

The G2-Like gene family in *Populus trichocarpa*: identification, evolution and expression profiles

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Abstract

The *G2-like* (GLK) transcription factors are plant-specific transcription factors (TFs) that perform extensive and significant roles in regulating chloroplast development. Here, genome-wide identification, classification, conserved motifs, *cis*-elements, chromosomal locations, MCSscanX, evolution and expression patterns of the *PtGLK* genes in the woody model plant *Populus trichocarpa* were analyzed in detail. In total, 55 putative *PtGLKs* (*PtGLK1*-*PtGLK55*) were identified and divided into 11 distinct subfamilies according to the gene structure, motif composition and phylogenetic analysis. Synteny analysis showed that 22 orthologous pairs and highly conserved microsynteny between regions of *GLK* genes across *P. trichocarpa* and *Arabidopsis* were identified. Furthermore, analysis of the duplication events and divergence times provided insight into the evolutionary patterns of *GLK* genes. The previously published transcriptome data indicated that *PtGLK* genes presented distinct expression patterns in various tissues and different stages. Additionally, several *PtGLKs* were significantly upregulated under the responses of cold stress, osmotic stress, and MeJA and GA treatments, implying that they might take part in abiotic stress and phytohormone responses. Overall, our results provide comprehensive information on the *PtGLK* gene family and elucidate the potential functional characterization of *PtGLK* genes in *P. trichocarpa*.

1. Introduction

Chloroplasts are responsible for the light energy response of photosynthesis, and contain the green pigment chlorophyll, which is basically relied upon by all plant life [1,2]. Recent research has shown that chloroplasts originated from primary endosymbiotic events related to these cyanobacteria [3,4]. Thus, the regulation of photosynthetic organs assembly depends on the synergy of the nucleus and chloroplast. Plastids not only function in photosynthesis but also in the synthesis of amino acids, fatty acids, purines and pyrimidine bases, terpenes and various pigments, and hormones, as well as the key aspects of nitrogen and sulfur assimilation [5–7]. In addition, proplastids in subepidermal meristem cells (or leaf sheaths in dark cotyledons) convert to mesophyll chloroplasts under light [8]. Conversely, members of the Golden 2-like (GLK) family can regulate the appearance of chloroplasts in the transition and maturity stages, and *GLK* genes are essential in angiosperm chloroplast development [2, 9–10]

GLK transcription factor was first identified in maize (*Zea mays* L.), and was proven to be a new transcriptional regulator that functions on cellular differentiation in the leaves of maize [11]. The *GLK* genes belong to the GARP superfamily of nuclear transcription factors [12], which are defined by GOLDEN2 in maize, RESPONSE REGULATOR-B (ARR-B) proteins in *Arabidopsis* [13], and the PHOSPHATE STARVATION RESPONSE1 (PSR1) protein in *Chlamydomonas* [14]. Most GLK proteins contain two domains: a Myb-DNA-binding domain (DBD; containing a helix-loop-helix motif) and a C-terminal box (containing a GCT box) [15,16].

GLK genes are crucial for the formation and development of chloroplasts, and participating in various biotic and abiotic stress defense processes of organisms [17,18]. In *Arabidopsis*, *AtGLK1* and *AtGLK2*

genes were found to be involved in the production of chloroplast redundantly [19,20]. Overexpression of *AtGLK1* can cause resistance to *Fusarium graminearum* [21,22] and improve sensitivity on the virulent oomycete pathogen *Hyaloperonospora arabidopsidis* (Hpa) [1]. In addition, *SIGLK2* affects the photosynthesis of developing fruits and contributes to the characteristics of mature fruits in tomato (*Solanum lycopersicum*) [23]. Moreover, owing to the increased expression of chloroplast development and fruit-photosynthesis-related genes, the carbohydrates and carotenoids in ripe fruit were found to be enhanced in the overexpression of *SIGLK2* [24]. *ZmGLK1* is considered as a regulator of the development of chloroplasts in mesophyll cells of C4 tissues, while *GLK* gene pairs plays a redundant role in C3 species and promote the development of chloroplasts in maize [14,25].

Poplar is an important model plant in the study of woody plants, with the characteristics of rapid growth and easy genetic transformation. The accomplishment of the poplar genome sketch provides potential in gene identification and gene function analysis. The *GLK* genes have been identified and described in maize [26], *Arabidopsis* [17], tomato [27], and tobacco [28]. Nevertheless, there has been no comprehensive study on the *GLK* family genes of *P. trichocarpa*. In this study, 55 putative *PtGLK* genes were identified and classified into 11 groups, taking maize *GLKs*, *Arabidopsis GLKs*, and their related conserved domains as references. A comprehensive bioinformatics analysis was carried out to study gene structure, domain composition, chromosome distribution, syntheses analysis, and expression patterns. Promoter *cis*-elements and expression level of genes in response to abiotic stress (cold and osmotic) and phytohormone (MeJA and GA) treatments were also examined. The information derived from this study offers a valuable resource for further study on the characterization and function of the poplar *GLK* gene family.

2. Materials And Methods

2.1. Plant material treatment, RNA extraction, qRT-PCR analysis, and gene expression analysis

The material used in this study was poplar 84 K (*Populus alba*×*Populus glandulosa*) which is an aspen hybrid poplar from Korea. Poplar trees were grown under the settings of 16 h light/8 h dark and were maintained at 25°C and 80% relative humidity in a greenhouse. Three-month-old poplar seedlings were treated with osmotic stress, cold stress, and MeJA and GA treatments. For cold stress, the seedlings were positioned in a 4°C growth chamber and sampled at 0 h, 1 h, 3 h, 6 h, 12 h and 24 h after stress imposition. For osmotic stress, the seedlings were accumulated at 0 h, 1 h, 2 h, 3 h, 6 h, 12 h and 24 h after being sprayed with 20% polyethylene glycol (PEG) 6000. For phytohormone treatments, a solution of 100 µM jasmonic acid (JA) and 300 mg/L gibberellic acid (GA) were sprinkled onto poplar plants on the basis of the requirements and sampled randomly at 0 h, 1 h, 3 h, 6 h, 12 h and 24 h after the phytohormone treatments were applied. Seedlings irrigated at 28°C in an artificial growth chamber and sprinkled with distilled water were used as negative controls and were sampled at 0 h.

Total RNA was extracted from the treated samples using an RNAPrep Pure Plant Kit (TransGen Biotech, Beijing, China) on the basis of the manufacturer's instructions [29]. The expression level of 18S rRNA was used as an internal control to standardize the expression values of each gene, and the relative expression level of each gene was counted via the $2^{-\Delta\Delta Ct}$ method. The primers of the 11 *PtGLK* genes were designed by the NCBI Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to amplify 150–250 bp PCR products (Table S4). The heatmap of *PtGLK* gene expression was generated using the Amazing Heatmap module in TBtools for the poplar FM (female catkins, prior to seed release), F (female catkins, post-fertilization), M (male catkins), ML (mature leaf), REF (washed fibrous roots < 0.5 cm diameter from field-grown trees), RTC (roots from plants in tissue culture), G43h (seedlings were germinated 43 h post-imbibition), ApB (actively growing shoot apex), AxB (axillary bud), YFB (newly initiated female floral buds), YMB (newly initiated male floral buds), Xylem1 (developing phloem), Phloem3 (developing phloem/cambium), and PC (phloem, cortex, epidermis) [30,31].

2.2. Identification of *PtGLKs*

Poplar GLK sequences were acquired from the Phytozome12.1 database (<https://phytozome.jgi.doe.gov>). The previously reported GLK protein sequences of *Arabidopsis* [19] were used for the purpose of identifying the poplar GLK proteins for a BLAST alignment of the poplar protein database. More than 30% similarity and an E-values below 0.001 were set as the parameters to determine the poplar candidate GLK proteins. Then the domains of all poplar GLK proteins were investigated using Pfam (<http://pfam.xfam.org/>) to determine the putative proteins. The gene IDs, physical positions, sequences of the genes and proteins, and the coding sequences (CDS) were downloaded from the *P. trichocarpa* genome database (<https://genome.jgi.doe.gov/portal/Poptr1/Poptr1.home.html>). The detailed physical parameters of *PtGLK* genes, including molecular weight (MW) of amino acids, isoelectric point (pI), and length of the CDS, were predicated using ExPASy (http://www.expasy.ch/tools/pi_tool.html) [32].

2.3. Multiple sequence alignment and phylogenetic analysis

The protein sequences of poplar GLK proteins were aligned with the ClustalW tool [33]. The alignment of the *PtGLK*-domain-containing sequence was displayed by DNAMAN (<https://www.lynnon.com/dnaman.html>). The phylogenetic tree based on the complete *PtGLK* sequences was produced with MEGA 7.0 using the neighbor-joining method with 1000 bootstrap replications. Additionally, a combined phylogenetic tree of GLK protein sequences from *P. trichocarpa*, *Z. mays*, and *Arabidopsis* was also constructed via the same N-J method.

2.4. Gene and protein structure analysis

The exon/intron structures of *PtGLK* genes were decided by the Gene Structure Display Server (GSDS) platform (<http://gsds.cbi.pku.edu.cn/>) using the complete genomic sequence and CDS retrieved from the Phytozome 12.1 database (<https://phytozome.jgi.doe.gov>) [34]. The conserved motifs presented in *PtGLK* proteins were analyzed by the online MEME tool (<http://meme-suite.org/tools/meme>) [35] according to the following rules: optimum width of motifs at 5–50, and maximum number of motifs at 10 residues for *PtGLK* proteins. Motif annotation was identified using the Pfam tools. The predict protein homology

model was analyzed using the Phyre2 website (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>), and alignment of the PtGLK protein sequences was determined via Hidden Markov Models (HMM) with the HMM-HMM search in intensive mode [36].

2.5. Chromosomal location, synteny analysis and duplication events

Chromosomes location information was acquired from the *P. trichocarpa* genome database on the basis of the genome annotation file (gff file), and all *PtGLKs* were mapped onto the poplar chromosomes by MapInspect software (<http://www.softsea.com/review/MapInspect.html>). The possible gene duplication landscape was identified by the Multiple Collinearity Scan Toolkit (MCScanX) software [37]. Segmental duplication and tandem duplication were determined according to the means covered by Wang et al. (2010) [38]. The syntenic maps were subsequently displayed using the Dual Systemy Plotter software (<https://github.com/CJ-Chen/TBtools>) [39].

Ka and Ks were computerized by KaKs Calculator 2.0 on the basis of the pairwise alignments of paralogous and orthologous gene sequence with Clustalx 2.11 [40,41]. The Ka/Ks ratios were then calculated using DnaSP5 to investigate the gene duplication events [42].

2.6. Putative promoter region analysis of PtGLK genes

The 2000 bp upstream sequences of 55 *PtGLK* genes were selected as the putative promoter regions to determine the *cis*-elements. The putative *cis*-regulatory elements were identified by PlantCARE (<http://www.dna.affrc.go.jp/PLACE/>), and those that responded to abiotic stresses and hormone treatments were screened out [43,44].

3. Results

3.1. Identification of PtGLK genes in *P. trichocarpa*

To identify the *PtGLK* gene family in *P. trichocarpa*, previously reported Arabidopsis AtGLK1 (AT2G20570) and AtGLK2 (AT5G44190) protein sequences [19] were used as BLASTP sequences in extensive searches and alignment of usable protein databases in the poplar genome bank. A total of 55 *PtGLK* genes (*PtGLK1-PtGLK55*) were identified, all these were used to affirm the existence of the Myb-DNA-binding domain (DBD) through the Pfam database. To further examine the similarity among the PtGLK protein domains, multiple alignments of 55 PtGLK protein domain sequences were conducted with the DNAMAN 8 platform (Fig. 1). The result indicated that the *PtGLKs* were conserved across two regions of the Myb-DNA-binding domain with the HLH structure of the first helix containing the initial sequence PELHRR and the second helix containing NI/VASHLQ, which was consistent with the *GLK* members in *Z. mays* [26,46], *Arabidopsis* [5], tomato [27,45], and tobacco [28].

