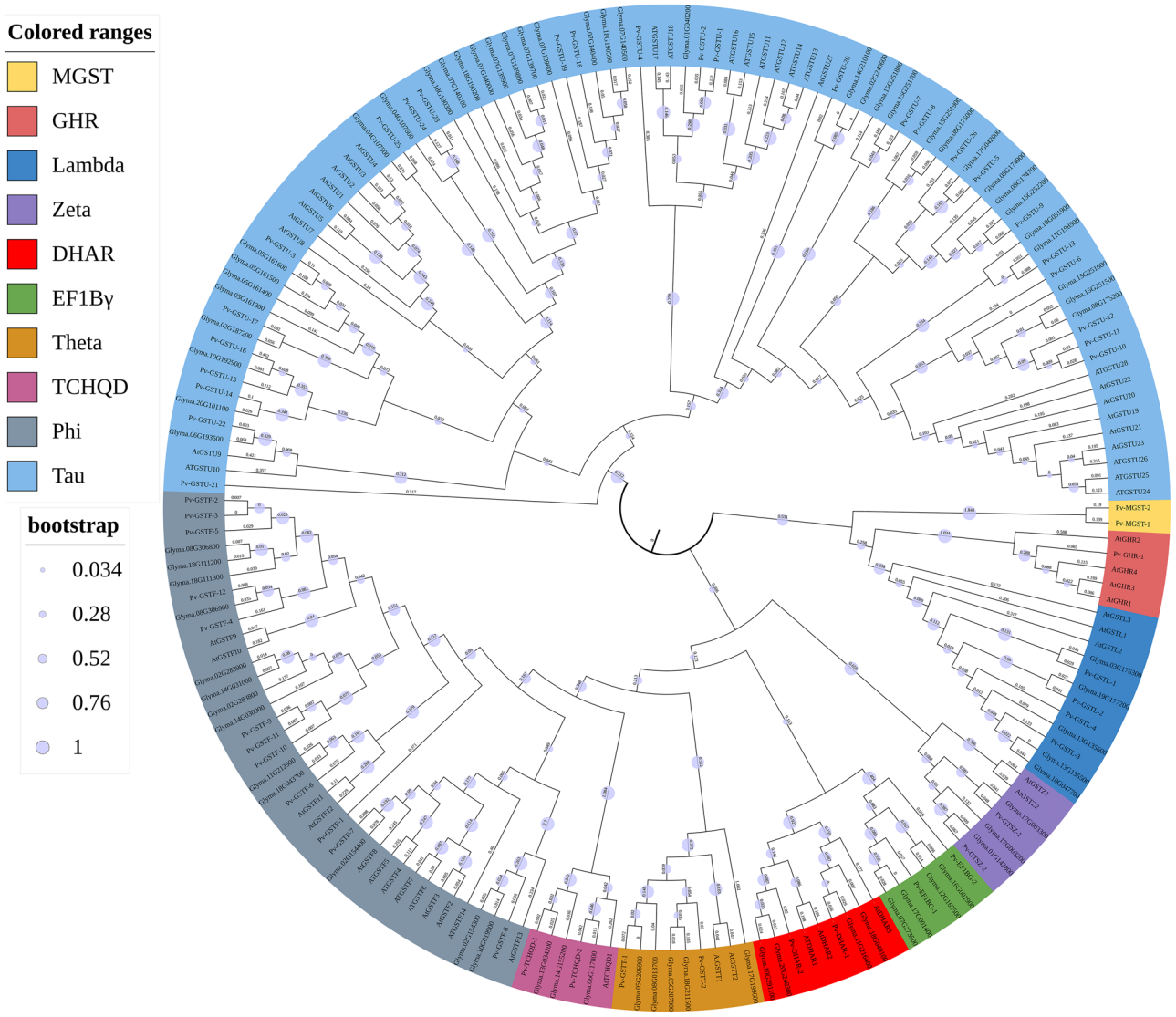

Fig. 2 Phylogenetic tree plotted with GST proteins of *A. thaliana*, *G. max*, and *P. vulgaris* plants. Colors indicate the subclasses of GSTs (names were given in the image); circles indicate bootstrap values

plant species' presence and arrangement of *GST* genes differ. The evolutionary links and conservation of *GST* genes across different plant species were thought to be clarified by this comparative mapping.

Studies were carried out on the genomes of *P. vulgaris*, *G. max*, and *A. thaliana* obtained using the Phytozome v12.1 database, and the genes that were found to show orthology were correlated in the light of the obtained data and illustration is given in Fig. 5. This visual representation aids in understanding the patterns of orthologous genes and their distribution within the genomes of the three plant species, facilitating further comparative genomic analyses and offering insights into the evolutionary dynamics of the *GST* gene family.

Promoter Analysis and Subcellular Location of *Pv-GST* Genes

In order to learn about the regulatory mechanisms and potential roles of *Pv-GST* genes in response to stress, the 2000 bp upstream region of each gene was scanned and promoter analysis was performed in these regions, and the findings were given in Fig. 6. Literature reviews revealed that ABA-dependent and ABA-independent pathways were associated with dehydration stress in plants. Notably, the ABA-responsive element (ABRE) and the dehydration-responsive element (DRE) were highlighted in the literature as being directly related to dehydration stress (Zhu 2002; Shinozaki et al. 2003). The gene containing the most abundant ABRE

