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Genome-wide identification and comprehensive analysis of the AP2/ERF gene family in *Prunus sibirica* under low-temperature stress

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Abstract

Background   AP2/ERF transcription factors are involved in the regulation of growth, development, and stress response in plants. Although the gene family has been characterized in various species, such as *Oryza sativa*, *Arabidopsis thaliana*, and *Populus trichocarpa*, studies on the *Prunus sibirica* AP2/ERF (PsAP2/ERF) gene family are lacking. In this study, PsAP2/ERFs in *P. sibirica* were characterized by genomic and transcriptomic analyses.

Results   In the study, 112 PsAP2/ERFs were identified and categorized into 16 subfamilies. Within each subfamily, PsAP2/ERFs exhibited similar exon-intron structures and motif compositions. Additionally, 50 pairs of segmentally duplicated genes were identified within the PsAP2/ERF gene family. Our experimental results showed that 20 *PsAP2/ERFs* are highly expressed in leaves, roots, and pistils under low-temperature stress conditions. Among them, the expression of *PsAP2/ERF21*, *PsAP2/ERF56* and *PsAP2/ERF88* was significantly up-regulated during the treatment period, and it was hypothesised that members of the PsAP2/ERF family play an important role inlow temperature stress tolerance.

Conclusions   This study improves our understanding of the molecular basis of development and low-temperature stress response in *P. sibirica* and provides a solid scientific foundation for further functional assays and evolutionary analyses of PsAP2/ERFs.

Keywords   AP2/ERF transcription factor family, *Prunus Sibirica*, Genome-wide identification, Low-temperature stress, Expression pattern

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Background

The AP2/ERF transcription factors (TFs) are among the largest TF families in plants [1]. They have one or more AP2 structural domains (consisting of 60–70 amino acids) [2]. Depending on the type and number of conserved structural domains, AP2/ERF TFs can be categorized into five subfamilies: AP2 (APETALA2), RAV (related to abscisic acid insensitive3/Viviparous1), ethylene-responsive factor (ERF), dehydration-responsive element binding (DREB), and soloist. The gene family size varies among species, with 163 members in *Arabidopsis thaliana* [3], 167 in *Oryza sativa* [4], 158 in *Actinidia eriantha* [5], 119 in *Chinese jujube* [6], 200 in *P. trichocarpa* [7], and 208 in *Citrus maxima* [8].

AP2/ERF TFs play a pivotal role in biological activities of plants, with differences in cellular functions among AP2/ERF subfamilies [9]. Many ERF family proteins aid in biological and metabolic regulation and the abiotic stress response [10]. For example, AP2 subfamily proteins have important regulatory functions during development, including embryonic development, seed development, and flower organ development [11]. RAV TFs regulate leaf physiological characteristics and stress responses [12]. DREBs play a pivotal role in stress response and photosynthesis [13]. Meanwhile, under abiotic stress, DREBs activate the expression of many target genes, including stress-inducible *RD29A* and Abscisic acid (ABA) [14]. C-repeat Binding Factor (CBF) proteins are a core part of the DREB subfamily [15]. CBF TFs are activated in plants under low-temperatures and transcriptionally regulate the expression levels of approximately 12% of low-temperature responsive genes. The role of CBFs in cold resistance in plants has been reported [16]. For example, *OsDREB1A* is associated with temperature stress in rice [17], and *AtCBF1−4* in transgenic *A. thaliana* enhance cold tolerance [18].

The woody plant *Prunus sibirica* is a member of the family Rosaceae, with a natural distribution centered in northeastern China, eastern Siberia, and northern, eastern, and southeastern Mongolia [19]. It is a pioneer species for afforestation of deserted mountains and for retaining soil and water [20]. It is also a companion species in sand-barren forests, offering protection against wind, sand, and water erosion; further, it improves ecosystem functioning [21]. At the same time, the hard, wear-resistant wood of *P. sibirica* is a good material for furniture and agricultural tools, and the colorful petals make it an important ornamental tree for landscaping. *P. sibirica*, as a cultivated apricot, has been used in various industries, including food and beverage, oilseed, traditional Chinese medicine, chemical industry, cosmetics, and other industries. Additionally, apricot shells are used as high-grade raw materials for preparing activated carbon [19].

Temperature is a key determinant of plant development, reproduction, and distribution [22]. Low-temperatures affect plant physiological indicators, metabolism, and growth and can lead to apoptosis [23]. In particular, *P. sibirica* is characterized by an early flowering time; frost damage affects the flowering period and stunts floral organs, including pistil abortion and stylar dysplasia, and flower deformation, thereby reducing yields and limiting the economic value of the species [24]. Hence, mining potential antifreeze-related genes in reproductive organs can facilitate the selection of productive and frost-resistant *P. sibirica* varieties, thus increasing the value of their products.

To deepen our understanding of AP2/ERF TFs in *P. sibirica*, in this study, we performed a genomic analysis, gene function prediction, and transcriptomics analysis using qRT-PCR. The results of this study provide a foundation for further studies on gene functions and molecular evolutionary mechanisms of AP2/ERF TFs in *P. sibirica*. Furthermore, they provide theoretical guidance for breeding high-quality *P. sibirica* varieties and germplasm improvement through genetic engineering.

