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Genome-wide Analysis and Characterization of *Eucalyptus grandis* TCP Transcription Factors

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ABSTRACT

Teosinte branched/Cycloidea/Proliferating cell factors (TCPs), a small transcription gene family, serve in developmental processes such as branching, flowering, and growth of plants. In this study, the TCP transcription gene family of eucalyptus, which is considered important for its medicinal and industrial uses, was bioinformatically investigated. A total of 16 *Eucalyptus grandis* TCP (Egra-TCP) genes were found to be distributed on chromosomes 1, 2, 4, 6, 7, 9, 10 and 11. Several segmentally-duplicated gene couples including Egra-TCP-7/Egra-TCP-11, -13 and -16, Egra-TCP-6/Egra-TCP-12 and -15, Egra-TCP-12/Egra-TCP-15 and Egra-TCP-11/Egra-TCP-13 were discovered. Egra-TCPs were divided into three main clades based on phylogenetic analysis, motif, and gene structure. While Egra-TCP-10 has the highest molecular weight with 47.19 kDa, the lowest was Egra-TCP-1 with 21.68 kDa. Twelve Egra-TCP genes were found to have no introns, while the Egra-TCP-7, -15, and -16 genes had a single intron. The orthologous relationships among *E. grandis*/*Arabidopsis thaliana* and *E. grandis*/*Vitis vinifera* were identified through a synteny analysis. Digital gene expression profiles of Egra-TCP genes in tissues such as xylem, phloem, shoot tips, young and mature leaf revealed a high expression pattern. The findings of this study contributes to existing knowledge in the biotechnology field by providing contributing to our understanding of the molecular basis of the TCP gene family in the eucalyptus plant.

Keywords: bHLH domain, CYC/TB1 clade, Digital gene expression, Phylogenetic analysis, TCP transcription factors

1. Introduction

Teosinte branched/Cycloidea/Proliferating cell factor (TCP) is a plant-specific transcription factor (TF). TCPs consists of teosinte branched 1 from corn, Cycleoidea from *Antirrhinum majus*, and PCF (proliferating cell nuclear antigen factor) from *Oryza sativa*. The members of TCP contain a DNA binding motif called the TCP domain, and a basic helix loop helix conserved at the N-terminal end (Ilhan et al. 2018; Zheng et al. 2018). TCP TF plays a role in embryonic development, leaf development, branching, flowering, circadian rhythm, hormone, and stress response mechanisms in plants (Liu et al. 2018; He et al. 2020). The gene family of TCP is divided into two subgroups, class I includes PCF or TCP-P, while class II includes CYC/TB1 and CIN (CINCINNATA) or TCP-C (Ilhan et al. 2018; Ding et al. 2019; Leng et al. 2019; Lin et al. 2019). Members of the class I group differ from class II in the 4 amino acid sequences. Some class II members, however, contain an arginine-rich R domain, which is thought to be involved in protein-protein interaction (Wang et al. 2018; Jiu et al. 2019).

Class I TPCs such as TCP20 in *Arabidopsis* and PCF1/PCF2 in rice (*Oryza sativa*) are involved in increasing plant growth and cell proliferation (Xu et al. 2014). When plants with mutant TCP genes were compared with wild-type plants, no phenotypical difference was detected. It is also known that TCP20 is involved in leaf senescence and jasmonic acid synthesis pathway, while TCP16 is involved in other stages of floral development (Li et al. 2005; Danisman et al. 2012; Wang et al. 2018). In *Arabidopsis*, some TCPs, such as TCP7, 8, 22, and 23, have near expression levels in young leaves (Aguilar-Martinez & Sinha 2013).

Class II TCPs are examined in 2 subgroups, the CYC/TB1 and CIN (Martin-Trillo & Cubas 2010; Feng et al. 2019; Jiu et al. 2019). Unlike class I, class II TCPs have been found to inhibit mutant cell growth and proliferation (Jiu et al. 2019). *Arabidopsis* and tomato CIN clade mutants showed that leaf blade cells divide for a longer period of time and show larger leaves and shape changes when compared to wild types (Jiu et al. 2019; Leng et al. 2019). The TB1 gene inhibits lateral branching and growth in maize and increases lateral branching in mutants (Doebley et al. 1997). Class II TCP gene family members have roles in the response mechanism to abiotic stress conditions (Ilhan et al. 2018). For this reason, some of the TCP TF is targeted by miR319 (Palatnik et al. 2003; Nag et al. 2009; Xu et al. 2014).

Eucalyptus, the most dominant genus in the Australian flora, belongs to the *Myrtaceae* family. The genus contains more than 800 species and dominates the much of Australia (Macphail & Thornhill 2016). With its rapid growth and superior tree characteristics, eucalyptus is used for tree planting in more than 100 countries on six continents. *Eucalyptus grandis* and *E. globulus* are preferred in breeding programs worldwide (İlhan 2018). Eucalyptus offers renewable resources for the paper industry, biomaterials, and bioenergy production; its high concentrations of mono- and sesquiterpenes also provide ecological functions as well as medical and industrial uses (Myburg et al. 2014).

So far, TCP TF members have been detected in *Arabidopsis* (Li 2015), *O. sativa* (Yao et al. 2007), *Solanum licopersicum* L. (Parapunova et al. 2014), cotton (Zheng et al. 2018), apple (Xu et al. 2014), sorghum (Francis et al. 2016), common bean (Ilhan et al. 2018), soybean (Feng et al. 2018), wheat (Zhao et al. 2018), grape (Jiu et al. 2019; Leng et al. 2019; Min et al. 2018), alfalfa (Wang et al. 2018), carrot (Feng et al. 2019). While the importance of TCP genes on growth and development is widely-known, a detailed analysis has yet to be performed on *Eucalyptus grandis*. In this study, a comparative bioinformatics and in silico gene expression analysis in different tissues of the TCP gene family members in *Eucalyptus grandis* was performed,and putative Egra-TCPs were identified by genome-wide scans. Additionally, phylogenetic relationships, chromosomal distribution, gene structures, conserved motif, and cis-acting element analyzes were performed. The findings from this study will allow for a better understanding of the potential functions and classification of Egra-TCPs. In addition, it aid future functional studies of the eucalyptus plant.