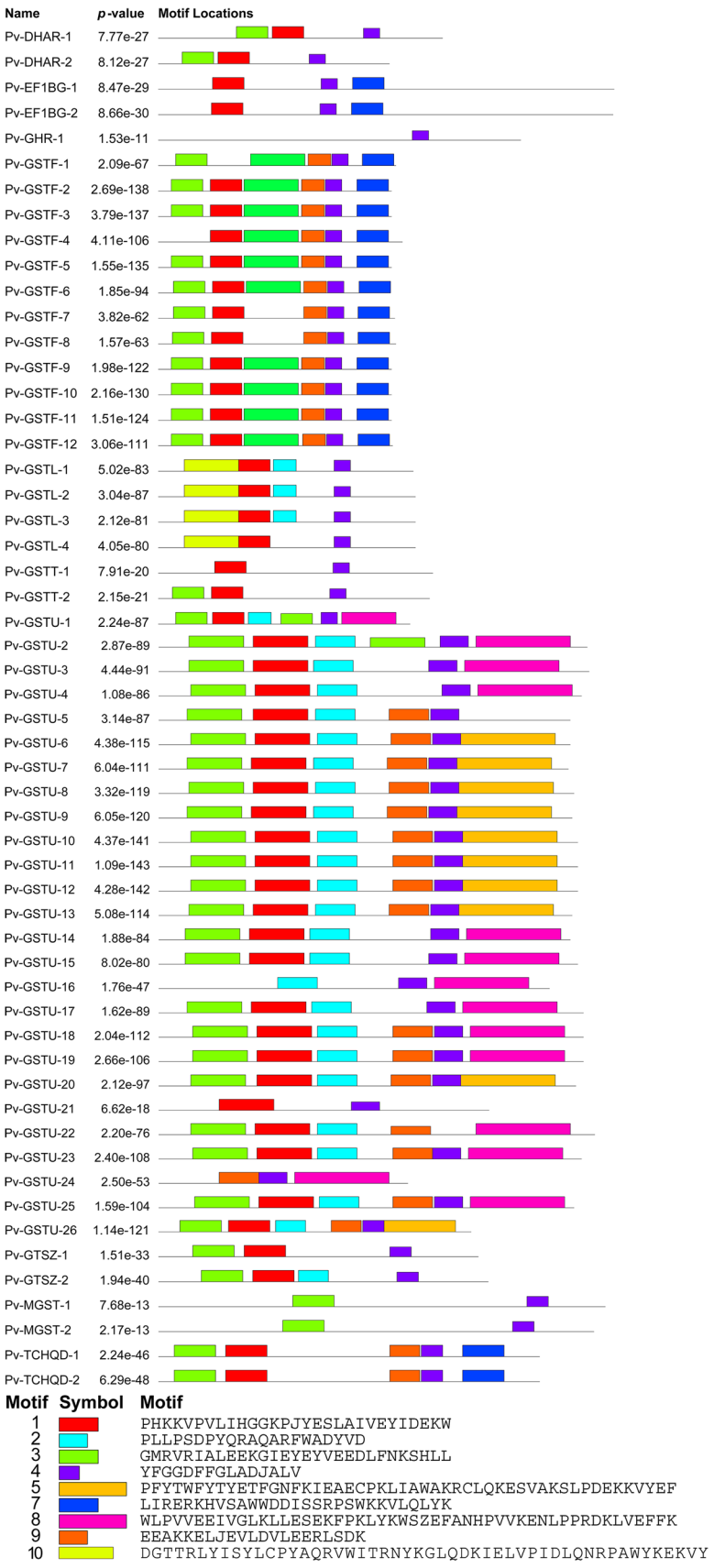

Fig. 3 Figurative representation of possible motifs found in *Pv-GST* genes. The 10 different motifs were shown in different colors. The colored boxes indicated that the genes contain that motif in their alignment and the lengths of the motifs were also schematized

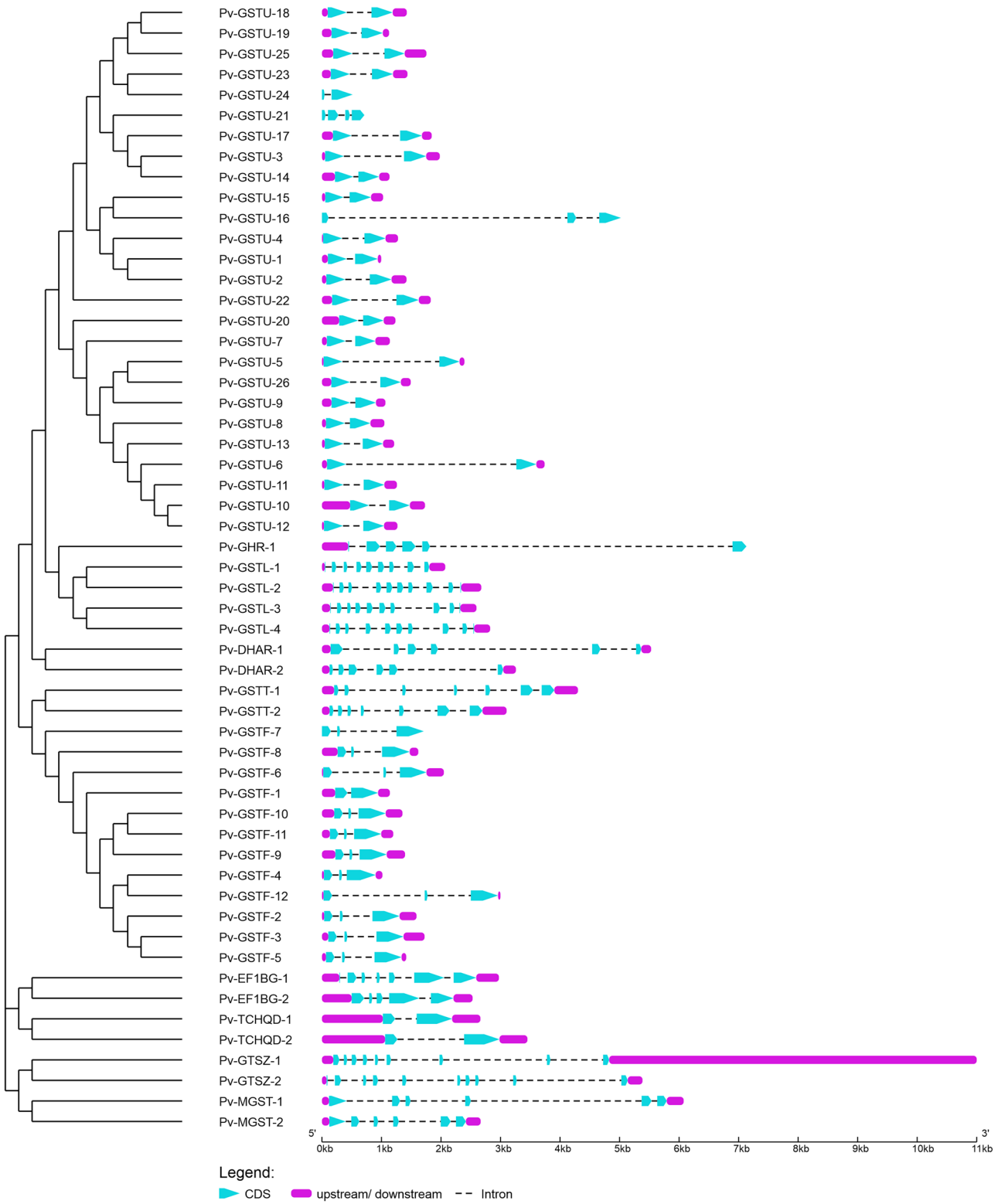

Fig. 4 Representation of exons and introns of *Pv-GST* genes. The phylogenetic tree was generated with MEGA7 program in Newick notation tree format and used as a guide for sorting *Pv-GST* genes in this illustration