Base information about the *PtGLK* genes is presented in Table 1, including accession number, gene location, protein length, molecular weight (MW), exon numbers, and physicochemical parameters. The

PtGLK genes exhibited an inclusive conservation of amino acid sequence lengths and molecular weights. The encoded amino acid sequences ranged from 282 aa to 486 aa, and the predicted molecular weight (MW) varied from 28.87 to 53.24 kDa. Moreover, the theoretical isoelectric point (pI) ranged from 5.55 to 9.46.

Table 1
Detailed information about 55 predicted *PtGLK* genes in *P. trichocarpa*.

Gene name	Sequences ID	Position	MW (Da)	PI	CDS Length (dp)	Size (aa)	Exons
PtGLK1	Potri.001G133400	1:10847419–10850001(-)	31627.53	8.81	849	282	6
PtGLK2	Potri.001G137600	1:11217022–11222607(-)	42631.70	9.23	1137	378	6
PtGLK3	Potri.001G228500	1:24773549–24785927(+)	44752.57	9.46	1194	397	7
PtGLK4	Potri.001G243600	1:26128861–26132400(+)	43710.04	5.60	1173	390	1
PtGLK5	Potri.001G280000	1:29369697–29372077(+)	40494.88	9.17	1095	364	5
PtGLK6	Potri.001G314800	1:32569346–32574020(+)	33183.56	6.19	924	307	6
PtGLK7	Potri.002G130200	2:9870763–9874363(+)	37341.82	9.13	1026	341	6
PtGLK8	Potri.002G257800	2:24613058–24620174(+)	55859.36	5.67	1527	508	9
PtGLK9	Potri.003G096300	3:12224274–12229729(+)	42662.42	8.32	1137	378	6
PtGLK10	Potri.003G100100	3:12532374–12535858(+)	31744.78	9.35	852	283	7
PtGLK11	Potri.004G010000	4:578584–581272(-)	33504.16	9.29	900	299	6
PtGLK12	Potri.004G057900	4:4830585–4835870(-)	44811.55	9.13	1182	393	8
PtGLK13	Potri.004G082400	4:6782882–6788780(-)	54009.82	8.91	1461	486	6
PtGLK14	Potri.004G144800	4:16752076–16754518(-)	46587.06	6.78	1263	420	5
PtGLK15	Potri.005G134600	5:10350601–10352768(+)	40982.06	6.61	1092	363	5
PtGLK16	Potri.006G000800	6:65468–69307(-)	36861.30	5.82	1008	335	7
PtGLK17	Potri.006G034900	6:2203147–2204103(-)	34337.29	6.29	957	318	1

Gene name	Sequences ID	Position	MW (Da)	PI	CDS Length (dp)	Size (aa)	Exons
PtGLK18	Potri.006G101000	6:7706396–7709168(+)	29535.26	8.45	774	257	6
PtGLK19	Potri.006G155200	6:13942764–13945934(+)	50341.05	6.90	1383	460	4
PtGLK20	Potri.006G191000	6:19805840–19809519(-)	46157.24	6.22	1236	411	7
PtGLK21	Potri.007G003200	7:217578–223435(+)	52901.02	5.89	1452	483	8
PtGLK22	Potri.007G039400	7:3211363–3213546(+)	39293.93	6.39	1056	351	5
PtGLK23	Potri.008G071700	8:4407226–4410438(-)	40441.08	6.47	1098	365	5
PtGLK24	Potri.008G081800	8:5130397–5132950(-)	39266.66	7.74	1071	356	6
PtGLK25	Potri.008G087600	8:5451126–5452933(-)	36023.53	6.28	969	322	6
PtGLK26	Potri.008G117500	8:7521481–7524148(+)	41765.06	7.02	1146	381	6
PtGLK27	Potri.008G142000	8:9567883–9571945(+)	33287.23	6.41	891	296	6
PtGLK28	Potri.009G075100	9:7303554–7305935(-)	36669.94	8.39	984	327	7
PtGLK29	Potri.009G106600	9:9281411–9284063(-)	44701.97	7.68	1221	406	5
PtGLK30	Potri.010G099600	10:12276444–12280250(-)	33351.41	6.52	894	297	6
PtGLK31	Potri.010G128900	10:14543665–14546219(-)	42886.00	7.04	1173	390	5
PtGLK32	Potri.010G167901	10:17078789–17080240(+)	36251.75	6.56	984	327	6
PtGLK33	Potri.010G174100	10:17485942–17488292(+)	39535.00	8.24	1071	356	6
PtGLK34	Potri.010G185700	10:18281593–18283962(+)	40985.52	8.17	1080	366	5
PtGLK35	Potri.011G023600	11:1756622–1758677(+)	29795.92	6.90	783	260	6

Gene name	Sequences ID	Position	MW (Da)	PI	CDS Length (dp)	Size (aa)	Exons
PtGLK36	Potri.011G067150	11:5850100–5859105(-)	45526.25	9.05	1215	404	8
PtGLK37	Potri.012G042100	12:3747827–3754998(-)	47609.77	7.74	1311	436	6
PtGLK38	Potri.013G048000	13:3422495–3427517(-)	48374.34	5.47	1296	431	7
PtGLK39	Potri.013G060200	13:4416769–4421033(-)	46986.79	6.32	1269	422	6
PtGLK40	Potri.014G000700	14:86919–101555(-)	46972.34	5.25	1272	423	8
PtGLK41	Potri.014G037200	14:2349029–2352687(+)	37514.97	6.76	1029	342	6
PtGLK42	Potri.015G031600	15:2434069–2440389(+)	48391.68	8.50	1332	443	6
PtGLK43	Potri.016G001100	16:49984–54790(-)	34335.52	5.56	945	314	7
PtGLK44	Potri.016G001200	16:54862–58064(-)	33886.38	6.07	945	314	6
PtGLK45	Potri.016G032600	16:1838128–1840398(-)	34582.52	6.20	972	323	1
PtGLK46	Potri.016G047900	16:3088672–3091778(+)	43015.82	7.20	1143	380	7
PtGLK47	Potri.016G048000	16:3093112–3095536(+)	35155.62	8.15	942	313	6
PtGLK48	Potri.016G117000	16:12149752–12152009(+)	28871.56	7.14	753	250	6
PtGLK49	Potri.016G121800	16:12632080–12634309(-)	42465.18	9.06	1137	378	5
PtGLK50	Potri.017G054800	17:4223359–4227988(+)	33428.77	5.79	930	309	6
PtGLK51	Potri.017G137600	17:13885991–13891467(+)	53240.15	8.42	1449	482	6
PtGLK52	Potri.018G074200	17:8850610–8853341(-)	50668.48	6.66	1392	463	4
PtGLK53	Potri.018G151400	18:16178867–16182619(-)	49807.24	6.37	1356	451	8

Gene name	Sequences ID	Position	MW (Da)	PI	CDS Length (dp)	Size (aa)	Exons
PtGLK54	Potri.019G020900	19:3280854–3285857(-)	48638.99	5.55	1311	436	8
PtGLK55	Potri.019G032700	19:4541705–4544412(+)	46728.47	8.46	1263	420	7

3.2. Phylogenetic analysis of the GLK genes and the determination of gene structures

To analyze the evolutionary relationship of the poplar GLK family, a neighbor-joining phylogenetic tree was produced by aligning 55 PtGLK protein sequences with 59 and 42 protein sequences from *Z. mays* [26] and *Arabidopsis*, respectively [5]. The detailed information of *ZmGLK* genes and *AtGLK* genes are listed in Table S1. In the phylogenetic tree, the GLK family members were classed into 13 groups according to the evolutionary relationships and motif analysis of poplar GLK proteins, and PtGLKs were assigned into 11 groups (G1-G11), but not G12 and G13. The numbers of PtGLK members in different groups was unbalanced, with groups 1 to 11 containing 11, 18, 1, 2, 2, 3, 2, 5, 5, 2, and 6 proteins, respectively (Fig. 2).

A separate phylogenetic tree only with PtGLK proteins was formed to provide additional insight into the structure characteristics of *PtGLK* genes, and all PtGLK proteins were grouped into 11 subfamilies which is consistent with the phylogenetic tree of *P. trichocarpa*, *Z. mays*, and *Arabidopsis*. Exon/intron organization analysis of the *PtGLK* genes, which were defined by the arrangement of *PtGLK* genes, could gain additional insight into the development of poplar GLK family members. The number of exons in the subfamilies ranged from 1 to 9 (Fig. 3). More than half of the *PtGLK* genes (75%) had six or more exons, and only five genes (9) had four or fewer exons. The vast majority of the *PtGLK* genes that assembled into the same subfamily exhibited similar or identical exon/intron distributions, including the number of exons and their length. In total, phylogenetic analysis and conservative gene structure provide reliable grouping classification results for *PtGLK* members in the same group. Additionally, the exon/intron structure of each segmentally duplicated pair showed homologous exon/intron distributions.

3.3. Identification of conserved sequence motifs and determination of homology modeling in poplar GLK genes

The conserved motifs of 55 PtGLK proteins within each subfamily were analyzed by MEME software. Eight distinct motifs were identified, and detailed sequence information of each motif is displayed in Table S2. With the Conserved Domain Database, six putative motifs were functional comments, being defined as Myb-SHAQKYF for motifs 1, 3, 5, 6, and 8, and Myb-CC-LHEQLE for motif 2 (Fig. 4). Nevertheless, no functional notes were given to the remaining two putative motifs. Members of the protein family gathered in the same subfamily displayed similar or identical motif components and

spatial distributions, which revealed the functional similarities of these proteins. For example, all the PtGLK proteins contained a Myb DNA-binding domain (motif 3), with an HLH structure (with the first helix containing initial sequences PELHRR and invariably comprising 14 amino acids and the second helix containing an initial NI/VASHLQ motif). Except for the conserved GLK Myb-DNA-binding domain, the members within each subfamily had specificity motifs that probably represent their variety functions in plant development and in response to abiotic stress (Figure S1). For instance, motif 2 (Myb-CC-LHEQLE) only appeared in subfamilies 3, 4, 5, 6, 7, 8, 9, 10, and 11.

To further investigate the potential structures of the PtGLK proteins, we made use of Phyre2 to predict the homology modeling and aligned the protein sequences with the HMM-HMM search in intensive mode [46]. The result showed that each PtGLK protein could be modeled with confidence, and 12 PtGLKs (PtGLK17, PtGLK18, PtGLK19, PtGLK22, PtGLK26, PtGLK28, PtGLK29, PtGLK30, PtGLK31, PtGLK35, and PtGLK48) among them had 100% of their predicted lengths modeled with > 40% confidence (Fig. 5).

3.4. Chromosomal locations and synteny analysis of PtGLK genes in *P. trichocarpa*

A total of 55 *PtGLKs* were acquired and were distributed to the 19 poplar chromosomes (Chr1-Chr19) (Fig. 6). The number of *PtGLKs* per chromosome ranged from one to seven. For example, chromosome 16 contained seven *PtGLK* genes, with the largest number, followed by chromosome 1, with six, and chromosomes 6, 8, and 10 with five. Conversely, chromosomes 12 and 15 possessed only one *PtGLK* gene each. In addition, the potential duplication events were analyzed by the MCScanX program to search the mechanism for the *PtGLK* gene family. A total of 22 duplicated pairs of *PtGLK* genes were defined as segmental duplication gene pairs, but not tandem duplication gene pairs, in a syntenic map (Fig. 7A, and Table 2). Moreover, the analysis showed that there was an unevenly distribution mode among the 22 segmental duplicated pairs on the 19 chromosomes. These results suggested that segmental duplication events probably play a primary role in the amplification of the poplar GLK gene family.