2. Material and Methods

2.1. Identification of TCP proteins in the eucalyptus genome

The Pfam Accession Number for TCP TF (PF03634; https://www.ebi.ac.uk/interpro/entry/pfam/PF03634/) was obtained from the Pfam database. Protein sequences of the TCP gene family in the genome of Eucalyptus (Myburg et al. 2014), *Arabidopsis thaliana* (Lamesch et al. 2012), and *Vitis vinifera* (Jaillon et al. 2007) were retrieved from Phytozome Database v13 (https://phytozome-next.jgi.doe.gov/) using the Pfam Accession Number PF03634. Both blastp in the Phytozome Database v13 and a hidden Markov model (HMM) (http://www.ebi.ac.uk) were used to scan the *E. grandis* genome with default parameters to find all probable TCP proteins in the eucalyptus genome. TCP protein sequences belonging to maize (*Zea mays*), common snapdragon (AmCYC, AmCIN) and rice (*Oryza sativa*) (OsPCF1: LOC_Os04g11830, OsPCF2: LOC_Os08g43160) were obtained form Ilhan et al. (2018). The molecular weight and theoretical isoelectric point (pI) of the obtained Egra-TCP proteins were determined using the "ProtParam tool" (https://web.expasy.org/protparam/) according to Kasapoğlu et al. (2020).

Structure, physical locations, gene duplications, identification of conserved motifs, and phylogenetic analyzes of Egra-TCP genes

The Gene Structure Display Server v2.0 (http://gsds.gao-lab.org/) was utilized to define on the exon and intron regions of the Egra-TCP proteins (Hu et al. 2015). Genomic and coding DNA sequences have been used to predict the position information of Egra-TCP genes. Using the Phytozome Database v13, the chromosomal locations and sizes of the Egra-TCP genes were determined. All TCPs were mapped onto the Eucalyptus chromosomes by Circos (Krzywinski et al. 2009; a syntonic map was subsequently displayed by TBtools) (Chen et al. 2020).

The "Multiple EM for Motif Elimination (MEME) Tool" was used to identify additional conserved motifs of the Egra-TCP proteins (Bailey et al. 2006). The parameters of the MEME tool were set as previously described (Ilhan et al. 2018). Identified motifs were scanned using the default settings of the InterPro database (Quevillon et al. 2005). In addition, for conserved region sequence analysis, sequence logo analyzes of the bHLH domains were drawn using the WEBLOGO online web tool (Crooks et al. 2004).

Phylogenetic analyzes were performed according to the neighbor-joining (NJ) method with 1000 replicated bootstrap values. A protein sequence alignment of the Egra-TCP was performed using ClustalW (Thompson et al. 1997). In addition, a phylogenetic tree was

obtained using the MEGA v7 program (Kumar et al. 2016). The tree was shaped using the Interactive Tree of Life (iTOL) interface (Letunic & Bork 2011).

2.2. Subcellular localization and promoter analyses of eucalyptus TCP gene family

PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to perform cis-acting element analysis in 5' upstream regions (Supplementary File 1) encompassing roughly 2 kilobases DNA segments of each sequence of the Eucalyptus TCP gene members (Lescot et al. 2002), after which TBTools software was used to draw the phenogram (Chen et al. 2020). Subcellular localizations were estimated via WoLFPSORT (Horton et al. 2007).

2.3. The Orthologous Relationships

Using MCScanX (Wang et al. 2012) with default parameters, gene duplication events between *E. grandis*, *A. thaliana,* and *V. vinifera* were determined. The substitution rates of Ka (non-synonymous substitution rate), Ks (synonymous substitution rate), and Ka/Ks between duplicate pairs of Egra-TCP genes were calculated and synteny maps were drawn using TBTools.

2.4. In silico gene expression analysis

Illimuna RNAseq datasets were obtained using the Phytozome Database v13. Expression profiles of the Egra-TCP genes were analyzed in special tissue libraries obtained from six different plant tissues (Floem, immature xylem, xylem, mature leaf, shoot tips and young leaf). In silico expression profiles were calculated with Cufflinks in units of FPKM (Expected number of fragments per kilobase of sequenced transcript per million base pairs) (Trapnell et al. 2013). The values of FPKM were converted to logarithm base 2, and a heatmap was created using the CIMMiner (https://discover.nci.nih.gov/cimminer/).

3. Results and Discussion

3.1. Identification of E. grandis TCP Genes

The sequences of TCP gene family members were obtained from *Eucalyptus grandis*, *Arabidopsis thaliana,* and *Vitis vinifera* genomes using the Phytozome Database v13 with the TCP gene family accession number acquired from the Pfam server. The TCP domains in the obtained sequences were confirmed through an HMM analysis. Through these analyzes, 16 TCP genes were identified in eucalyptus. The amino acid numbers, theoretical pIs, and instability indices of these genes are provided on Table 1.

The lengths of the eucalyptus TCP proteins range from 194 to 464. The longest amino acid sequence was Egra-TCP-10 with 464, while the shortest sequence was Egra-TCP-11 with 194 amino acids. Similarly, the highest molecular weights of Egra-TCP proteins were obtained in Egra-TCP-10 with 47.19 kDa, while the lowest was in Egra-TCP-1 with 21.68 kDa. While the theoretical pIs vary between 6.44and 10.07, the lowest value was determined in Egra-TCP-7 and the highest value was determined in Egra-TCP-4. The instability indices were altered from 30.63 (Egra-TCP-11) to 72.42 (Egra-TCP-4). According to these results, the instability index of Egra-TCP-11 was lower than 40, indicating that this protein is relatively stable.