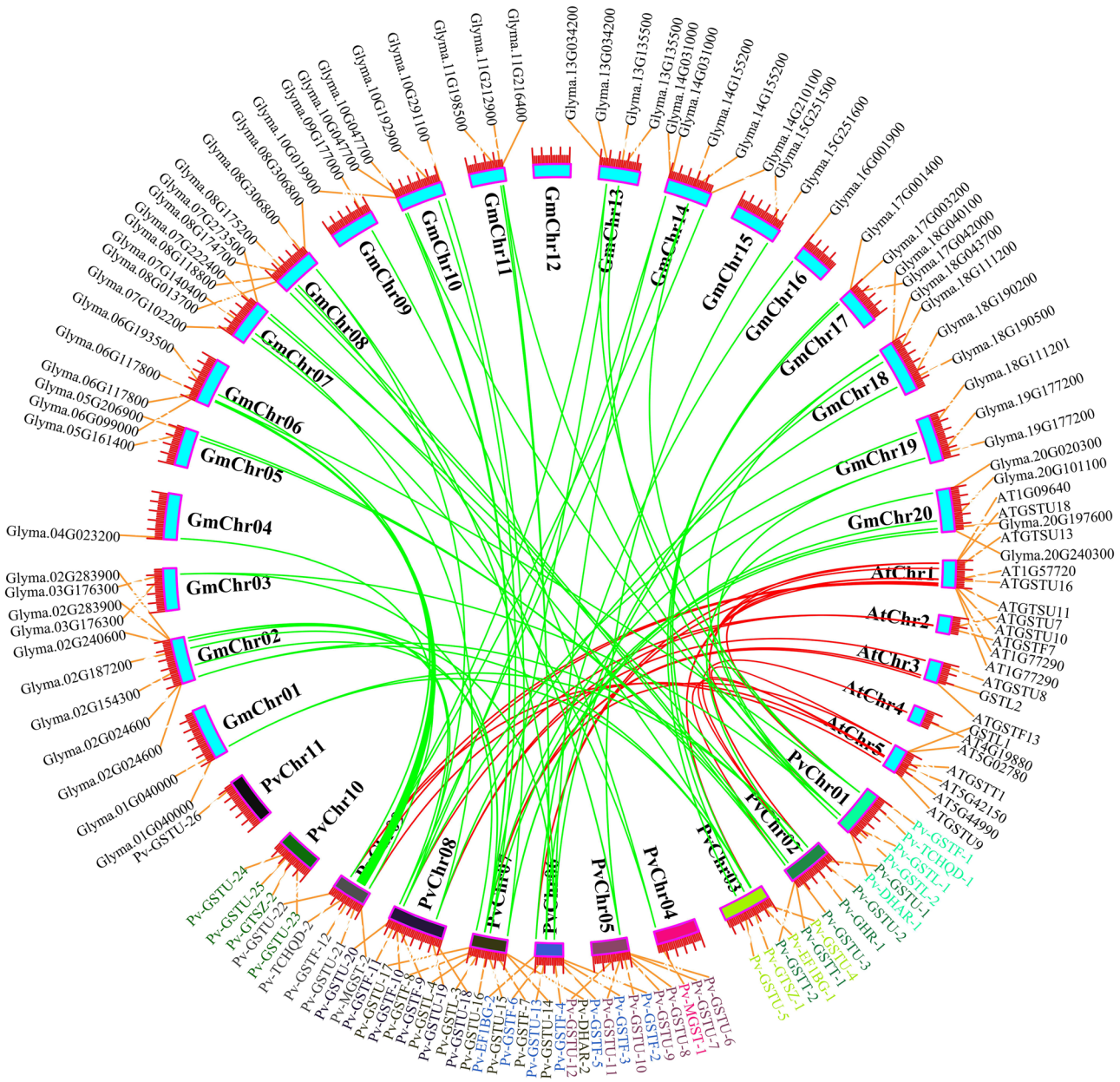

Fig. 5 Comparative mapping of the *P. vulgaris*, *G. max*, and *A. thaliana GST* genes (green lines show the relationship between *G. max-P. vulgaris* genomes, red lines show the relationship between *A. thaliana-P. vulgaris* genomes; some gene names were colored according to their chromosome color)

element was found to be *Pv-GSTU-18* (10) and three genes containing DRE-core (*Pv-EF1BG-1*, *Pv-GSTF-2*, and *Pv-GSTU-13*) and two genes containing DRE1 (*Pv-GSTU-2* and *Pv-GSTU-3*) were identified. The *cis*-element Myb-binding site, which is a response to various biotic and abiotic stresses, was found to be present in the promoter region of 22 *Pv-GST* genes and the gene containing this *cis*-element the most was found to be *Pv-GSTU-4*.

The subcellular locations of the *GST* gene products obtained as a result of the scans were shown in Supplementary Table 3. Although it was possible to encounter the *GST* gene family at every intracellular level in plant tissues in general, based on the information obtained in this study, it was concluded that all *Pv-GST* gene products were soluble GSTs. Except for the *Pv-DHAR-1*, *Pv-GSTF-1*, *Pv-MGST-1*, and *Pv-MGST-2* genes, the products of all 51 other genes were predicted to be localized in the cytoplasm, with the *Pv-DHAR-1* gene product concentrated in the chloroplast and *MGST* genes products in the mitochondria and *Pv-GSTU-4*'s product in the nucleus. It was also predicted that the majority of *Pv-GSTU*

Fig. 6 Figure showing *cis-acting* elements found in the 2000 bp upstream regions of the *Pv-GST* genes

gene products could be found simultaneously in the cytoplasm, chloroplast, and mitochondria. The Pv-GSTT-1 and Pv-GSTT-2 proteins were not localized in mitochondria and chloroplasts but were concentrated in peroxisomes.