To further determine the evolutionary orthologous relationships of *PtGLKs*, two comparative syntenic maps of *P. trichocarpa* related to *Arabidopsis* and *Z. mays* were also drawn (Fig. 7B). As shown in Tables 3, 22 and 11 orthologs of *P. trichocarpa* between *Arabidopsis* (Pt-At) and *Z.mays* (Pt-Zm) were identified, respectively. Moreover, highly conserved microsynteny was found among the regions of *PtGLK* genes between *P. trichocarpa* and *Arabidopsis*, especially in Pt8 and At1 and in Pt10 and At1, with seven and four synteny genes, respectively.

3.5. Evolutionary and divergence patterns of the GLK gene family

For each *PtGLK* gene pair, the Ka/Ks ratios were calculated to evaluate divergence times and selective pressure for the duplicated *PtGLK* genes. To further search the evolutionary events and divergence profiles of *GLK* genes between *P. trichocarpa* and *Arabidopsis*, statistical analysis of the Ka/Ks ratios and the Ks values were conducted. As shown in Fig. 8 and Table 2, the average frequency distribution of

the calculated Ks values of paralogous pairs (Pt-Pt) was approximately 0.24, suggesting that *PtGLK* genes went through a large-scale duplication event was about 17 million years ago (MYA). Compared with a prior study indicating the timing of a whole-genome duplication in *P. trichocarpa* at 7–12 MYA [47], this result indicated that the large-scale duplication of *PtGLK* genes occurred earlier [42]. Additionally, the frequency distributions of Ks values for the orthologous pairs from the *P. trichocarpa* and *Arabidopsis* genomes averaged ~ 2.25 (Fig. 8, Table 3), suggesting that the divergence time of the *GLK* genes was 118 MYA. With reference to a previous study, it can be inferred that the divergence times between *P. trichocarpa* and *Arabidopsis* were 102–113, this result indicated that the *PtGLK* genes went through gene evolution before the separation of *Z. mays*. The Ka/Ks ratios peak in the poplar genome (Pt-Pt) and between the *P. trichocarpa* and *Arabidopsis* genomes (Pt-At) were distributed between 0.14–0.50 (Table 2) and 0.06–0.20 (Table 3), respectively, which suggests that *PtGLK* genes have probably experienced highly positive purifying selection between *P. trichocarpa* and *Arabidopsis* genomes, as well as being paralogous in the poplar genome.

Table 2
Ka/Ks and divergence analysis of paralogous *PtGLK* gene pairs (Pt-Pt) in *P. trichocarpa*.

Paralogous	Ka	Ks	Ka/Ks	Selection pressure	Duplicate type
<i>PtGLK2/9</i>	0.07	0.22	0.29	Purifying selection	Segmental duplication
<i>PtGLK1/10</i>	0.06	0.21	0.31	Purifying selection	Segmental duplication
<i>PtGLK5/28</i>	0.11	0.23	0.50	Purifying selection	Segmental duplication
<i>PtGLK6/50</i>	0.03	0.17	0.17	Purifying selection	Segmental duplication
<i>PtGLK7/41</i>	0.10	0.16	0.65	Purifying selection	Segmental duplication
<i>PtGLK12/36</i>	0.12	0.24	0.49	Purifying selection	Segmental duplication
<i>PtGLK13/51</i>	0.05	0.24	0.22	Purifying selection	Segmental duplication
<i>PtGLK14/29</i>	0.07	0.23	0.29	Purifying selection	Segmental duplication
<i>PtGLK15/22</i>	0.08	0.26	0.31	Purifying selection	Segmental duplication
<i>PtGLK16/43</i>	0.04	0.16	0.28	Purifying selection	Segmental duplication
<i>PtGLK17/45</i>	0.03	0.24	0.14	Purifying selection	Segmental duplication
<i>PtGLK18/48</i>	0.06	0.33	0.18	Purifying selection	Segmental duplication
<i>PtGLK19/52</i>	0.06	0.30	0.21	Purifying selection	Segmental duplication
<i>PtGLK20/46</i>	0.08	0.30	0.27	Purifying selection	Segmental duplication
<i>PtGLK21/40</i>	0.07	0.31	0.23	Purifying selection	Segmental duplication
<i>PtGLK24/33</i>	0.05	0.18	0.28	Purifying selection	Segmental duplication
<i>PtGLK25/32</i>	0.06	0.18	0.36	Purifying selection	Segmental duplication
<i>PtGLK26/31</i>	0.07	0.29	0.25	Purifying selection	Segmental duplication
<i>PtGLK27/30</i>	0.09	0.27	0.34	Purifying selection	Segmental duplication
<i>PtGLK37/42</i>	0.08	0.24	0.33	Purifying selection	Segmental duplication
<i>PtGLK38/54</i>	0.10	0.20	0.50	Purifying selection	Segmental duplication
<i>PtGLK39/55</i>	0.07	0.28	0.24	Purifying selection	Segmental duplication

Table 3
Ka/Ks and divergence analysis of orthologous *GLK* gene pairs (Pt-At)
in *P. trichocarpa* and *Arabidopsis*.

Paralogous	Ka	Ks	Ka/Ks	Selection pressure
<i>PtGLK26/AtGLK1</i>	0.32	1.68	0.19	Purifying selection
<i>PtGLK31/AtGLK1</i>	0.33	1.66	0.20	Purifying selection
<i>PtGLK26/AtGLK3</i>	0.30	1.95	0.15	Purifying selection
<i>PtGLK31/AtGLK3</i>	0.30	2.52	0.12	Purifying selection
<i>PtGLK2/AtGLK4</i>	0.33	1.80	0.19	Purifying selection
<i>PtGLK9/AtGLK4</i>	0.30	2.46	0.12	Purifying selection
<i>PtGLK26/AtGLK6</i>	0.28	3.07	0.09	Purifying selection
<i>PtGLK25/AtGLK7</i>	0.31	1.93	0.16	Purifying selection
<i>PtGLK32/AtGLK7</i>	0.29	1.73	0.17	Purifying selection
<i>PtGLK24/AtGLK8</i>	0.24	1.66	0.15	Purifying selection
<i>PtGLK33/AtGLK8</i>	0.22	2.04	0.11	Purifying selection
<i>PtGLK17/AtGLK17</i>	0.27	4.41	0.06	Purifying selection
<i>PtGLK45/AtGLK17</i>	0.27	3.27	0.08	Purifying selection
<i>PtGLK55/AtGLK19</i>	0.25	2.25	0.11	Purifying selection
<i>PtGLK54/AtGLK20</i>	0.44	1.82	0.24	Purifying selection
<i>PtGLK21/AtGLK23</i>	0.33	2.10	0.16	Purifying selection
<i>PtGLK40/AtGLK23</i>	0.35	1.94	0.18	Purifying selection
<i>PtGLK4/AtGLK27</i>	0.36	2.29	0.16	Purifying selection
<i>PtGLK8/AtGLK31</i>	0.35	1.71	0.20	Purifying selection
<i>PtGLK13/AtGLK35</i>	0.33	2.00	0.17	Purifying selection
<i>PtGLK41/AtGLK38</i>	0.32	1.84	0.18	Purifying selection
<i>PtGLK1/AtGLK40</i>	0.38	3.29	0.12	Purifying selection

3.6. Expression profiles of *PtGLK* genes in different tissues and stages of *P. trichocarpa*

To characterize the dynamics of *PtGLK* gene expression, we studied gene expression patterns in various vegetative tissues and different stages of reproductive development using high-throughput RNA sequencing (RNA-seq) data from a public database produced in a previous study [48]. The *GLK*

expression patterns were analyzed in 14 tissues or development stages of *P. trichocarpa*, including: FM, F, M, ML, PC, G43h, YFB, ApB, AxB, REF, RTC, YMB, Xylem1, and Phloem3. Detailed information about the RNA-seq data for the 55 *PtGLK* genes are listed in Table S3. Hierarchical clustering of the heatmap showed that *PtGLK* genes had divergence expressed in a variety of poplar tissues and development stages (Fig. 9). According to the expression profiles in 14 tissues, the poplar GLK family genes were divided into seven clusters (C1-C7). The four genes (*PtGLK21*, *PtGLK43*, *PtGLK45*, *PtGLK54*) clustered in C2 showed high expression levels in Xylem1 and Phloem3 tissues. A total of 20 genes grouped in C4/C5 were highly expressed in FM, F, and M. Additionally, many genes in C3 (except *PtGLK21*, *PtGLK36*, and *PtGLK41*) displayed high expression levels in Phloem3. In contrast, the majority of the 20 genes (C4, C5) presented lower expression levels in Phloem3. Taken together, the results showed that *PtGLKs* presented diverse expression profiles in different tissues and senescence stages, providing preliminary insight into the further functional exploration.

3.7. Analysis of cis-regulatory elements in the promoter regions of *PtGLK* genes

To study the *cis*-regulatory elements (CREs) in the promoter regions of the *PtGLK* genes, we searched the 2000 bp of the genomic sequence upstream from the translation start site as the putative promoter regions within the PlantCARE [48]. Analysis of the promoters of *PtGLKs* in *P. trichocarpa* revealed that various potential CREs corresponding to defense and stress, light responsiveness, cold responsiveness, osmotic responsiveness, MeJA responsiveness, GA responsiveness, IAA responsiveness, and SA responsiveness were identified. Detailed elements are listed in Fig. 10 and Table S5. The numbers of CREs were also significantly different in the promoters of different poplar *GLK* gene family members. The promoters of *PtGLK30* contained the highest variety of CREs (MBS, G-box, Box4, ARE, ABRE, TCT-motif, TCCC-motif, TCA-element, P-box, GT1-motif, LTR, AE-box, TGACG-motif, MRE, and CGTCA-motif), while *PtGLK43* contained only seven kinds of CREs. Moreover, all *PtGLKs* contained one or more abiotic stress elements, this result revealed that the expression of most *PtGLK* genes was associated with abiotic stress. Additionally, a total of 36 *PtGLKs* (65.5%) had two or more phytohormone induction elements, and *PtGLK24*, *PtGLK46* and *PtGLK57* included all five phytohormone induction elements, such as IAA-, ABA, GA-, MeJA- and SA- induction elements (Fig. 10). The analysis of CREs displayed that the number and distribution of CREs in different *PtGLK* genes were dissimilar, suggesting that each *PtGLK* gene was controlled by differing groups of TFs and that the expression of *PtGLKs* could respond to different abiotic stresses and phytohormone treatments.

3.7. *PtGLK* gene expression profiles in response to abiotic stress and phytohormone treatments

Several *GLK* genes have been studied to be related to the regulation of abiotic stresses and phytohormone response in maize [26], tobacco [27], and tomato [28]. To explore whether *PtGLK* genes also had the same function, the dynamic expression of 11 *PtGLK* genes (*PtGLK1*, *PtGLK3*, *PtGLK6*, *PtGLK16*, *PtGLK17*, *PtGLK21*, *PtGLK32*, *PtGLK36*, *PtGLK38*, *PtGLK48*, and *PtGLK53*) as representatives of

each subfamily were randomly selected and detected in response to abiotic stresses and phytohormone treatments (Table S6). As shown in Fig. 11, there were five, six, five, and three genes, and the change of their expression levels were greater than or equal to fivefold in comparison with 0 h, showing themselves as significantly changed genes in response to cold stress, osmotic stress, MeJA, and GA treatments, respectively. Among them, *PtrGLK3*, *PtrGLK21*, *PtrGLK32*, and *PtrGLK53* were up-regulated both by cold and osmotic stresses, and *PtrGLK1*, *PtrGLK21*, and *PtrGLK53* were up-regulated under both MeJA and GA treatments. In addition, *PtrGLK38* (> 60-fold that of 0 h), *PtrGLK53* (> 70-fold that of 0 h), *PtrGLK3* (> 60-fold that of 0 h), and *PtrGLK53* (> 30-fold that of 0 h) were the most highly expressed after 12 h of cold stress, osmotic stress, MeJA, and GA treatments, respectively. We also found that only the expression of *PtrGLK53* was strong in response to all the four different treatments. Above all, these results implied that *PtGLKs* were in response to most of the applied abiotic stress and hormone treatments.