Table 1- The information about TCP gene family members found in Eucalyptus genome

Gene ID	*Phytozome ID*	*Chromosome location*	*aa lenght*	*MW (Da)*	*pI*	*Instability index*	*Classifies*	*Subcellular localization (WolfPSORT)*	*NCBI accession no*
Egra-TCP-1	Eucgr. A01143.1	Chr01:25174892..25175768 (+)	206	21680.53	7.70	62.41	unstable	nucl: 5, mito: 5, cyto: 2, chlo: 1, plas: 1	XP_010046620.1
Egra-TCP-2	Eucgr. A02843.1	Chr01:43511787..43513420 (+)	343	35580.41	9.05	63.56	unstable	nucl: 14	XP_010025203.1
Egra-TCP-3	Eucgr. B00471.1	Chr02:4499527..4501083 (-)	427	44907.43	6.70	67.88	unstable	nucl: 14	XP_010029552.1
Egra-TCP-4	Eucgr. B03427.1	Chr02:52680182..52680991 (+)	270	28668.77	10.07	72.42	unstable	nucl: 11, mito: 2, cyto: 1	XP_010046658.1
Egra-TCP-5	Eucgr. B03529.1	Chr02:55205056..55206564 (+)	286	30207.42	6.45	52.72	unstable	nucl: 14	XP_010044850.1

Table 1- Continued

Gene ID	*Phytozome ID*	*Chromosome location*	*aa lenght*	*MW (Da)*	*pI*	*Instability index*	*Classifies*	*Subcellular localization (WolfPSORT)*	*NCBI accession no*
Egra-TCP-6	Eucgr. B00608.1	Chr02:6164848..6167321 (+)	405	44419.28	6.60	60.86	unstable	nucl: 11, chlo: 2, extr: 1	XP_010045604.1
Egra-TCP-7	Eucgr. B00699.1	Chr02:7267363..7269590 (-)	383	42610.25	6.44	48.51	unstable	nucl: 13, cyto: 1	XP_010031709.1
Egra-TCP-8	Eucgr. D02422.1	Chr04:37891043..37892367 (+)	300	32028.58	7.26	57.79	unstable	nucl: 14	XP_010053872.1
Egra-TCP-9	Eucgr. F01204.1	Chr06:16221067..16223636 (-)	414	44803.27	6.66	63.06	unstable	nucl: 13, cyto: 1	XP_010060659.1
Egra-TCP-10	Eucgr. F02587.1	Chr06:37967375..37969007 (-)	464	47191.71	8.09	60.85	unstable	nucl: 14	XP_010064787.1
Egra-TCP-11	Eucgr. G02354.1	Chr07:44218919..44219500 (+)	194	21807.44	9.62	36.63	stable	nucl: 13, cyto: 1	XP_010068762.1
Egra-TCP-12	Eucgr. I02038.1	Chr09:29927883..29930010 (+)	338	37109.33	6.85	61.83	unstable	nucl: 10, chlo: 1, cyto: 1, extr: 1, vacu: 1	XP_010029390.1
Egra-TCP-13	Eucgr. J01466.1	Chr10:18021225..18022148 (-)	308	35211.63	9.27	59.34	unstable	nucl: 12.5, cyto_nucl: 7, chlo: 1	XP_010034424.1
Egra-TCP-14	Eucgr. K01089.1	Chr11:13919096..13921146 (-)	358	39055.27	7.23	59.46	unstable	nucl: 14	XP_010035834.1
Egra-TCP-15	Eucgr. K02535.1	Chr11:33244993..33246287 (+)	342	38224.07	8.44	49.06	unstable	cyto: 8, nucl: 4, plas: 1.5, golg_plas: 1.5	XP_010037244.1
Egra-TCP-16	Eucgr. K02654.1	Chr11:33796218..33797790 (-)	367	42209.24	8.38	52.99	unstable	nucl: 14	XP_010037346.1

3.2. Physical locations and structure of Egra-TCP genes, gene duplications, identification of conserved motifs, and phylogenetic analyzes

The positions of the Egra-TCP genes in the genome were obtained from the Phytozome Database v13. All Egra-TCP genes were mapped to eucalyptus chromosomes using Circos (Figure 1). The Egra-TCPs are located on chromosomes 1, 2, 4, 6, 7, 9, 10, and 11, with most of their 5 genes on chromosome 2 and at least 1 gene on each of chromosomes 4, 7, 9, and 10.

The exon and intron numbers were determined through a structural analysis performed on the Egra-TCPs using the Gene Structure Display Server v2.0 (Figure 2). According to the gene structure analysis, 2 introns were detected in Egra-TCP-8 and 1 intron in Egra-TCP-7, -15, and -16 (TCP-C member genes). In addition, Egra-TCP-7 and -16 genes are in a similar group based on the phylogenetic tree. Again, Egra-TCP-7, -8, -15, and -16 genes were found to contain 2, 3, 2, and 2 exons, respectively. Twelve of these 16 genes are entirely intronless. Studies have shown that intronless genes are characteristic of a prokaryotic genome. Additionally, the existence of intronless genes in eukaryotic genomes has been known for past 20 years (Makeyev et al. 1999; Sugiyama et al. 1999). Many plant species have genes with no intron, including model organisms such as *A. thaliana*, *O. sativa*, and *Populus* (Yang et al. 2009). It has been reported that retrogenes are also intronless, and many retrogenes are found in eukaryotic genomes (Zhang et al. 2005). These intronless genes in the eukaryotic genomes are known to be important in comparative genomic and evolutionary studies (Zou et al. 2011; Ilhan et al. 2018). The intronless genes have been found in studies with TCP genes in different plant genomes. Approximately 77.8% of *Phaseolus vulgaris* TCP genes are intronless (Ilhan et al. 2018). Similarly, thirteen genes have been discovered in *Prunus meme* (Zhou et al. 2016), thirty-two in apple (Xu et al. 2014), forty in tobacco (Chen et al. 2016), thirty-two in *Gossypium raimondii* (Min et al. 2018), twelve in *V. vinifera* (Jiu et al. 2019), sixty-eight in *Gossypium barbadense* (Zheng et al. 2018), and fifty-one in *Brassica juncea* var. *tumida* (He et al. 2020).