3D Homology Modeling of Pv-GST Proteins

Blastp scanning was performed with the GST proteins in the Protein Data Bank (PDB), and then, the amino acid sequences of these proteins were converted into clear 3D visuals. Figure 7 displays images of the proteins identified in this investigation. Based on the information provided by 3D homology modeling, it was determined that all Pv-GST proteins contain a quadruple helix structure that provides substrate specificity. Based on the 3D models, Pv-GSTF members were very similar to each other, while Pv-GSTU members share the same domains except Pv-GSTU-24. The similarity of the quaternary structure formed by the alpha helix structures of Pv-GSTU members, especially the alpha helix structures shown in green–red-yellow-orange colors, was seen and this can be suggested as evidence that the tau class is different from other GST classes. These illustrations offer useful data on the structural layout and potential functional sites of Pv-GST proteins. To predict proteins' catalytic activity, ligand binding sites, and interactions with other molecules, it is helpful to understand the spatial organization of key domains and residues within proteins.

Our comprehension of the structural characteristics of Pv-GST proteins was improved by 3D homology modeling, which also aids in the examination of their potential functional significance in *P. vulgaris*. It serves as a basis

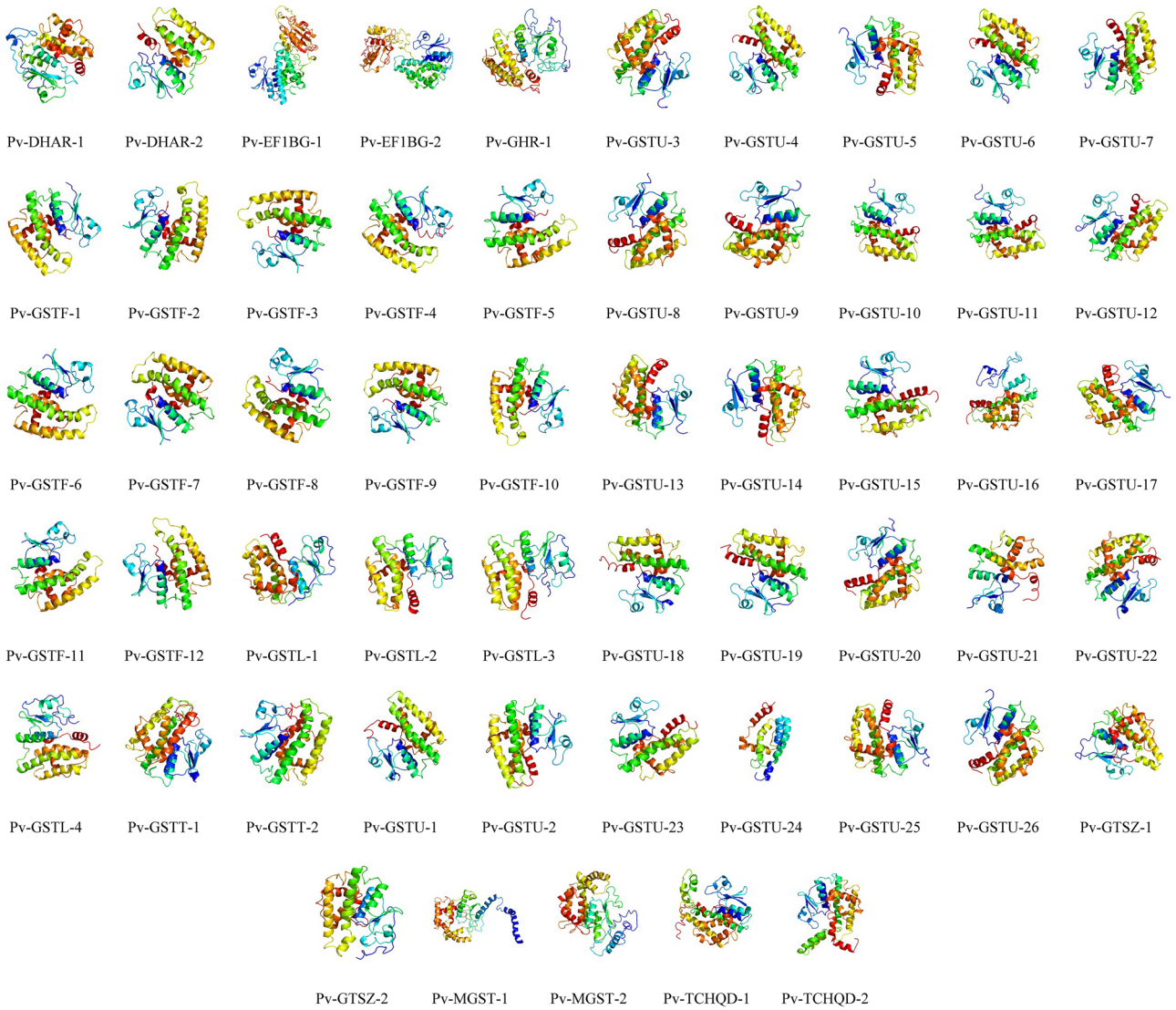

Fig. 7 3D structure modeling of Pv-GST proteins

for further research into the molecular and biochemical characteristics of these proteins and their functions in diverse physiological processes.

Protein–Protein Interactions of Pv-GST Proteins

The obtained protein sequence information was entered into the STRING database, and a visual diagram of protein interactions was created. In the protein–protein interactions given in Fig. 8, it was determined that the proteins that interacted most with other proteins were Pv-EF1BG-1, Pv-EF1BG-2, Pv-DHAR-1, and Pv-DHAR-2; this indicates their potential roles in forming complex networks and participating in diverse molecular interactions within the cellular context. Pv-MGST-1 was found to interact only with Pv-MGST-2, and this interaction may represent functional cooperation or regulation between two members of the Pv-MGST subfamily.