4. Discussion

4.1. *PtGLKs* in *P. trichocarpa*

The *GLK* genes have only been found in photosynthetic eukaryotes, such as green algae and higher plants, and they participate in the development of chloroplasts [16,49]. In previous research, detailed characteristics and functions of *GLK* genes were identified in *Arabidopsis* [17], maize [26], tobacco [28], and tomato [27]. However, the poplar *GLK* transcription factor has not yet been described up until now. In the current study, 55 putative *PtGLK* genes were identified in the poplar genome. The numbers of poplar *GLK* subfamily members were 13, 1, and 10 more than *Arabidopsis* (42), tomato (54), and sorghum (45), respectively. The greater number of *PtGLK* genes contain far more genes than those in these three species, which showed that the poplar genome size is substantially larger and is consistent with the genome duplication event [50,51].

According to the phylogenetic analysis, the predicted poplar *GLK* subfamily members were classified into 11 groups (G1-G11), and all 11 groups contained different genes from maize and *Arabidopsis*, suggesting that the *PtGLK* genes had diversified before these four species evolved. What is more, there were no orthologous genes in maize G12 and G13, suggesting a divergence among *Z. mays* and *P. trichocarpa*. In addition, *PtGLKs* belonging to the same subfamilies exhibited highly similar characteristics on the basis of their domain and gene structures, which indicated that the *PtGLKs* groupings were relatively reliable.

4.2. Expansion of the *PtGLKs* suggests functional diversification

Analysis of the chromosome location showed that *PtGLKs* were extensive and in-homogeneously distributed in 19 poplar chromosomes, which could be owing to insertion, deletion, duplication, and reversion [52,53]. Among the 55 *PtGLKs*, 22 segmental duplication events occurred, but not tandem duplication events. Segmental duplication events were the main pathway for expansion of the poplar *GLK* gene family. Otherwise, segmental duplication has been shown to be more universal than tandem

duplication and plays a crucial role in the long-term evolution in much of the research [54–57]. The synthesis analysis of *P. trichocarpa* and *Z. mays* genome sequences made clear that there was a notable collinearity between *P. trichocarpa* and monocots maize, which coincided well with the evolutionary relationship between dicotyledons and monocotyledons.

To better explore the profiles of macroevolution and evaluate the evolutionary times in *P. trichocarpa*, the Ks and Ka for paralogous (Pt-Pt) and orthologous (Pt-At) gene pairs were evaluated, and the Ks and Ka/Ks values were also calculated for each gene pair. The Ks values indicated that a large-scale duplication event occurred ~ 17 MYA in *P. trichocarpa* and that the divergence times for orthologous gene pairs (Pt-At) was approximately 118 MYA. Aggerbeck et al. showed that a whole-genome duplication event in *P. trichocarpa* occurred 12–18 MYA and the divergence time between *P. trichocarpa* and *Arabidopsis* was 102–113 MYA [58,59]. When compared with these previous reports, our results suggest that the poplar GLK gene family went through an earlier large-scale duplication event and diversified before the separation of *Arabidopsis*. In addition, the Ka/Ks ratio can be used to define the effect of selective pressure selection on coding sequences [55]. Here, the Ka/Ks ratios for the Pt-Pt and Pt-At gene pairs were both < 1, suggesting that the *PtGLK* genes probably have went through strong purifying selection during evolution [33,60].

4.3. PtGLKs play an important role in poplar development

To predict possible functions of *PtGLK* genes in the growth and development of *P. trichocarpa*, we examined the expression patterns of 55 *PtGLK* genes in view of a previous reported transcriptome data. Most *PtGLK* genes showed high expression levels in xylem, which implied that they may have a function in the development of xylem. Generally speaking, compared with genes located in different subfamilies, genes in the same subfamily often have the same domains and similar functions. Previous studies show that two *Arabidopsis* genes (At5G44190.1 and At2G20570.2) were identified as functioning in leaf senescence [61]. In the current results, a total of 10 *PtGLK* genes (*PtGLK4*, *PtGLK14*, *PtGLK15*, *PtGLK17*, *PtGLK19*, *PtGLK22*, *PtGLK26*, *PtGLK31*, *PtGLK45*, and *PtGLK52*) were classified with the *Arabidopsis* GLK genes (At5G44190.1 and At2G20570.2) in G1 (Fig. 2), which suggested that these genes in different species were alike functionally and structurally. Therefore, it is speculated that these 10 putative *PtGLK* genes were involved in poplar Phloem3 senescence. The RNA-seq data showed that the transcript abundance of 17 *PtGLK* genes in group three decreased, which was closely related to the increase of leaf senescence level, indicating that these 17 *PtGLK* genes may play an essential role in the process of poplar leaf senescence. In addition, previous reports showed that the expression of *ZmGLK47* was high in all maize tissues and played a significant role in the formation and evolution of chloroplasts [26]. As the ortholog pair of *ZmGLK47* in *P. trichocarpa*, the *PtGLK4*, *PtGLK14*, *PtGLK15*, *PtGLK17*, *PtGLK19*, *PtGLK22*, *PtGLK26*, *PtGLK29*, *PtGLK31*, *PtGLK45*, and *PtGLK52* shared the same protein structure and conserved domains and also exhibited the same expression patterns.

4.4. Potential functions of PtGLKs in abiotic stress and phytohormone signaling responses

Plant genomes have various stress-related genes, allowing plants to respond to diverse environmental conditions [62]. The GLK family has been reported to play a significant role in abiotic stress and phytohormone treatment response, such as cold stress, osmotic stress, salinity stress, ABA, MeJA, GA, and SA [26, 27]. Additionally, the *cis*-elements of the promoter, to a large extent, determine the stress-responsive gene expression profiles which contribute to plants adaption to adverse conditions, and are associated with a variety of stimuli-responsive genes [63,64]. Therefore, we investigated the expression of 11 selected *PtGLK* genes under the two stress treatments and two phytohormone treatments. Preliminary research showed that orthologous genes of different species were conservative in gene functions, while paralogous genes presented different functions, because of gene duplication [65]. For instance, we found that the expression of *ZmGLK1* and *PtGLK32* (the ortholog of *ZmGLK1* in *P. trichocarpa*) displayed similar patterns in response to cold and osmotic stress [26]. However, the expression of *PtGLK17* and its ortholog in maize, *ZmGLK50*, exhibited opposite patterns, which suggested that *PtGLKs* could have lost or obtained new functions during evolution (Fig. 10). These results revealed that paralogous pairs probably play similar roles in the course of plant growth and development.

5. Conclusions

In this study, 55 members of the poplar GLK family were identified, which could be divided into 11 subfamilies on the basis of gene structures and conserved domains. Furthermore, the systematic analysis of chromosomal locations, synteny analysis, and evolutionary pattern provided valuable insight into the biological functions of the poplar GLK family members. The expression profiles of poplar GLK family genes indicated that *PtGLKs* were involved in different tissues and various stages of poplar growth and development. The expression levels of *PtGLKs* under different abiotic and phytohormone treatments provides a basis for understanding the role of *PtGLKs* in the stress and phytohormone response. Taken together, these results will provide valuable resources to further explore the potential functional characteristics of *PtGLKs* in *P. trichocarpa*.

Abbreviations

CDD	conserved domain database
DBD	Myb DNA-binding domain
DRE	osmotic-responsive element
GA	gibberellic acid
GLK	Golden2-like
LTRE	cold-responsive element
MeJA	methyl jasmonate
MW	molecular weight
MYA	million years ago
PEG	polyethylene glycol 6000
pI	isoelectric point
Ptr	<i>Populus trichocarpa</i>
SA	silica acid
FM	female catkins
F	female catkins
M	male catkins
ML	mature leaf
REF	washed fibrous roots
RTC	roots from plants in tissue culture
AxB	axillary bud
YFB	newly initiated female floral buds
YMB	newly initiated male floral buds
Xylem1	developing phloem
PC	Phloem, Cortex, Epidermis
Phloem3	developing phloem/cambium

Declarations

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Kh. X.; writing—original draft preparation, Rh.W.; writing—review and editing, Rh.W.; visualization, L.D.; supervision, L.D.; project administration, Rh.W.; funding acquisition, L.D.