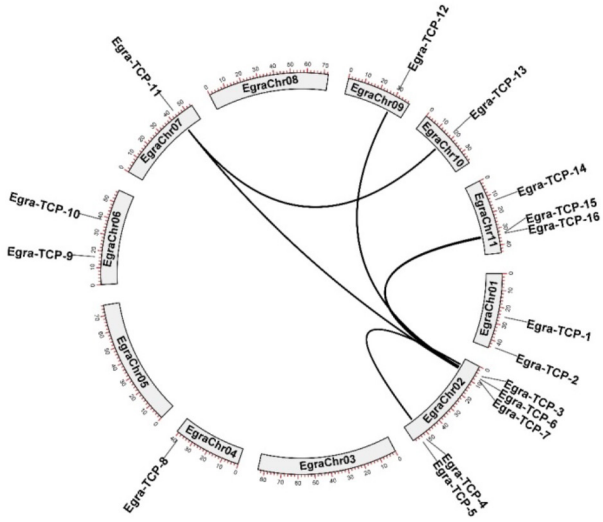

Figure 1- Chromosomal distribution and gene duplication events of Egra-TCP genes
The black curves indicate segmentally-duplicated genes. The lengths of eucalyptus chromosomes can be predicted according to the scale given

One of the most crucial evolutionary mechanisms that produce genetic diversity and functional novelty is gene duplication (Huo et al. 2018). Tandem and segmental duplications have an important role in the proliferation of plant gene families and the acquisition of new gene functions in the evolutionary process (Kondrashov et al. 2002; Cao et al. 2021). Gene duplication events have been a sunject of study in order to better determine the evolutionary relationships of members of the *E. grandis* TCP gene family. A total of 6 duplicate gene pairs were identified between Egra-TCPs using the MCScanX (Table 2, Figure 1). All of the duplicating gene pairs identified were found to be whole genome duplication or segmental duplication genes. Previous studies on the TCP gene family reported that there are both segmental and WGD duplications in apples (Xu et al. 2014), and tandem duplications in addition to these duplication events in *Arabidopsis* and rice (Yao et al. 2007). Moreover, it has been suggested that these duplications occurred during the evolution of the angiosperm (Yao et al. 2007). New gene functions obtained during these widespread plant duplication events lead to significant evolutionary changes (Navaud et al. 2007).

TBTools software was used to compute the Ka, Ks and Ka/Ks values, as a good indicator of the selection pressure at the protein level. A Ka/Ks value less than 1 means purifying selection, while a value greater than 1 means positive selection. If Ka/Ks are equal to 1, it indicates neutral selection in the evolutionary process (Juretic et al. 2005; İlhan 2018; Kizilkaya et al. 2020). The fact that Ka/Ks is less than 1 among WGD or segmental duplication gene pairs found among Egra-TCP genes in this study suggests that they are under purifying selection in the evolutionary process (Table 2). As seen in Table 2, the Ka/Ks value could not be calculated between the Egra-TCP-7/Egra-TCP-11 gene pair.

The "MEME tool" was performed to discover more Egra-TCP conserved motifs (Bailey et al. 2006; Aygören et al. 2022). In the preserved motif analysis, 15 preserved motifs were detected. The length of the determined motifs varied between 2 and 50. The most motifs were detected in Egra-TCP-10 (10 motifs), while the least motifs were detected in Egra-TCP-5 and Egra-TCP-14 with 2 motifs. The sequence obtained from Motif-1 in InterProScan searches included the TCP domain. Motif-5 shows the CYC/TB1 and R domains (Figure 3, Supplementary File 1). Motif -1 is found in all Egra-TCP genes, while Motif-5 containing the R domain, is found in Egra-TCP-4, -7, -11, -13, -15, and -16 genes.

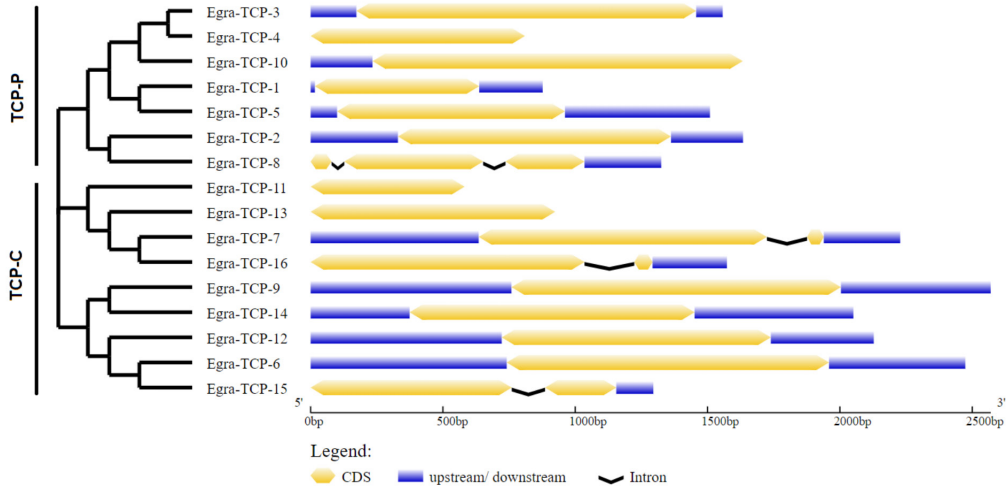

Figure 2- Exon and intron structures of Egra-TCP genes. The dark-blue and yellow boxes indicate UTRs and exon regions, respectively, and the black lines represent introns