By examining the predicted miRNA targets, it was possible to gain insights into the regulatory interactions between miRNAs and *GST* genes. miRNAs were small RNA molecules that can bind to the messenger RNA (mRNA) transcripts of

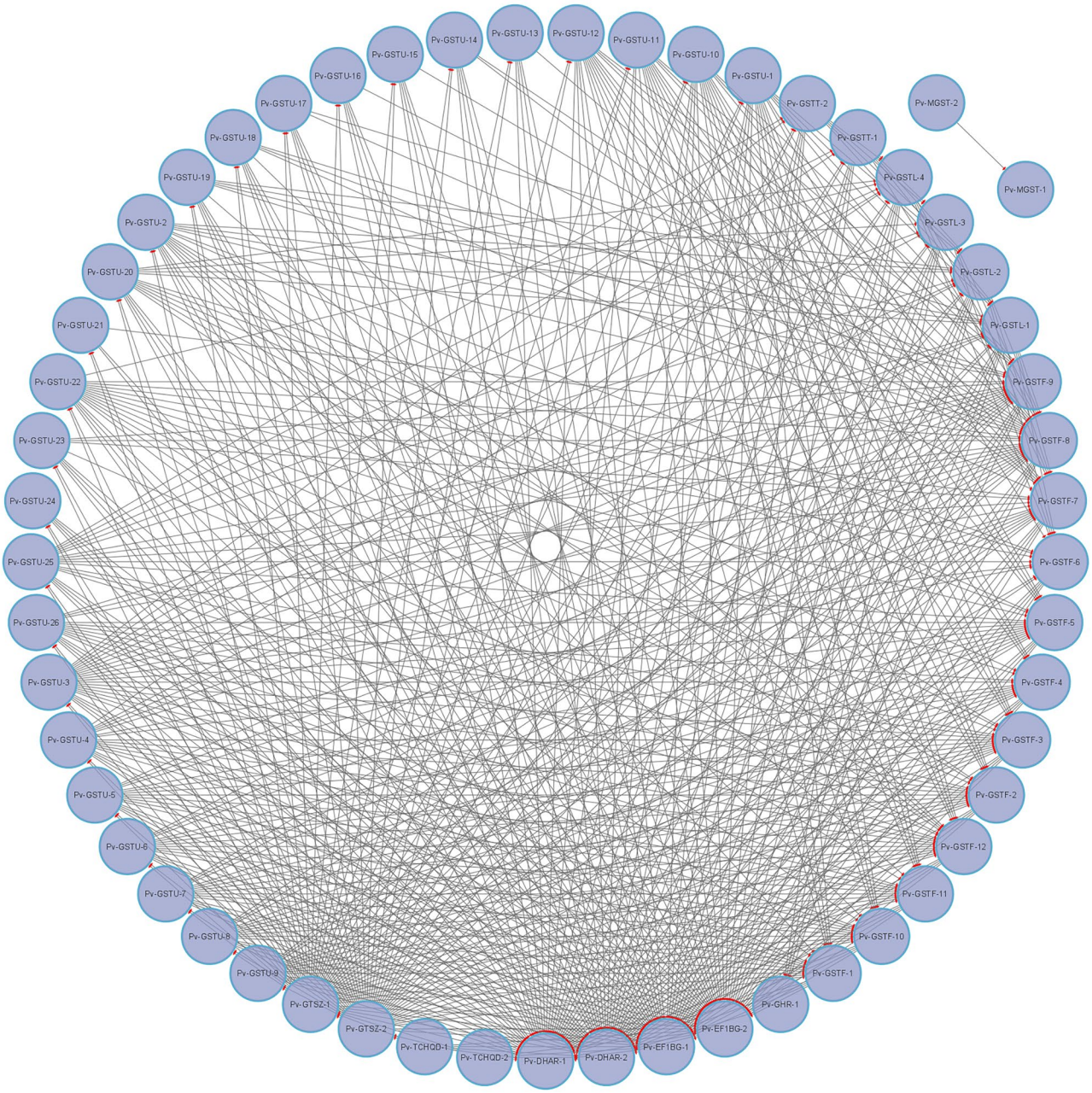

Fig. 8 Protein–protein interactions (PPI) of *Pv-GST* gene products. The red region shows how much that protein interacts with other Pv-GST proteins

target genes, resulting in posttranscriptional gene regulation, including mRNA degradation or translational repression (Bartel 2004). The identification of miRNAs associated with *GST* genes suggests potential regulatory mechanisms involved in modulating *GST* expression and function. miRNAs thought to be associated with GSTs were obtained from the psRNATarget database, and results were given in Supplementary Table 4. miR159a.1 targets 3 genes including *Pv-GSTU-18*, *Pv-GSTZ-1*, and *Pv-GSTZ-2*, while miR319c targets 4 genes including *Pv-GSTF-3*, *Pv-GSTF-5*, *Pv-GSTU-18*, and *Pv-GSTZ-2*. In addition, *Pv-GSTU-18* and *Pv-GSTZ-2* genes were also targeted by miR159a.1 and miR319. The *Pv-GSTF-8* gene was found to be targeted by miR2119.

In Silico Gene Expression Analysis

Determining the expression levels of genes in silico has the potential to identify molecular approaches to combat possible stress conditions. Specialized tissue libraries obtained from leaf samples of *P. vulgaris* plants treated with drought and salt stress were used (Fig. 9).

The gene expression analysis revealed interesting patterns of gene regulation in response to drought and salt stress conditions. *Pv-DHAR-1* exhibited upregulation in both drought and salt stress conditions, suggesting its potential involvement in stress response pathways. Similarly, *Pv-DHAR-2* showed upregulation under drought stress but downregulation under salt stress, indicating its differential response to these two stressors. The expression level of the *Pv-GSTU-2* gene was observed to increase under the two stress conditions, which may indicate a potential for this gene in response to both stress conditions. In a similar but opposite pattern, the expression levels of *Pv-GSTU-9* and *Pv-GSTU-22* genes were found to decrease under the two stress conditions.

Different conditions were observed to affect the expression levels of some genes, e.g., while the expression levels of *Pv-GSTU-23* and *Pv-GSTU-24* genes increased under drought stress, the expression levels of *Pv-GSTL-2*, *Pv-GSTL-3*, and *Pv-GSTL-4* genes increased under salt stress, and the changes of these genes under other stress conditions were not so significant. Similarly, the expression levels of *Pv-GSTF-10* and *Pv-GSTU-13* genes decreased under drought stress, and *Pv-GSTF-3*, *Pv-GSTU-22*, and *Pv-GSTU-25* genes decreased under salt stress. The responses of these different genes to different stress conditions indicate the stress conditions that the genes were associated with and may have the potential to be the subject of detailed and further research on the relationships of these genes. The expression levels of *Pv-GSTU-14* and *Pv-GSTU-26* genes decreased under drought stress while their expression levels increased under salt stress, suggesting that these genes may play a role in the response to drought stress. In addition, the expression levels of *Pv-GSTF-2* and *Pv-GSTF-12* genes did not change significantly under both stress conditions, suggesting that these genes may be involved in stress conditions other than drought and salt stress conditions. Although the expression levels of *Pv-EF1BG* genes were decreased with very small changes under both stress conditions, the expression levels of these genes were discovered consistently high in silico. This gene was a member of the GST superfamily encoding the gamma subunit of the eukaryotic translation elongation factor 1B (Sheehan et al. 2001). Due to the fact that *Pv-EF1BG* was a gene encoding a product associated with translational mechanisms, it was likely that this gene has a constant high level of expression under all conditions, considering the ongoing process of translational mechanisms. For further investigation, qRT-PCR assay of this gene was performed in this study and its expression level was analyzed.