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Figures

PtGLK1	KPRLRW	TADLH	DRFVDAV	TKLGG	PDKATPKSV	LRLMGLKGL	TLYHLKSHLQKYRLG	56			
PtGLK2	PRMRW	TTTTLH	ARFVHAV	ELLGG	HERATPKLV	LELMDVKDL	TLAHVKSHLQMYRT	54			
PtGLK3	KIRVRW	TQDLH	KRFVESV	NRLGG	AEKATPKG	ILREMDVHGL	TLHVKSHLQKYRT	55			
PtGLK4	RPRLW	VTPLQLH	KRFVDVVG	HLG	MKN	AVPKTIMQWM	NEGLTREN	VASHLQKYRL	54		
PtGLK5	PRLRW	IPDLH	LCFVQAV	ERLGG	YERATPKLV	LQLMNIKGL	SLIAHVKSHLQMYRSK	55			
PtGLK6	KPRLRW	TAELHER	FVDVA	QALGG	PDKATPKT	IMRTMGVKGL	TLYHLKSHLQKYRLG	56			
PtGLK7	PRMRW	TTTTLH	ARFVHAV	QLGG	HERATPKSV	LELMDVNDL	TLAHVKSHLQMYRT	54			
PtGLK8	KPRMRW	TPELHEA	FVEAVN	...NLG	GATPKGVLKLM	KVDSLT	IYHVKSHLQKYRT	52			
PtGLK9	PRMRW	TTTTLH	ARFVHAV	ELLGG	HERATPKSV	LELMDVKDL	TLAHVKSHLQMYRT	54			
PtGLK10	RPRLRW	TADLH	DRFVDAV	TKLGG	PDKATPKSV	LRLMGLKGL	TLYHLKSHLQKYRLG	56			
PtGLK11	PRLRW	APDLH	HCFVHAVER	LGGED	RATPKMV	LQIMDVKGL	TIHVKSHLQMYRSM	55			
PtGLK12	PRLRW	TPDLH	LSFVHAVER	LGGQEKATPKLV	LQLMNV	RGLSLIAHVKSHLQMYRSK	55				
PtGLK13	PRMRW	TSTLH	ARFVHAV	ELLGG	HERATPKSV	LELMDVKDL	TLAHVKSHLQMYRT	54			
PtGLK14	KHRROWSPELH	RQFVNAL	QQLGGAQVATPKQ	IRELMQV	DGLTNDEVKSHLQKYRLH			56			
PtGLK15	KQRROWSPELH	RQFVDAL	QQLGGYQVATPKQ	IRELMQV	DGLTNDEVKSHLQKYRLH			56			
PtGLK16	KQRLRW	THELHER	FVDVA	QALGG	PDPRATPKG	VLRVMGVQGL	TIYHVKSHLQKYRLA	56			
PtGLK17	RPRLW	VTPLQLH	KRFVDVA	HLG	IKNA	VPKTIMQLM	NVDGLTREN	VASHLQKYRL	54		
PtGLK18	KERLRW	TQELH	DRFEEAVN	QLGG	PDPRATPKG	ILRAMGIS	GLTIYHVKSHLQKYRTS	56			
PtGLK19	KARROWSPELH	RRFVNAL	HMLGGSQVATPKQ	IRELMKV	DGLTNDEVKSHLQKYRLH			56			
PtGLK20	KTRIRW	TQDLH	EKFVECVN	RLLGGAEKATPKA	ILNLM	SDGLTIFHVKSHLQKYRIA		56			
PtGLK21	KPRMRW	TPELHER	FVEAVNK	LDAEKATPKG	VLKLMNVKGL	TIYHVKSHLQKYRLA		56			
PtGLK22	KQRROWSPELH	RRFVDAL	QQLGGCQVATPKQ	IREHMQV	DGLTNDEVKSHLQKYRLH			56			
PtGLK23	PRLRW	TPDLH	LCFVHAVER	LGGQDRATPKLV	LQMMNTKDL	NIAHVKSHLQMYRSK		55			
PtGLK24	KPRLRW	TVELHER	FVDVA	QALGG	PDKATPKT	IMRVMGVKGL	TLYHLKSHLQKFRLG	56			
PtGLK25	KPRLRW	TQELH	QRFVEAVN	QLGG	ADRATPKSL	LMRVMEIPGL	TLYHLKSHLQKYRLG	56			
PtGLK26	KQRROWSPELH	RRFLHAL	RQLGGS	HAATPKQ	IRELMKV	DGLTNDEVKSHLQKYRLH		56			
PtGLK27	PRLRW	TPELH	RQFVHA	IERLGG	QDKATPKLV	LQMDVKGL	TIHVKSHLQMYRS	54			
PtGLK28	PRLRW	TPDLH	LCFVQAV	ERLGG	HERATPKLV	LQLMNF	KGLSLIAHVKSHLQMYRSK	55			
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PtGLK31	KQRROWSPELH	RRFLHSL	QQLGGS	HAATPKQ	IRELMKV	DGLTNDEVKSHLQKYRLH		56			
PtGLK32	KPRLRW	TQELH	QRFVEAVN	QLGG	ADRATPKSL	LMRVMEIPGL	TLYHLKSHLQKYRLG	56			
PtGLK33	KPRLRW	TVELHER	FVDVA	TQLGG	PDKATPKT	IMRVMGVKGL	TLYHLKSHLQKFRLG	56			
PtGLK34	PRLRW	TPDLH	LCFVHAVER	LGGQDRATPKLV	LQMMNIKGL	SLIAHVKSHLQMYRSK		55			
PtGLK35	KPRLRW	TLELHER	FVDVATL	LGG	PDKATPKA	IMRMGVKGL	TLYHLKSHLQKFRLG	56			
PtGLK36	PRLRW	TPDLH	LSFVHAVER	LGGQEKATPKLV	FQLMNV	RELSIAHVKSHLQRYRSK		55			
PtGLK37	PRMRW	TSSLH	ARFVHAV	ELLGG	HERATPKSV	LELMDVKDL	TLSHVKSHLQMYRT	54			
PtGLK38	KPRMRW	TPELHEA	FVEAVN	QLGGSERATPKG	VLKLMNV	EGLTIYHVKSHLQKYRS		55			
PtGLK39	KPRLRW	TPDLHER	FIEAVN	QLGG	ADKATPKT	VMKLMGIPGL	TLYHLKSHLQKYRLS	56			
PtGLK40	KPRMRW	TTELHER	FLDAVN	KLDGAEKATPKG	VLKLMNV	EGLTIYHVKSHLQKYRLA		56			
PtGLK41	PRMRW	TTTTLH	ARFVHAV	RLGG	HERATPKSV	LELMDVNDL	TLAHVKSHLQMYRT	54			
PtGLK42	PRMRW	TSSLH	SRFVHAV	ELLGG	HERATPKSV	LELMDVKDL	TLAHVKSHLQMYRT	54			
PtGLK43	KQRLRW	THELHER	FVDVA	QALGG	PDPRATPKG	VLRVMGVQGL	TIYHVKSHLQKYRLA	56			
PtGLK44	KPRLRW	TADLH	DRFVDAV	SQGG	PNKATPKA	ILRTMNVKGL	TLFHLKSHLQKYRLG	56			
PtGLK45	RPRLW	VTPLQLH	KRFVDVA	HLG	IKNA	VPKTIMQLM	NVDGLTREN	VASHLQKYRL	54		
PtGLK46	KTRIRW	TQDLH	EKFVECVN	RLLGGAEKATPKA	ILKLM	SDGLTIFHVKSHLQKYRS		55			
PtGLK47	KKRIRW	TQGLHEK	FIKCVNS	LGGAAK	AKPKA	ILKMMET	KGLTIVQVKSHLQKYRS	55			
PtGLK48	KDRLRW	TQELH	DRFEEAVN	QLGG	PDPRATPKG	ILRAMGIS	GLTIYHVKSHLQKYRTS	56			
PtGLK49	PRLRW	TPELH	LCFVHAVER	LGGQERATPKLV	LQMMNVNGL	SLIAHVKSHLQMYRSK		55			
PtGLK50	KPRLRW	TAELHER	FVDVA	TQLGG	PDKATPKT	IMRTMGVKGL	TLYHLKSHLQKYRLG	56			
PtGLK51	PRMRW	TSTLH	ARFVHAV	ELLGG	HERATPKSV	LELMDVKDL	TLAHVKSHLQMYRT	54			
PtGLK52	KARROWSPELH	RRFVNAL	HMLGGSQVATPKQ	IRELMKV	DGLTNDEVKSHLQKYRLH			56			
PtGLK53	KQIRRW	TTELH	DLFVDAV	KS	LGGPDVATPKS	ILGIMNVKGL	SIYHVKSHLQKYRLA	56			
PtGLK54	KSRMRW	TPELHEA	FVEAVN	QLGGSERATPKG	VLKQMN	VEGLTIYHVKSHLQKYRT		55			
PtGLK55	KPRLRW	TTDLHER	FIEAVN	QLGG	ADKATPKT	VMKLMGIPGL	TLYHLKSHLQKYRLS	56			
Consensus	r	w	lh	f	a	pk	m	l	shlq	r	

Figure 1

Multiple sequence alignment of the PtGLK conserved domain.

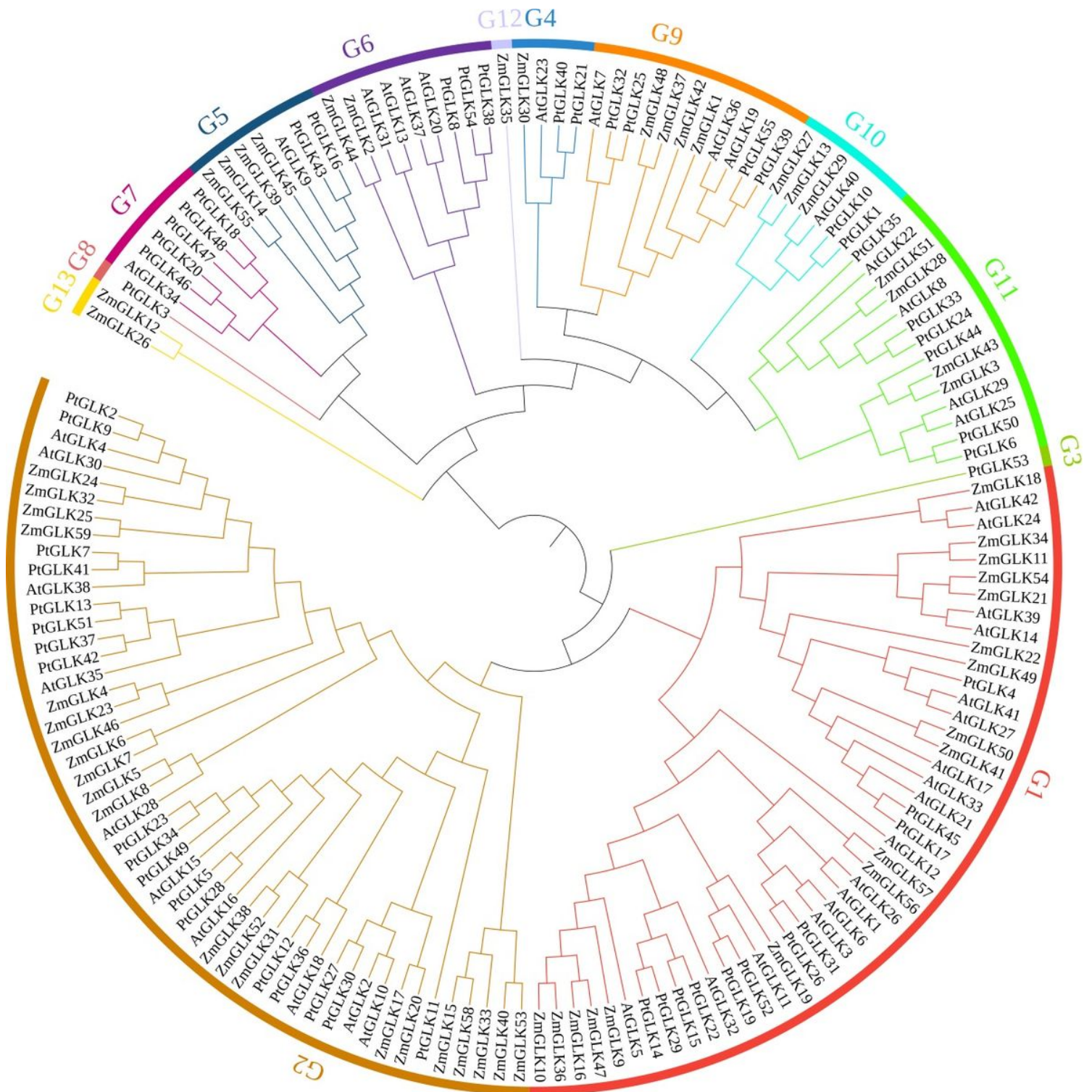


Figure 2

Phylogenetic relationships of GLK proteins of *P. tichocarpa*, *Z. mays*, and *Arabidopsis*. The tree was produced using the verging neighbor-joining method with MEGA7.0. Each specific color represented one group in the branches, and 13 groups were discovered in total.