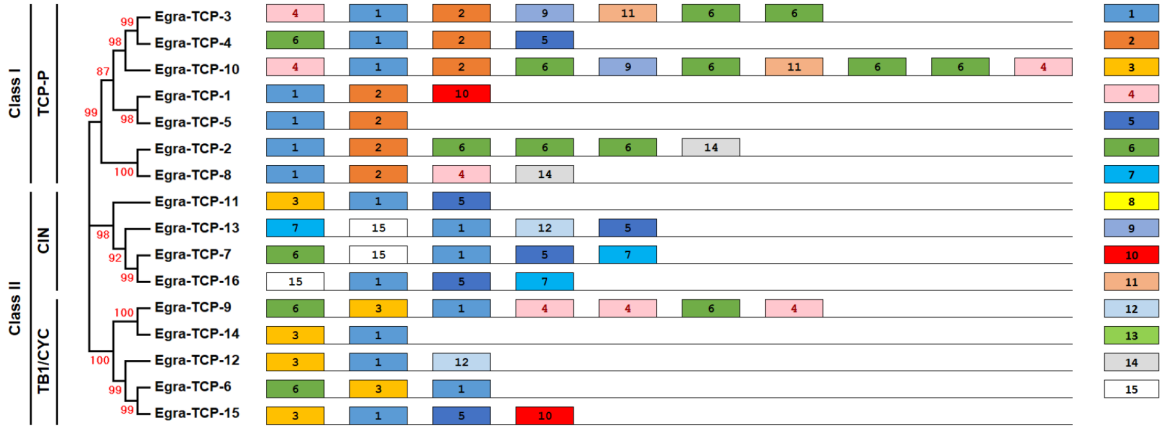

Figure 3- Additional motif analysis of Egra-TCPs. Fifteen motifs are displayed in different colors

Table 2- Ka, Ks, Ka/Ks values, Selection pressure, and duplication type of Egra-TCPs

Gene 1	*Gene 2*	*Ka*	*Ks*	*Ka/Ks*	*Selection pressure*	*Duplication type*
Egra-TCP-3	Egra-TCP-4	0.464	1.094	0.424	Purifying	WGD or Segmental
Egra-TCP-6	Egra-TCP-12	0.533	2.618	0.204	Purifying	WGD or Segmental
Egra-TCP-6	Egra-TCP-15	0.582	1.582	0.368	Purifying	WGD or Segmental
Egra-TCP-7	Egra-TCP-16	0.502	2.248	0.223	Purifying	WGD or Segmental
Egra-TCP-11	Egra-TCP-13	0.571	3.596	0.159	Purifying	WGD or Segmental

Ka : Non-synonymous substitution rate, Ks: Snonymous substitution rate

A sequence alignment analysis showed that all TCPs contain conserved basic helix loop helix. In addition, according to this analysis, the members of the PFC group have a deletion of four amino acids compared to the members of the TCP-P group in terms of the basic helix loop helix domain (Figure 4). This result is compatible with the phylogenetic analysis results.

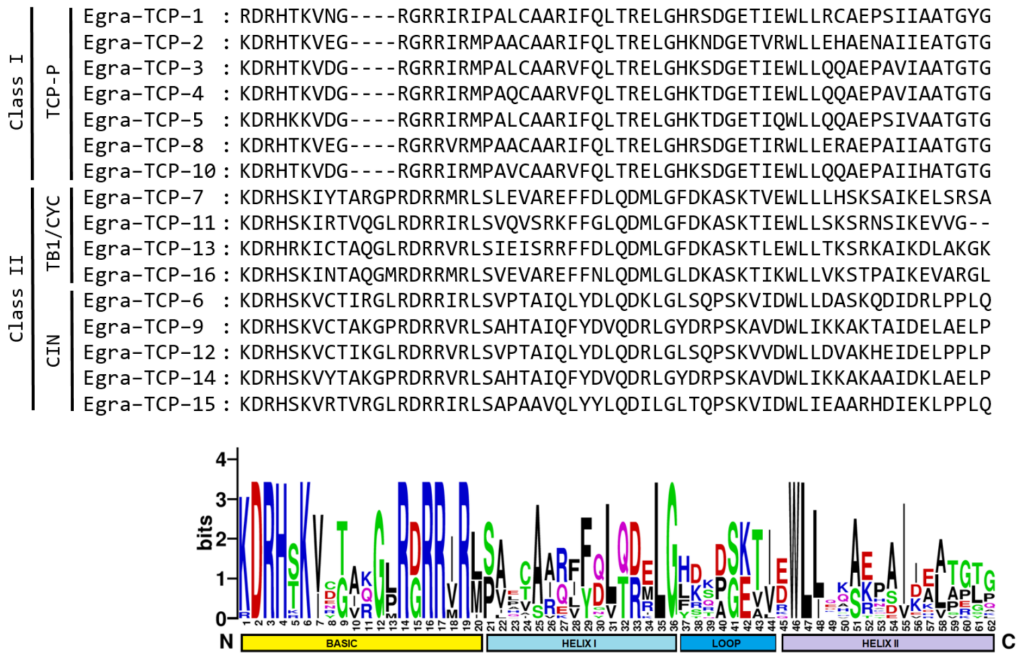

Figure 4- Sequence alignment of Egra-TCPs containing bHLH domain

To further examine the function of Egra-TCPs, the phylogenetic relationships were analyzed. TCP proteins from *Arabidopsis thaliana*, *Vitis vinifera*, maize (TB1), the common snapdragon (AmCYC, AmCIN) and rice (OsPCF) plants, and *E. grandis* were aligned based on multiple sequence alignment in MEGA v7 program with bootstrap values using the Neighbor-Joining method. A supported rootless tree is drawn. The tree was organized with iTOL (Figure 5).