Quantitative Real-Time PCR (qRT-PCR) Analysis

RNAseq data and CDS data were used to design primers to perform qRT-PCR. To determine the expression levels of *Pv-GST* genes, qRT-PCR was performed with leaf tissues from two bean cultivars (Elkoca-05 and Serra), and the expression levels of *Pv-EF1BG-2*, *Pv-GSTU-2*, *Pv-GSTU-3*, *Pv-GSTU-14*, and *Pv-GSTU-26* genes were investigated and given in Figs. 10 and 11 (primer sequences of these genes given in Supplementary Table 5). The expression changes of these genes under drought stress (by 20% PEG6000), salt stress (by 150 mM NaCl), and melatonin treatments (200 µM) were evaluated. In drought stress, only *Pv-GSTU-2* of the 5 *Pv-GST* genes in Elkoca-05 showed a significant decrease in expression level, while no change was observed in the other genes. In melatonin treatment, a significant increase was detected in the expression levels of all genes except *Pv-GSTU-14*. When both treatments were combined, the expression levels of *Pv-EF1BG-2*, *Pv-GSTU-14*, and *Pv-GSTU-26* increased. In Serra, a significant decrease in the expression levels of other *Pv-GST* genes except *Pv-GSTU-26* was determined under drought stress. When melatonin was applied alone, a significant decrease was observed in the expression levels of *Pv-EF1BG-2*, *Pv-GSTU-2*, and *Pv-GSTU-3* genes, while an increase was observed in other genes. When it was applied with drought, there was a significant decrease in the expression levels of *Pv-GSTU-2* and *Pv-GSTU-3* genes, while no change was detected in other genes. Under salt stress, a significant decrease was observed in the expression levels of *Pv-GST* genes except *Pv-GSTU-2* in Elkoca-05. When only melatonin was applied, a significant decrease was observed in the expression levels of *Pv-EF1BG-2* and *Pv-GSTU-14*, while a significant increase was detected in the expression levels of other genes. When melatonin was applied together

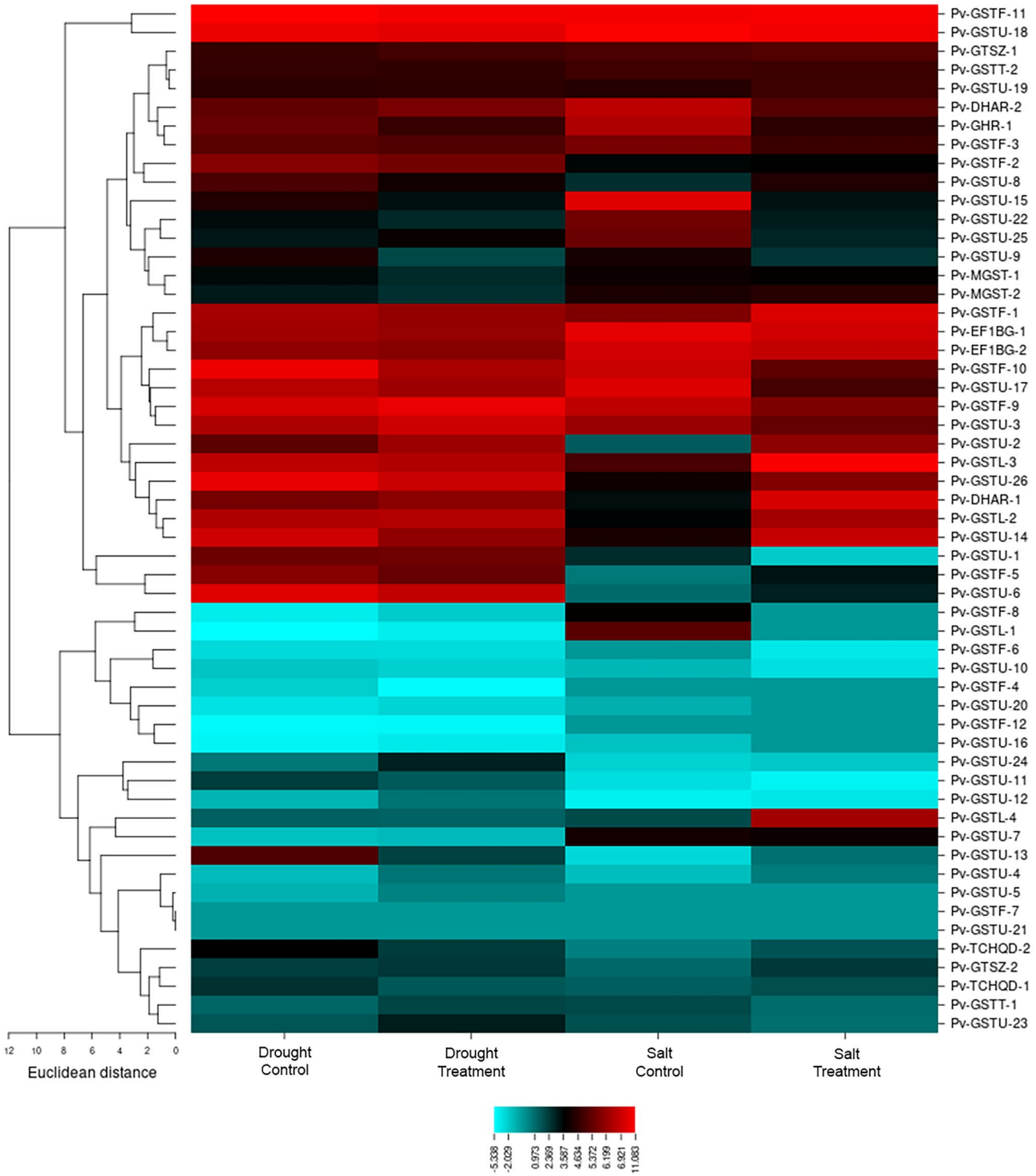

Fig. 9 Expression analysis (heatmap) in leaf tissues of *P. vulgaris* plant under different stress conditions. The colors show the expression levels of the genes. The expression level increases from blue to red. Black or near black colors indicate that the expression level does not change significantly. The stronger the difference in expansion between the two colors, the more the expression level of the gene changes

with salt, there was a significant increase in the expression levels of *Pv-EF1BG-2*, *Pv-GSTU-3*, and *Pv-GSTU-14* genes, while there was a decrease in the others. In Serra, salt stress caused a decrease in the expression levels of *Pv-EF1BG-2*, *Pv-GSTU-2*, and *Pv-GSTU-26*. Melatonin treatment caused a significant decrease in the expression levels of *Pv-GSTU-2*, *Pv-GSTU-3*, and *Pv-GSTU-26*. Both salt and melatonin treatment significantly increased