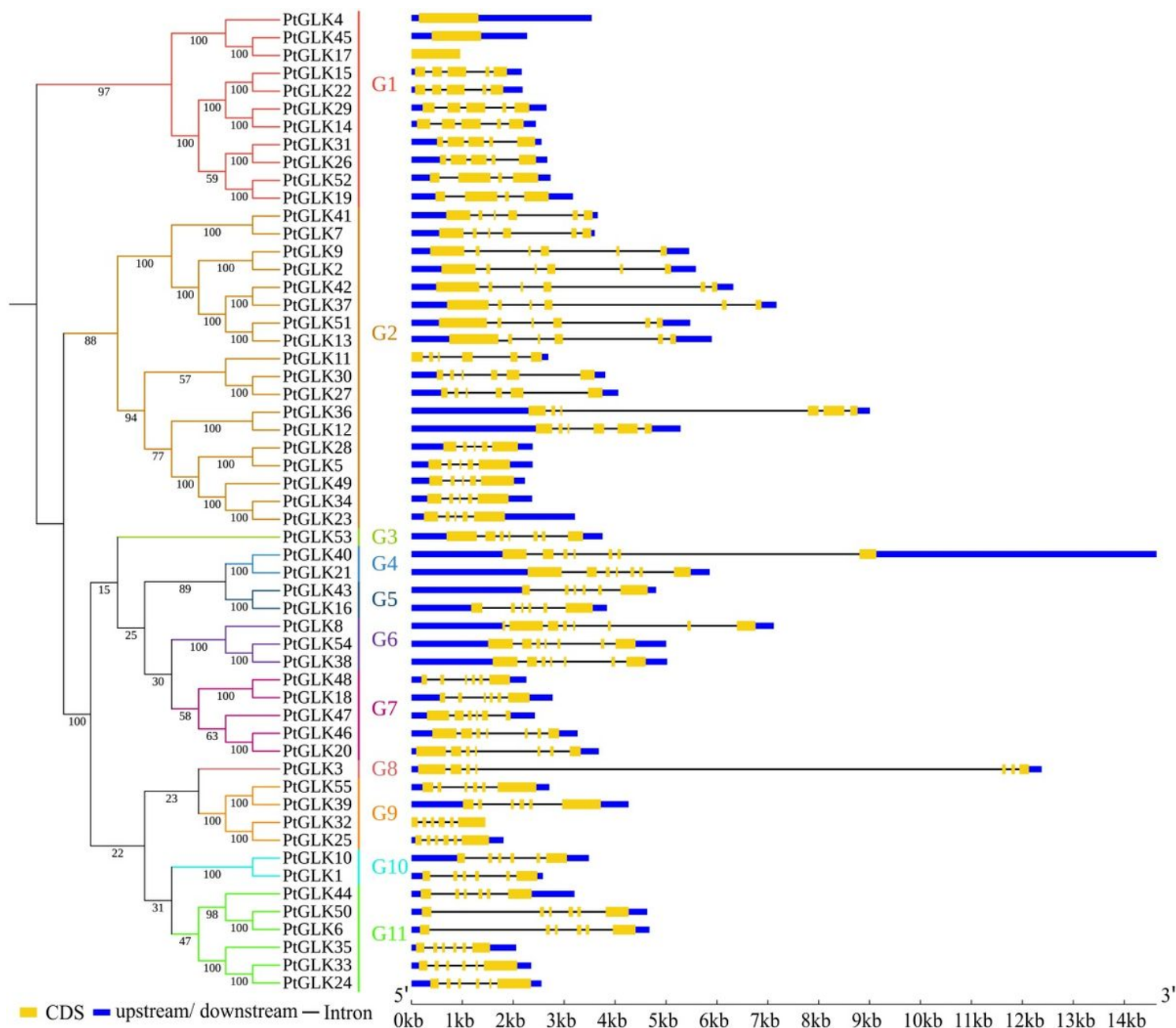


Figure 3

Phylogenetic relationship and gene structures of *PtGLKs*. Left: the neighbor-joining (NJ) phylogenetic tree was constructed by MEGA 7.0 according to *PtGLK* protein sequences. Right: the exon/intron structures of 55 *PtGLK* genes were generated by online GSDS.

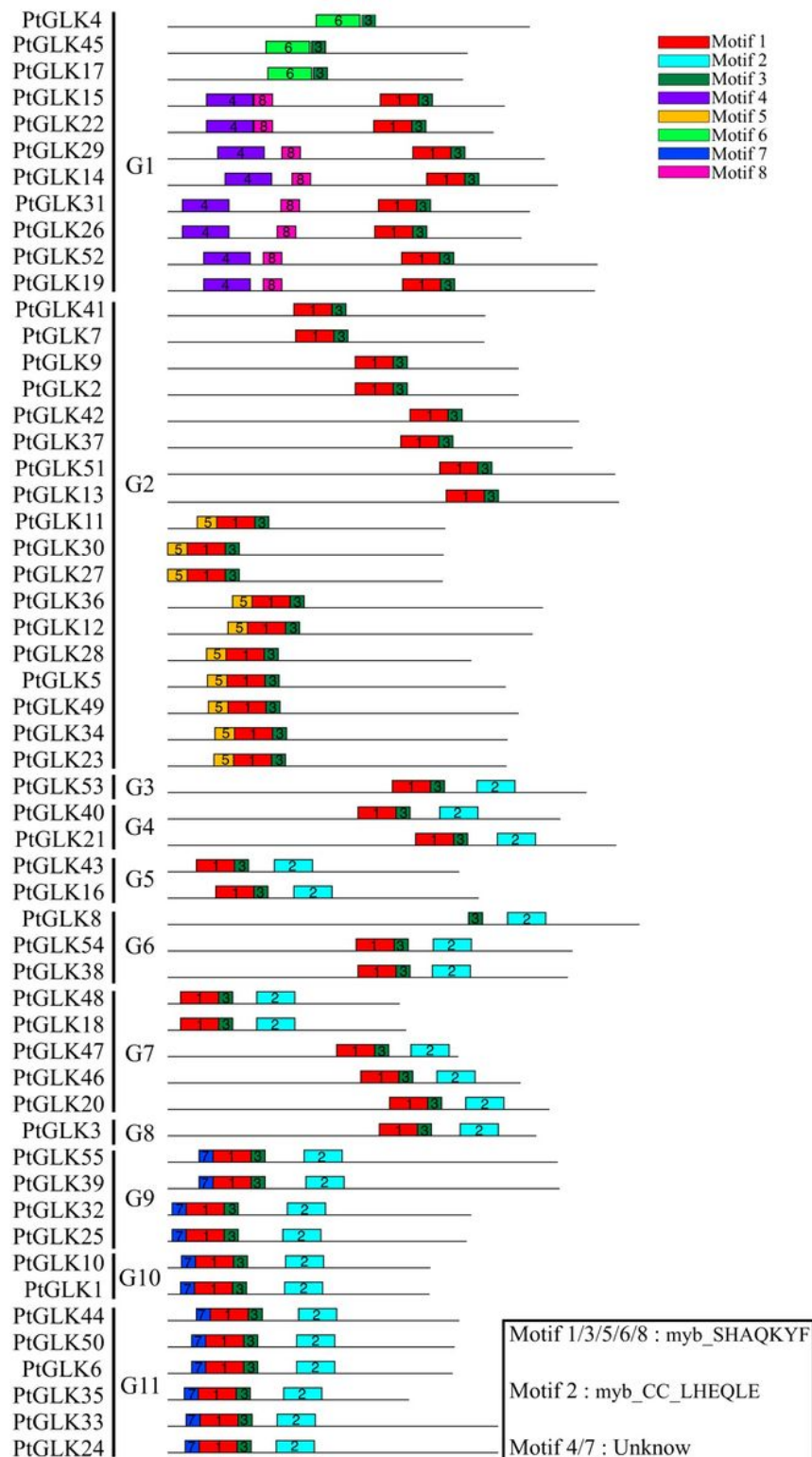


Figure 4

Schematic representation of 8 conserved motifs (1-8) in PtGLKs, ordered on the basis of online MEME analysis. The 8 motifs are represented in boxes of different colors, and the lengths of the motifs are exhibited proportionally.

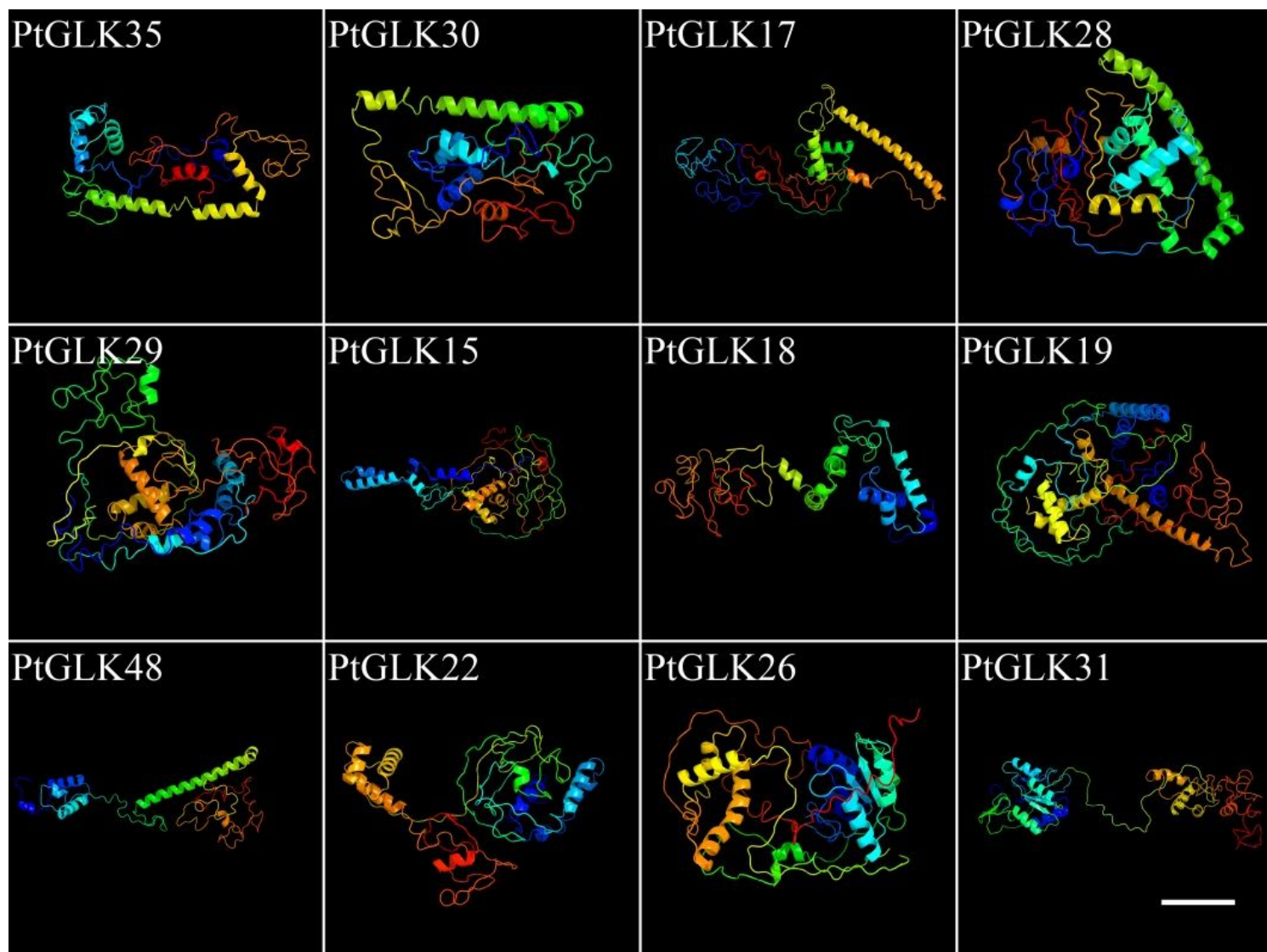


Figure 5

Predicted structures of PtGLK proteins. Bars: 20nm.