Egra-TCP proteins are divided into 2 different major groups, Class I and Class II. Class I clan is known as TCP-C clan, while Class II clan is known as TCP-P. Class I group TB1/CYC clades, including TB1 and AmCYC, contain Egra-TCP-7, -11, -13, and 16 proteins. The CIN clade of the Class II group contain AmCIN contains Egra-TCP-6, -9, -12, -14, and -15 proteins, while the TCP-C clade contains OsPFC-1, OsPFC-2, Egra-TCP-1, -2, -3, -4, -5, -8 and -10 proteins.

3.3. Subcellular Localization and promotor analysis of Egra-TCP gene family

The subcellular localization of the Egra-TCPs was estimated using the WoLFPSORT program (Table 1). The Egra-TCP-2, -3, -5, -8, -10, -14, and -16 proteins are estimated to be located in the nucleus, while some Egra-TCP proteins have been estimated to be located in organelles such as the nucleus, mitochondria, vacuoles, golgi apparatus, and chloroplasts. Previous studies predicted that TCP proteins are predominantly located in the nucleus (Leng et al. 2019; Lin et al. 2019).

To further examine the function and regulatory mechanism of the Egra-TCP genes, a promoter region analysis was performed. The approximately 2000 bp genomic sequence of the Egra-TCP genes has been uploaded to the PlantCARE database. Using the data obtained as a result of the analysis, a phenogram was drawn with the help of TBTools (Figure 6).

The cis-acting elements in the promoter sequence of the Egra-TCPs were collected in six groups:environmetal stress, development, promoter-related, hormone-related, light responsiveness, and site binding-related. Whereas each Egra-TCP had an average of 130 cis-acting elements, Egra-TCP-13 had the most, with 165; the lowest cis-acting number was Egra-TCP-4 with 106. The GCN4_motif, which is one of the elements involved in endosperm expression, was determined in Egra-TCP-2, 3, -7, -9, and -14 genes. The element associated with meristem expression, CAT-box, were found in Egra-TCP-1, -2, -9, 10, -11, -13, -14 and -16 genes. Hormone-related cis-acting elements such as ABRE, TCA-element, CGTCA-motif, TGACG-motif, GARE-motif, P-box, TATC box, ERE, AuxRR-core, and TGA-element have been detected in some of the Egra-TCP genes' promoter regions. The fact that many hormonal-related elements have been found in the promoter regions of the Egra-TCP genes suggests that these genes have important roles in growth and development. Elements such as ARE, MBS, MBS, and LTR were determined as elements related to environmental stress (Table S2 and S3). This supports our findings in previous studies (Ilhan et al. 2018).

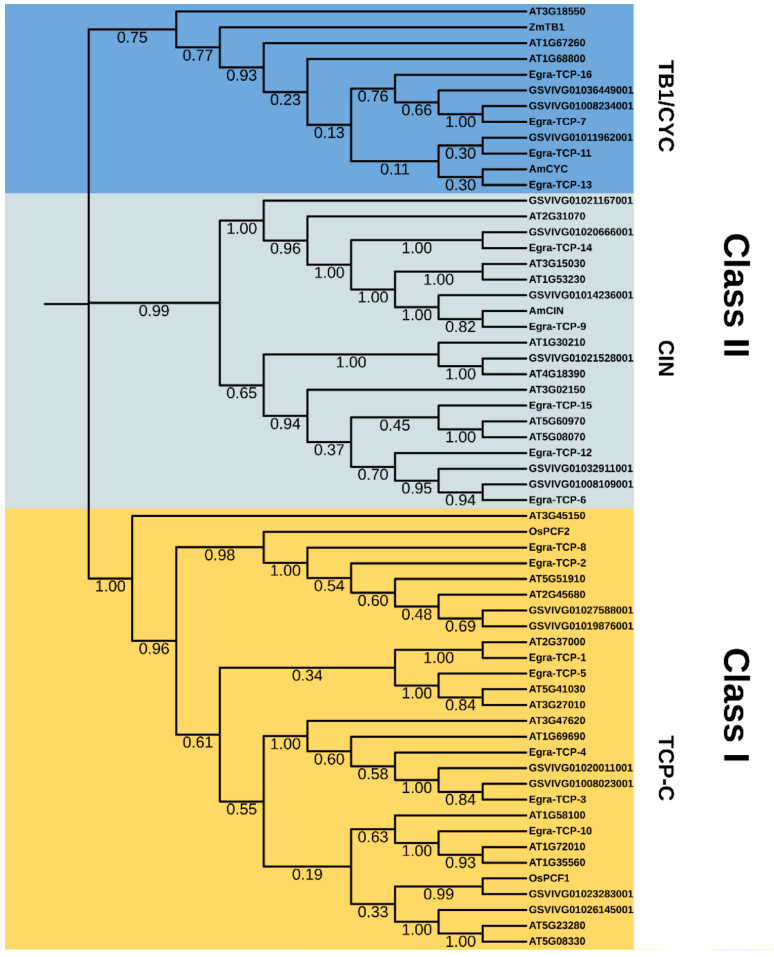

Figure 5- A phyologenetic tree of *Eucalyptus grandis*, *Arabidopsis thaliana*, and *Vitis vinifera* TCP proteins, maize (TB1), common snapdragon (AmCYC, AmCIN), and *O. sativa* (OsPCF1 and OsPCF2) proteins. The tree was drawn using the MEGA v7 and Neighbor-joining (NJ) algorithm with 1000 replicated-bootstrap values