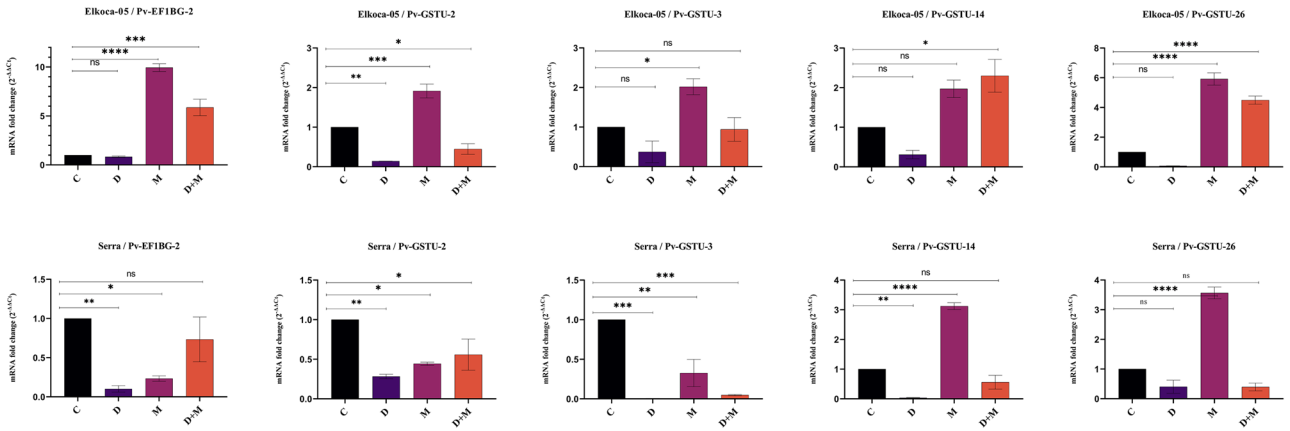

Fig. 10 Expression levels of 5 *Pv-GST* genes in leaf tissue of Elkoca-05 and Serra cultivars exposed to drought stress by 20% PEG6000 application (expression levels were calculated using the $2.^{-\Delta\Delta Ct}$ method; bars: mean + standard error ($n = 3$) and C: control, D: drought, M: melatonin, D + M: drought and melatonin treatment). Significant differences between control and treatments were determined by Dunnett test (*$p < 0.05$, **$p < 0.01$, and ns: non-significant)

the expression levels of *Pv-GSTU-2* and *Pv-GSTU-26*, decreased the expression levels of *Pv-GSTU-3* and *Pv-GSTU-14*, but did not cause any change in the expression level of *Pv-EF1BG-2*.

Discussion

Beans are one of the most frequently produced and consumed crops in the world, and as a result, they are very important economically. The glutathione S-transferase (*GST*) gene family is a large and diverse family of genes in plants that code for proteins essential for the detoxification of xenobiotics and endogenous compounds (Sharma et al. 2014). As they help in protecting plants against environmental stresses like drought, salinity, pollution, herbicides, and pathogens, GSTs are crucial for plant health, development, and survival (Frova 2003; Ahmad et al. 2020). In this study, *GST* genes were examined in silico since they struggle with many stress factors and are especially effective in growth and development by sessile organisms such as plants (Hao et al. 2021), and some genes that are thought to be important in in silico expression profiles were selected and their expression levels were evaluated by the qRT-PCR method.

According to the data obtained in this study, it was determined that there are 55 putative *GST* genes in common bean and these genes are distributed to all chromosomes. *GST* genes have also been identified in *Oryza sativa*, *Arabidopsis thaliana*, *Zea mays*, sugar orange, Japanese black pine,

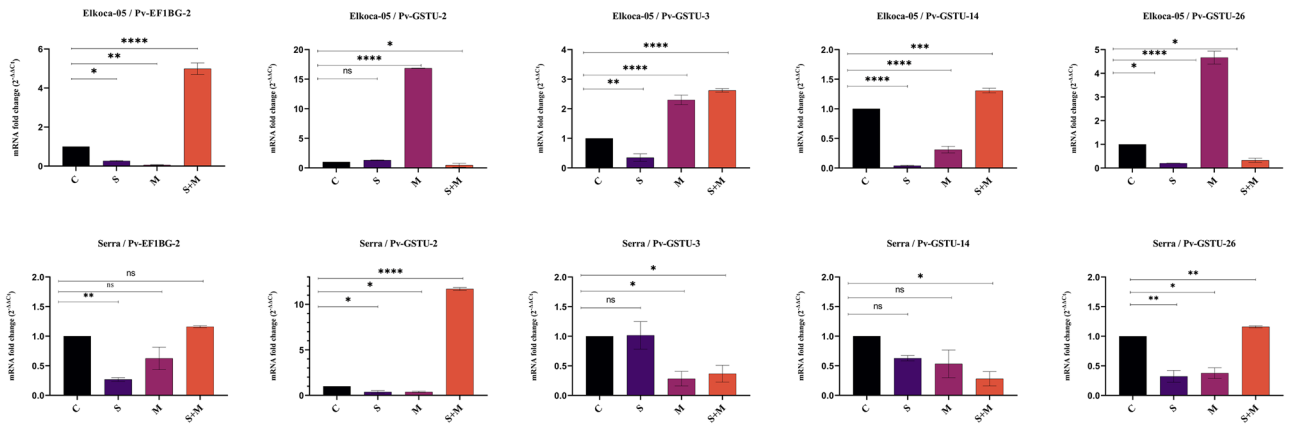

Fig. 11 Expression levels of 5 *Pv-GST* genes in leaf tissue of Elkoca-05 and Serra cultivars taken from a plant exposed to salt stress with 150 mM NaCl application (expression levels were calculated using the $2.^{-\Delta\Delta Ct}$ method; bars: mean + standard error ($n = 3$) and C: control, S: salt, M: melatonin, S + M: salt and melatonin treatment). Significant differences between control and treatments were determined by Dunnett test (*$p < 0.05$, **$p < 0.01$, and ns: non-significant)