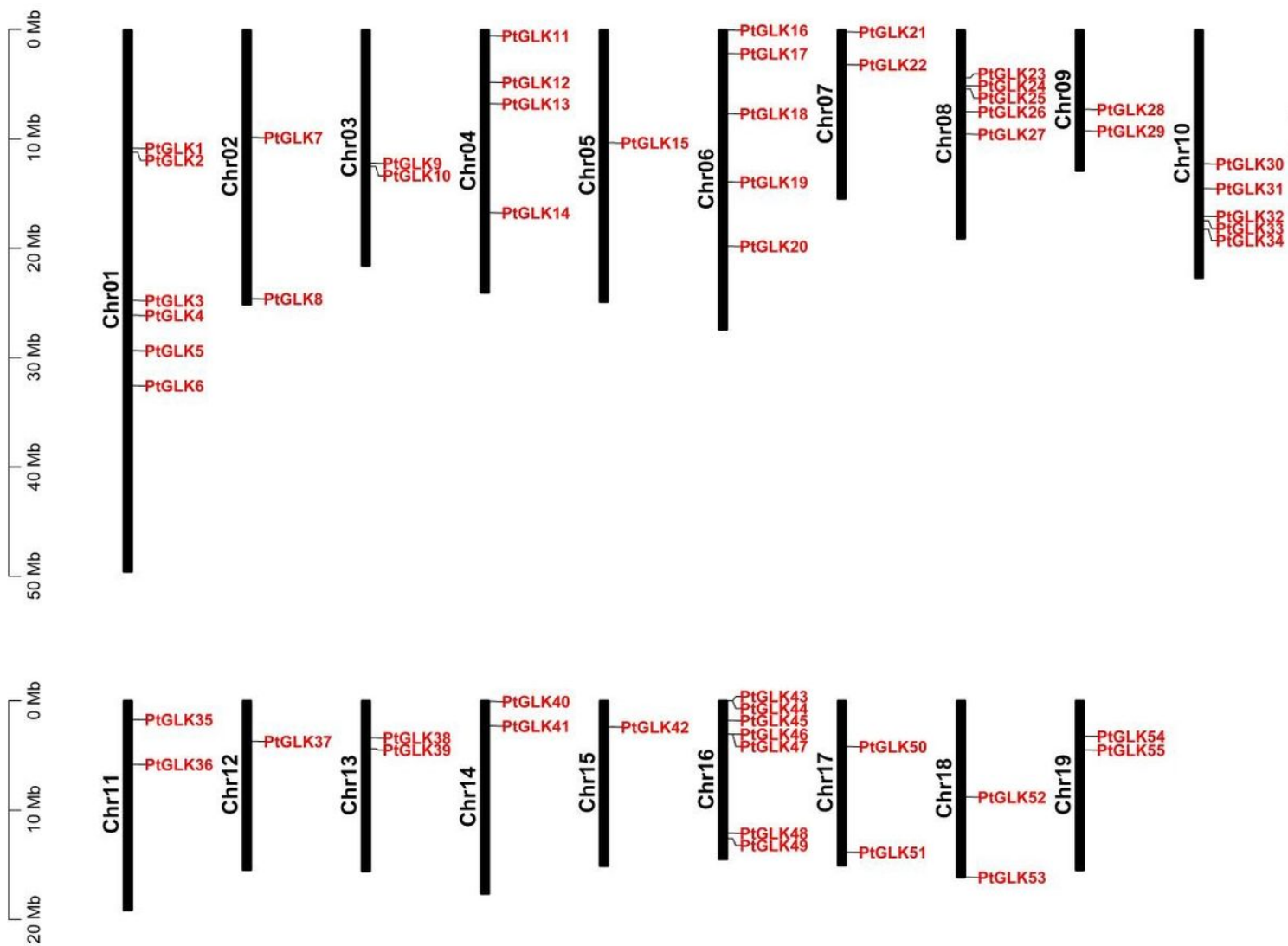


Figure 6

Chromosomal locations of *PtGLK* genes in *P. trichocarpa*.

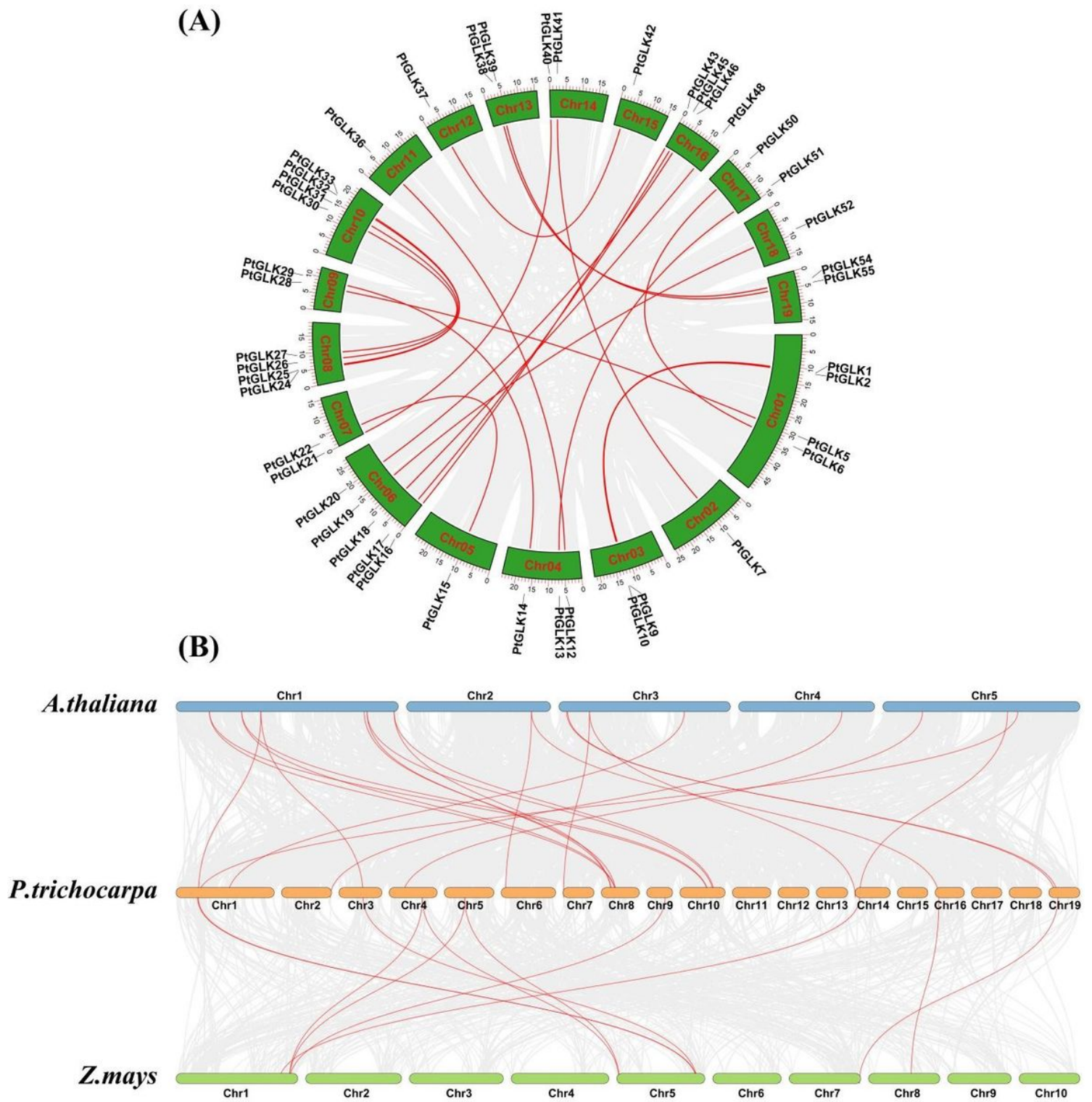


Figure 7

Duplication events of GLK proteins. (A) Synteny of *PtGLK* genes in *P. trichocarpa*. (B) Synteny of *GLK* genes between *P. trichocarpa* and two plant species (*Arabidopsis* and *Z. mays*).

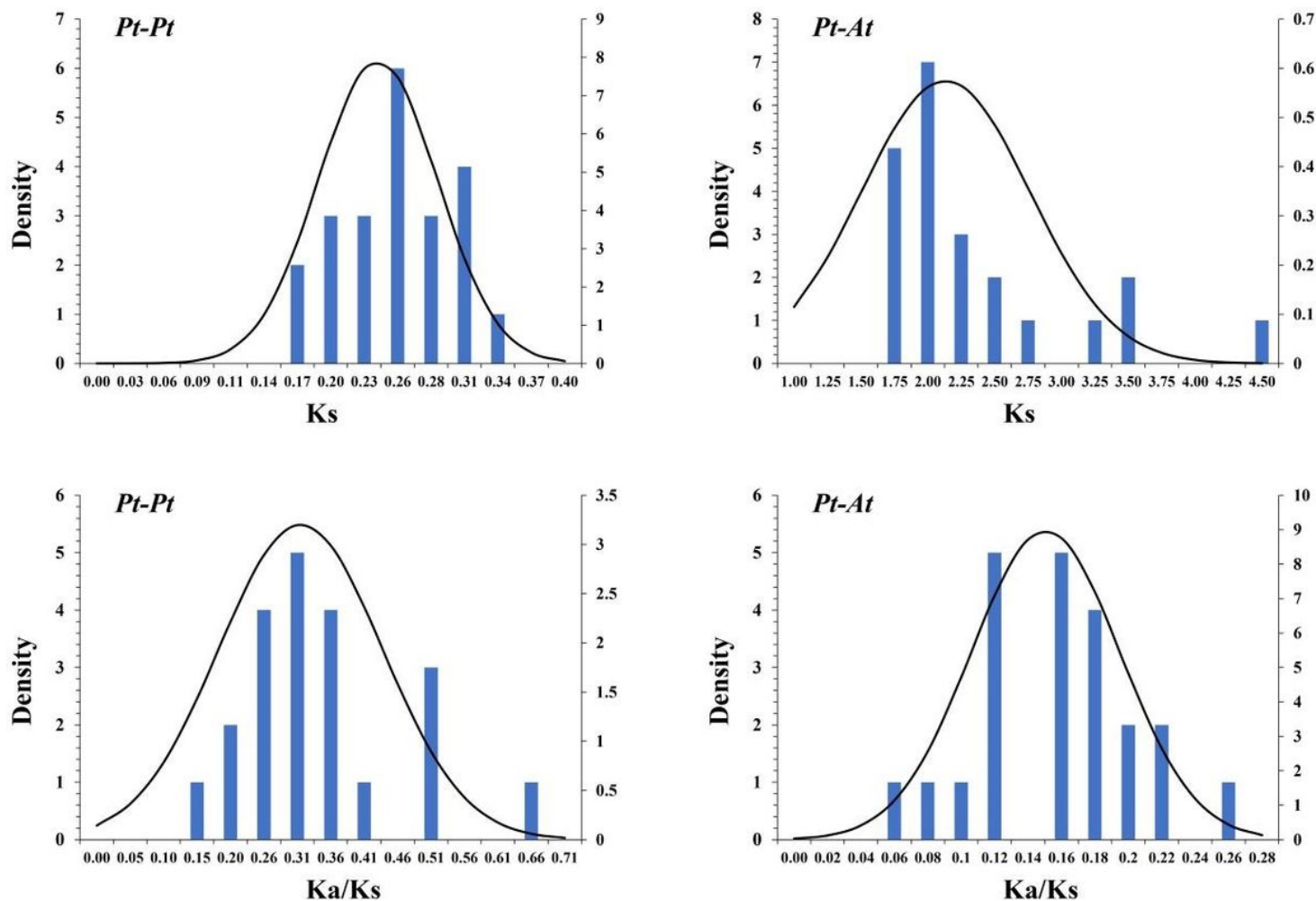


Figure 8

K_s and Ka/Ks value distribution of *PtGLK* genes in paralogous gene pairs (*Pt-Pt*) of the poplar genome and orthologous gene pairs between *P. trichocarpa* and *Arabidopsis*, viewed in terms of frequency distribution of relative K_s and Ka/Ks modes.