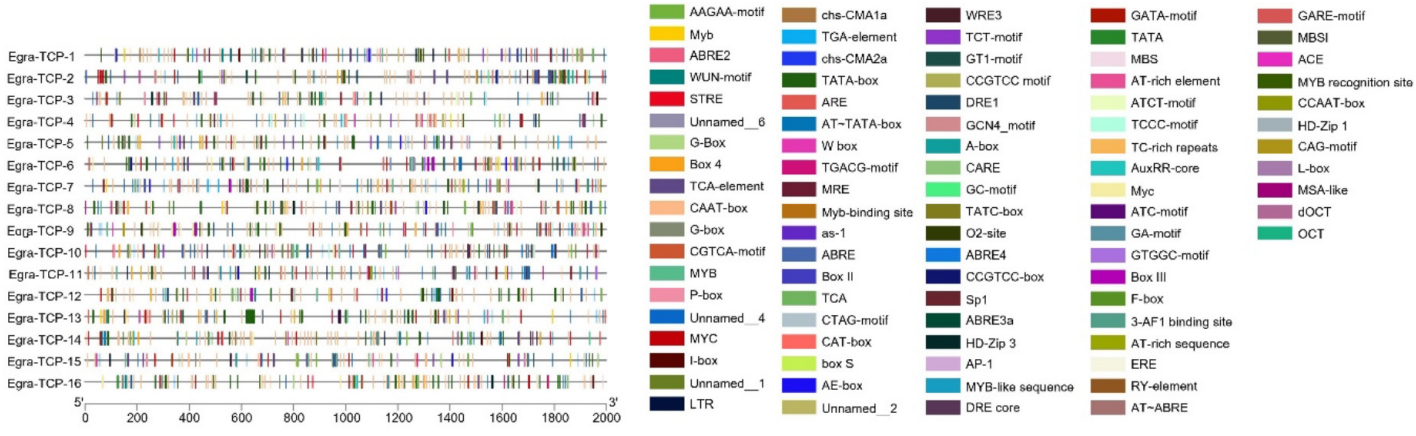

Figure 6- Cis-acting element analysis of Egra-TCP genes according to information obtained from the PlantCARE database

3.4. The Orthologous Relationships

TCP proteins are a conserved gene family among plant genomes. The orthologous association using the TCP genes *Eucalyptus grandis*, *Vitis vinifera,* and *Arabidopsis thaliana* was determined using MCScanX with default parameters. Our results showed orthologous pairs between the TCP genes of these three genomes (Table 3). In order to show the selection pressure in the evolutionary process, the Ka/Ks values of each orthologous gene pair were calculated. However, this value could not be calculated for some gene pairs. The calculated orthologous gene pair are under strong purifying selection pressure.

To predict the TCP's evolutionary pathways, a syntenic analysis of the TCP genes was performed in *Eucalyptus grandis*, *Vitis vinifera,* and *Arabidopsis thaliana* plants (Figure 7). The homology between *E. grandis* and Arabidopsis was higher than that between *E. grandis* and *V. vinifera*. In addition, when compared with the phylogenetic tree, these orthologous genes were determined to be in similar groups. Previous studies have revealed that TCP genes in similar groups show similar functions (Mondragon-Palomino and Trontin 2011; Citerne et al. 2013; Feng et al. 2018). For this reason, it is thought that these genes may have similar functions.

Table 3- Ka, Ks, Ka/Ks values, and Selection pressure among *E. grandis*, *V. vinifera*, and *A. thaliana* orthologous genes

Gene 1	***Gene 2***	***Ka***	***Ks***	***Ka/Ks***	***Selection Pressure***
E. grandis-V. vinifera					
Egra-TCP-2	GSVIVT01027588001	0.472	2.796	0.169	Purifying
Egra-TCP-2	GSVIVT01019876001	0.426	3.008	0.142	Purifying
Egra-TCP-4	GSVIVT01008023001	0.432	1.859	0.232	Purifying
Egra-TCP-4	GSVIVT01020011001	0.412	1.770	0.233	Purifying
Egra-TCP-7	GSVIVT01008234001	0.309	1.521	0.203	Purifying
Egra-TCP-8	GSVIVT01027588001	0.509	1.408	0.361	Purifying
Egra-TCP-9	GSVIVT01014236001	0.315	1.567	0.201	Purifying
Egra-TCP-12	GSVIVT01008109001	0.371	1.993	0.186	Purifying
Egra-TCP-12	GSVIVT01032911001	0.431	2.222	0.194	Purifying
Egra-TCP-13	GSVIVT01036449001	0.575	3.086	0.186	Purifying
Egra-TCP-14	GSVIVT01020666001	0.467	3.824	0.122	Purifying
Egra-TCP-14	GSVIVT01021167001	0.606	2.738	0.221	Purifying
Egra-TCP-15	GSVIVT01008109001	0.359	2.528	0.142	Purifying
Egra-TCP-16	GSVIVT01008234001	0.483	2.093	0.231	Purifying
E. grandis-A. thaliana					
Egra-TCP-7	AT1G68800.1	0.655	3.981	0.164	Purifying
Egra-TCP-7	AT3G18550.1	0.790	3.100	0.255	Purifying
Egra-TCP-13	AT3G18550.1	0.751	2.834	0.265	Purifying
Egra-TCP-15	AT5G60970.1	0.747	2.884	0.259	Purifying