Capsella rubella, *Solanum tuberosum*, *Gossypium raimondii*, *Gossypium arboreum*, *Glycine max*, *Malus domestica*, and *Medicago ruthenica*, 79, 55, 42, 23, 27, 49, 90, 59, 49, 74, 8, and 66, respectively. *Pv-GST* genes are divided as 10 subclasses and most of the *Pv-GST* genes are in tau (26 genes) and phi (12 genes) subclasses. According to Dixon et al. (2002), *GST* genes in organisms are mostly found in the tau and phi classes. The products of *GST* genes were predicted to be most abundant in the cytosol, followed by the chloroplast and mitochondria; in addition, the data presented in the study by Edwards et al. (2000) on the intracellular location of GSTs were in agreement. This may be evidence that *GST* genes may play a role in the defense against oxidative stress and cellular detoxification in plants. Moreover, it can be said that *Pv-GST* genes are highly conserved according to their structures, conserved motifs, and Ka/Ks values. The gene family expansions have occurred mostly as a result of various events of gene duplications such as tandem and segmental duplications. These events are important in plants because they can lead to the evolution of new genes and gene functions (Cannon et al. 2004). The expansion of the plant *GST* gene family is mainly caused by the expansion of the tau and phi subclasses (Islam et al. 2018). In *Pv-GST* genes, 4 tau subclass members, 2 lambda, and *TCHQD* genes were found to be segmental duplicated genes. When the phylogeny and synteny analysis were examined, many *Pv-GST* genes had one-to-one homologous matches to the *A. thaliana* and *G. max* genomes. Based on these analyses, it is hypothesized that GSTs may be involved in *P. vulgaris* growth and development, numerous essential processes, and the reaction to numerous stressors. 3D modeling of Pv-MGST proteins shows their differences from other GST proteins, containing specific compositions of alpha helixes shown in green–red-yellow-orange colors. Protein–protein interactions show that Pv-MGST proteins interact with each other, and the remaining Pv-GST proteins interact with other Pv-GST proteins. Microsomal GSTs (MGSTs) are evolutionarily distinct from other GSTs (Frova 2003; Islam et al. 2018). In addition, the fact that the in silico expression levels of *Pv-EF1BG* and *Pv-DHAR* genes are high in the control groups and that they are the most interacting proteins in protein–protein interactions are findings that support each other.

In this study, the expression levels of *Pv-EF1BG*, *Pv-GSTU-2*, *Pv-GSTU-3*, *Pv-GSTU-14*, and *Pv-GSTU-26* genes were examined under salt and drought stress and the results are given in graphics. According to these results, as well as concordance with the transcriptome data from time to time, discrepancies were also observed. As a result of qRT-PCR analysis, a decrease in the expression levels of genes was observed under drought stress, a decrease under salt stress, an increase only under melatonin treatment, an increase under salt and melatonin treatment, and both an increase and a decrease under drought and melatonin treatment. Arnao and Hernández-Ruiz (2006) reported that melatonin enhances the effects of many other antioxidants in plants and that melatonin has healing effects. By transferring *GsGST* from *Glycine soja* to tobacco, increased salinity and drought stress resistance in tobacco was found in a different study (Ji et al. 2010). Similar to this, Yang et al. (2019) showed that three orthologous GSTs from the plants *Populus trichocarpa*, *Populus yatungensis*, and *Populus euphratica* improved *Arabidopsis*' tolerance to salt and drought stress. Another study by Srivastava et al. (2019) found that overexpression of a rice tau-class *GST* gene (*OsGSTU30*) in *Arabidopsis* boosted the plant's ability to withstand drought and heavy metal stress. Dinler et al. (2014) reported that GST activity could increase with exogenous nitric oxide (NO) application, but GST activity could increase directly due to salt stress application. In parallel, in the study conducted by Rezaei et al. (2013), they showed that the expression levels of some members of the *GST* gene family changed under the same stress conditions in different varieties of the same plant species, and they mentioned that the expression level of *GST* genes in general increased under drought stress. Chen et al. (2012) showed that knockout mutants of the *atgsu17* gene in *Arabidopsis*, which has more number of leaves and smooth elliptical leaf shape, showed higher survival rate in water deficiency stress than transgenic *Arabidopsis* in which the same gene was overexpressed, and this study reported the activity of the *AtGSTU17* gene. In a study on maize, it was shown by Edwards (2000) that GSTs protect the plant against an herbicide, atrazine. It is well recognized that GSTs have a role in plant defense against a variety of stress conditions (Marrs 1996; Chen et al. 2012).

Bioinformatics analysis and genome-wide characterization of the *GST* gene family and the effect of *Pv-GST* genes on drought and salinity metabolism have been elucidated. The fact that GSTs are of critical importance for almost all organisms can also be said for *P. vulgaris*, since they are found at a certain level of expression in almost all cases.

Conclusions

After in silico analysis of the *P. vulgaris* genome, 55 *Pv-GST* genes, which are members of the *GST* gene family, were identified. The relationships of *P. vulgaris*, *A. thaliana*, and *G. max GST* genes were demonstrated by phylogenetic analysis. Expression changes of *GST* genes in leaf tissues were determined by the qRT-PCR method after application of drought and salt stress to common bean plants, and the results were visualized. It was revealed that the expression levels of *GST* gene family members, which are members of the glutathione pathway, which has a serious importance in plants, changed under salt and drought stress conditions. It is thought that the data obtained as a result of this study will be a guide for further molecular biology, physiology, and breeding studies in beans.

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Author Contribution Selman Muslu made bioinformatic analysis and prepared the manuscript; Ayşe Gül Kasapoğlu provided data and revised the manuscript; Ahmed Sidar Aygören and Ebru Güneş collected the data and made laboratory experiments. Esma Yiğider cultivated the plants and provided the samples. This manuscript is supervised by Emre İlhan and Murat Aydın. All authors approved the final form of manuscript.

Availability of Data and Materials All relevant data are within the manuscript and its Supporting Information files.

Declarations

Ethical Approval This article does not contain any studies with human or animal subjects.

Conflict of Interest The authors declare no competing interests.

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