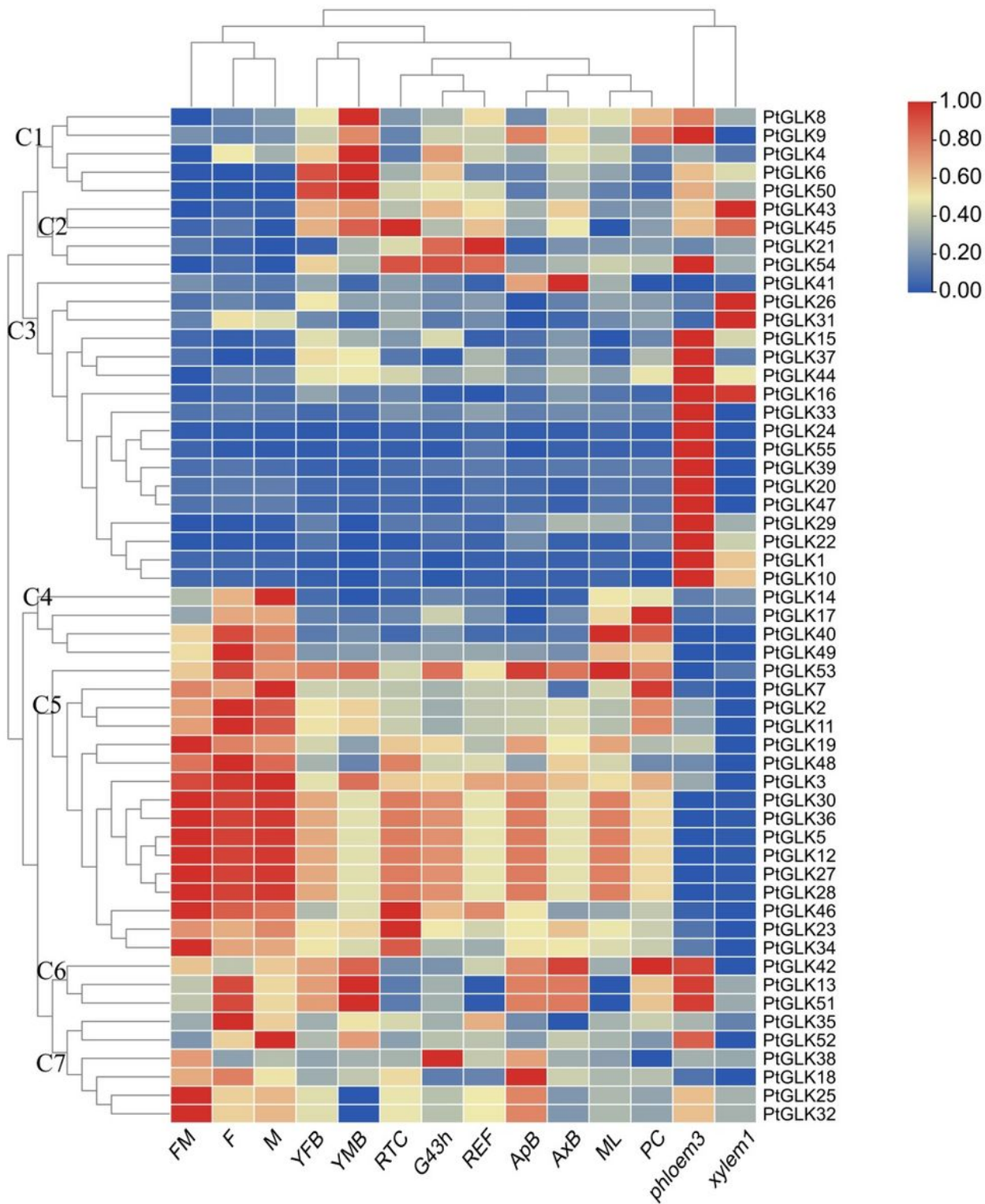


Figure 9

Expression profiles of *PtGLK* genes in different vegetative tissues and stages of reproductive development.

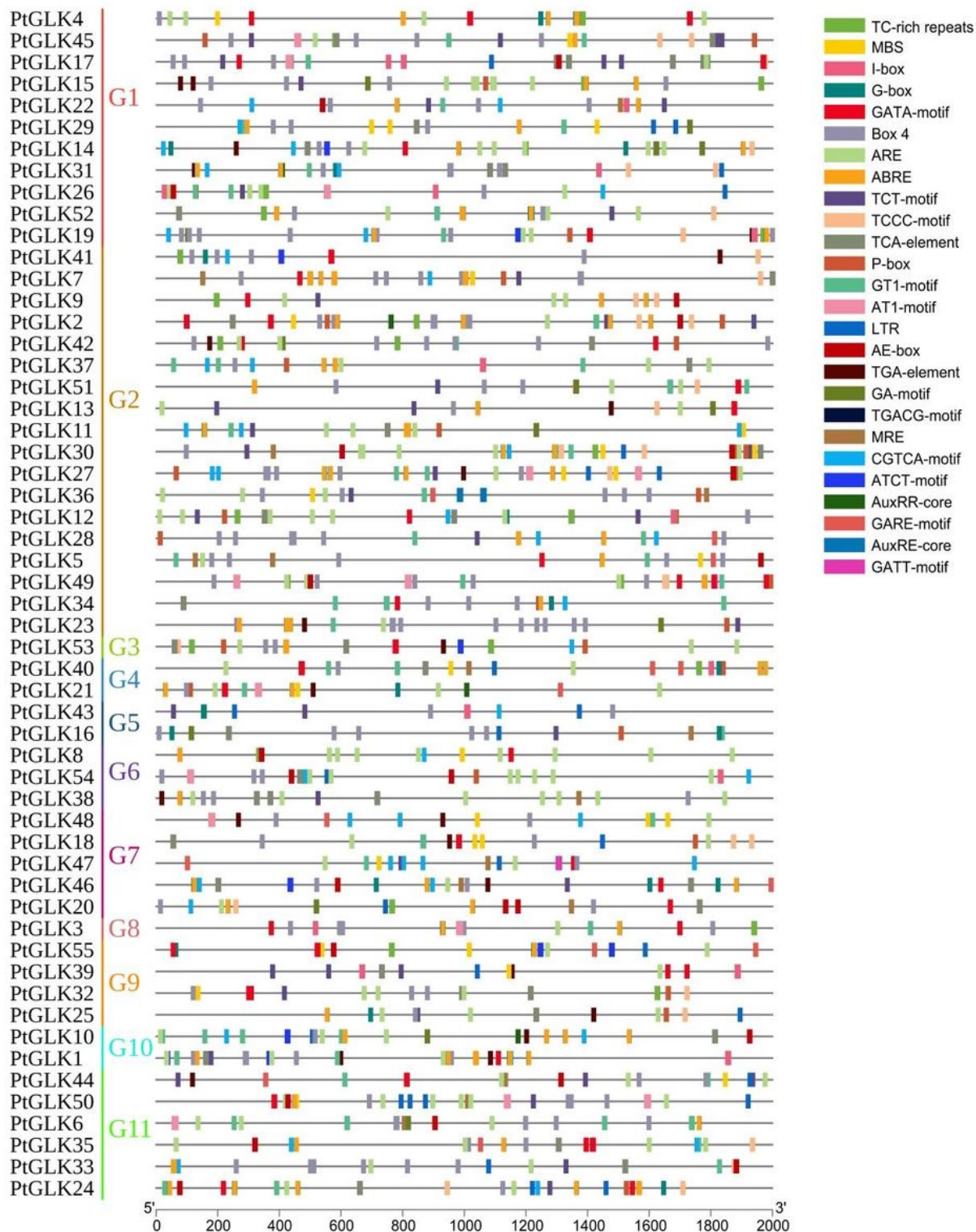


Figure 10

Analysis of *cis*-elements in promoter regions of *PtGLKs* according to information in the Plantcare database.

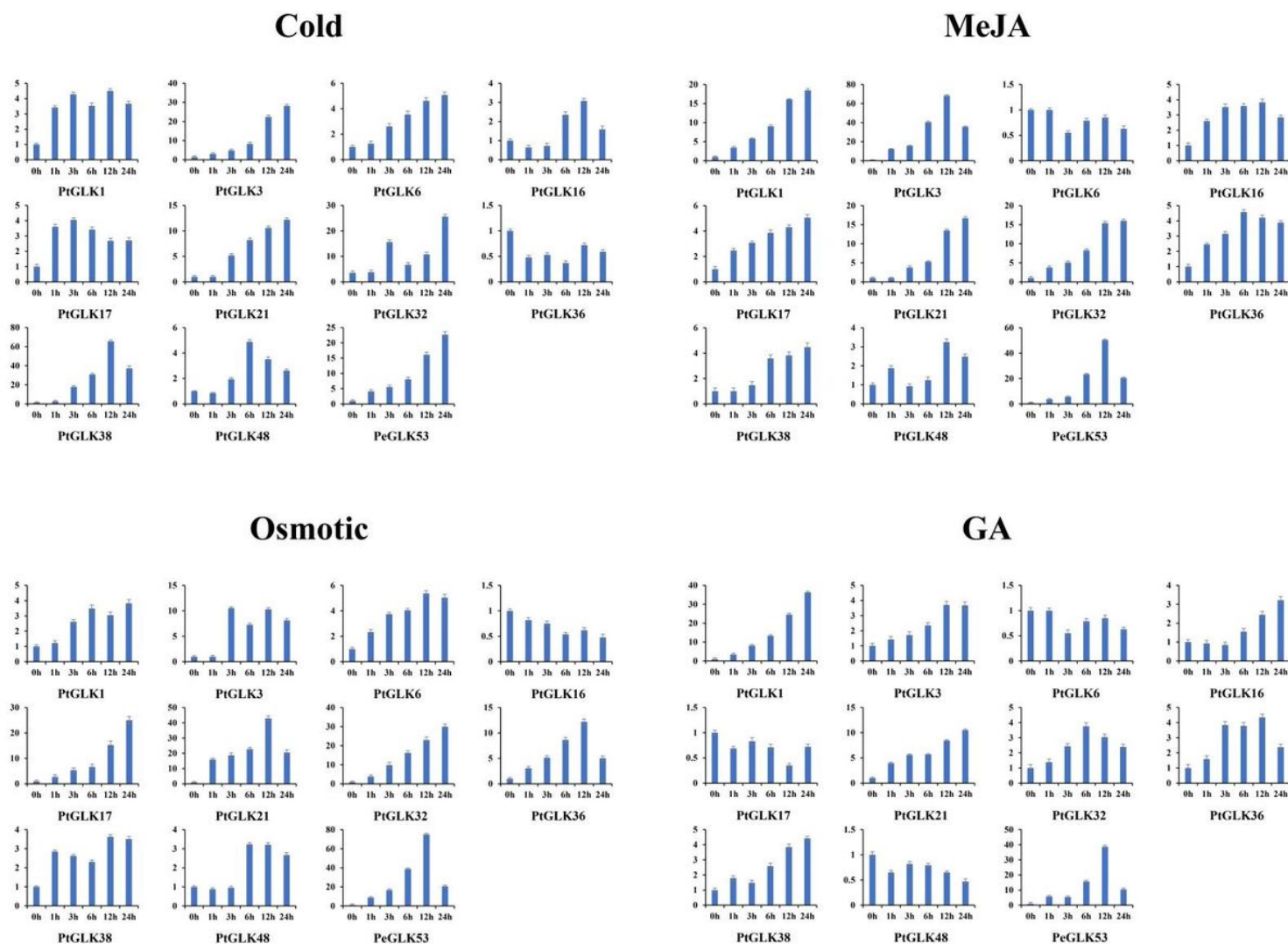


Figure 11

Expression patterns of 11 representative *PtGLK* genes in response to abiotic stresses and phytohormone treatments. The abiotic stresses and phytohormones used here were cold stress, osmotic stress, and MeJA and GA treatments. The relative expression levels were normalized to the reference gene *Pt18S*.

Supplementary Files

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