Ka : Non-synonymous substitution rate, Ks: Snonymous substitution rate

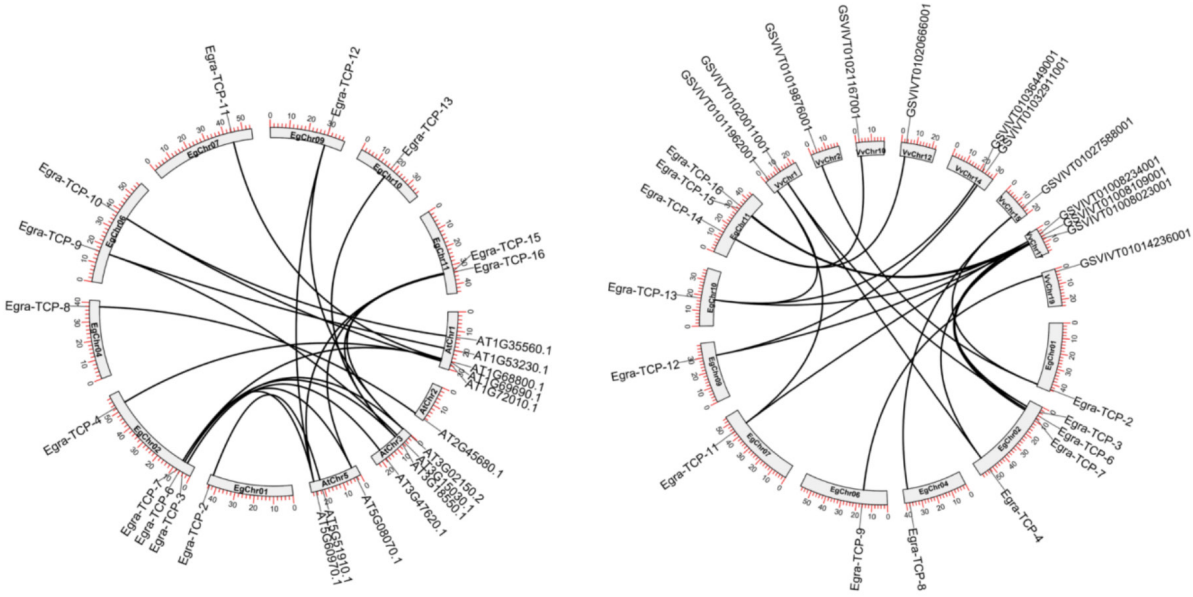

Figure 7- The orthologous relationships among *E. grandis*/*A. thaliana* (left) and *E. grandis*/*V. vinifera* (right). Black linker lines represented the syntenic relationships between *E. grandis*, *V. vinifera*, and *A. thaliana* TCP genes

3.5. In silico gene expression analyzes of TCP genes across various tissues

Different members of gene families are involved in different physiological processes among different tissues. In order to understand the expression differences and functions of TCP genes in plant growth and development, the expression values of tissue-specific TCP genes were analyzed with the data obtained from the Phytozome Database v13 (Figure 8).

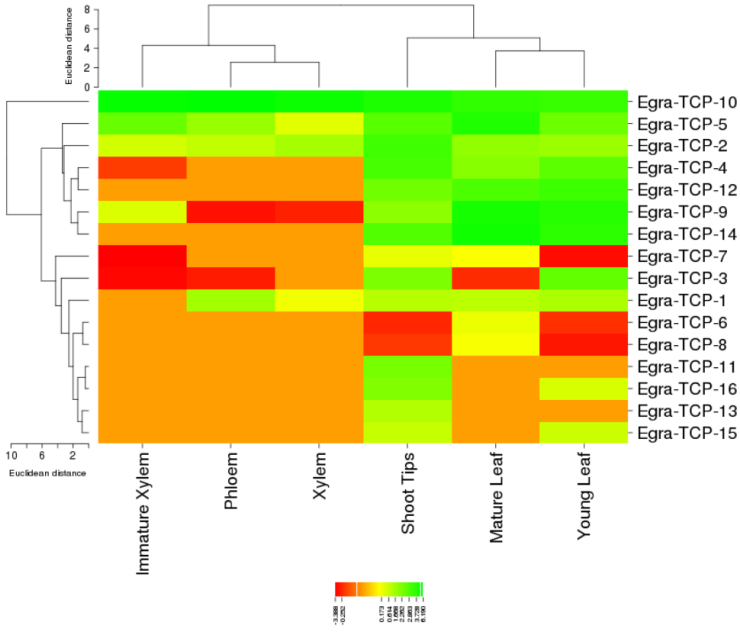

Figure 8- The expression levels of Egra-TCP genes in different tissues. The heatmap below indicates where the 16 Egra-TCPs are grouped in the six tissues. On the right are the names of the genes. Scale bars at the top display each gene's log 2 transformed FPKM values

It is seen that Egra-TCP-2, -5, and -10 have high expression levels in immature xylem, phloem, xylem, shoot tips, mature leaf, and young leaf tissues. While the remaining Egra-TCPs were not expressed in tissues such as immature xylem, phloem, and xylem, it was observed that they had little expression in shoot tips, mature leaf, and young leaf tissues. This result reveals that TCP genes are expressed tissue-specifically. Similar results have been recorded in other studies (Feng et al. 2018; Leng et al. 2019; Ling et al. 2020). A number of studies, however, have shown that TCP genes have different expression levels in different developmental stages of the plant (Pestana-Calsa et al. 2012; Xu et al. 2014; Min et al. 2018; Zheng et al. 2018; He et al. 2020). In addition, when the expression levels of duplicate Egra-TCP paralogs were compared, differences in gene expressions were detected, for example, the WGD or segmentally duplicated Egra-TCP-6/Egra-TCP-12 gene pair. While Egra-TCP-12 has a high expression level in shoot tips, mature leaf, and young leaf tissues, the expression level of Egra-TCP-6 is almost absent in shoot tips and young leaf tissues, and has been detected very little in mature leaf tissue. Tissue-specific expression differences are considered to be an indicator of functional transformations between genes (Makova and Li 2003; Li et al. 2005; Nag et al. 2009). The duplicated genes and the member of gene family and can acquire new biological functions during the evolution of the plant (Nag et al. 2009). Due to, Egra-TCP paralog gene pairs can acquire different functions in different tissues.

4. Conclusions

In this study, using in silico approaches in the eucalyptus genome, 16 TCP gene family members were identified. These genes are distributed in 7 different eucalyptus chromosomes. According to the gene expression analyses performed in immature xylem, phloem, xylem, shoot tips, mature leaf, and young leaf tissues, it was determined that Egra-TCPs have different expression levels in different tissues. In additiom, members of this gene family can take on different physiological functions in the growth and development processes of the plant. This contributes to our understanding of the functions and classification of TCP genes in the eucalyptus plant.

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Supplementary File 1 Link: http://glns.co/16hxg