Results

Identification and characterization of PsAP2/ERFs in *P. Sibirica*

A hidden Markov model (HMM) of the AP2/ERF structural domain (PF00847) was used to search for PsAP2/ERFs, revealing 112 candidates. All candidate AP2/ERF proteins contained intact AP2/ERF structural domains based on Pfam and NCBI-CDD analyses. The genes were named PsAP2/ERF1–PsAP2/ERF112 based on confidence levels (Table S1).

Physicochemical analyses based on sequence information showed that the 112 PsAP2/ERF proteins ranged from 109 to 781 amino acids in length, with a theoretical isoelectric point of 4.68 (PsAP2/ERF58) to 10.75 (PsAP2/ERF74) and a molecular weight of 30.48 kDa (PsAP2/ERF111) to 81.71 kDa (PsAP2/ERF17). Additionally, all PsAP2/ERFs had hydrophobicity indices of less than 0 and lipid indices of less than 100 and were identified as hydrophilic. Except for PsAP2/ERF22, PsAP2/ERF31, PsAP2/ERF76, PsAP2/ERF96, PsAP2/ERF90, PsAP2/ERF102, PsAP2/ERF106, and PsAP2/ERF111, all other members showed instability coefficients of greater than 40, indicating high instability. Prediction of subcellular localization suggested that PsAP2/ERFs are generally localized in the nucleus, with a small number localized in the cytoplasm (PsAP2/ERF111, PsAP2/ERF106, PsAP2/ERF37, PsAP2/ERF24, and PsAP2/ERF27) and in the chloroplast (PsAP2/ERF96, PsAP2/ERF56, and PsAP2/ERF21). Very few were localized in the mitochondria (PsAP2/ERF74 and PsAP2/ERF54) and in the plasma membrane (PsAP2/ERF20) (Table S1).

Phylogenetic analysis of PsAP2/ERFs, AtAP2/ERFs, and PtAP2/ERFs

We evaluated phylogenetic relationships among AP2/ERF proteins in *P. sibirica* and *A. thaliana.* The members of the AP2/ERF family could be categorized into four groups based on their overall structure. The AP2 subfamily (20 members) contained two AP2/ERF domains, the RAV subfamily (5 members) included an AP2/ERF domain and an additional B3 DNA-binding domain, the ERF subfamily contained only a single AP2/ERF domain, and the Soloist domain (1 member) contained specialized structural domains. In particular, adenine nucleotide translocator (ANT) proteins (7 members) are AP2 subfamily-specific plant-specific TFs. Because the ERF (39 members) and DREB (47 members) subfamilies had high structural domain similarity, they were classified as the same group; however, based on differences in their structural domains, the ERF subfamily was further divided into the DREB (group A) and ERF (group B) subfamilies. These subfamilies were further subdivided into twelve subgroups, A-1 to A-6 and B-1 to B-6, respectively, although no members of B-1 were detected in the ERF subfamily of *P. sibirica* (Fig. 1). Similar results were obtained in a comparison between the woody plants *Populus trichocarpa* and *P. sibirica* (Fig. S1).

Multiple sequence alignment, motif composition, and gene structure of PsAP2/ERFs

Multiple sequence alignments of the amino acid sequences of the conserved structural domains of PsAP2/ERF were analyzed. Although mutations were detected in some genes, all of the genes, except PsAP2/ERF112, contained complete or nearly complete structural domains (Fig. S2).

Divergence in gene structure can provide insight into gene family evolution. We constructed a phylogenetic tree of the 112 PsAP2/ERFs and systematically analyzed the conserved motifs and intron/exon structures (Fig. 2). PsAP2/ERF genes belonging to the same subfamily were clustered together (Fig. 2A). Ten conserved motifs of PsAP2/ERF were predicted (Fig. S3). As shown in Fig. 2B, motif 1 and motif 2 were present in all PsAP2/ERF genes and were identified as conserved structural domains. Motif 3 was shared by the DREB and ERF subfamilies. All members of the AP2 subfamily had motif 4 together with motif 5, while motif 9 was only found in the RAV subfamily. Motif 10 was only present in the DREB subfamily and could be used to distinguish the DREB subfamily from the ERF subfamily.

A better understanding of gene expression patterns can be obtained by analyzing introns and exons. The ANT and AP2 subfamilies contained more exons than those in the ERF subfamily. There were more untranslated regions (UTR) in the DREB subfamily than in other subfamilies.

Lastly, *PsAP2/ERF29*, *PsAP2/ERF37*, *PsAP2/ERF43*, *PsAP2/ERF44*, *PsAP2/ERF46*, *PsAP2/ERF65*, and 39 other *PsAP2/ERFs* did not contain UTRs (Fig. 2C).

Chromosomal localization, duplication, synteny, and K_a/K_s analysis of PsAP2/ERFs

The PsAP2/ERFs were assigned to chromosomes based on genome annotation information. The 112 genes encoding PsAP2/ERFs were randomly distributed across eight chromosomes, with 6 to 23 PsAP2/ERFs allocated to each chromosome (Fig. 3). Chromosome 1 (Chr1) contained the most PsAP2/ERFs (23), whereas chromosome Chr4 had the fewest (6). In turn, Chr2 and Chr5 had 18 PsAP2/ERFs, and Chr6, Chr3, Chr7, and Chr8 had 15, 14, 11, and 8 AP2/ERFs, respectively. Except for chromosomes 4 and 8, all other chromosomes showed clustering of members.

Gene duplication is a major driving force in genome evolution. Analyses of segmental and tandem duplications provide insight into gene family expansion. Duplication events between PsAP2/ERF members were detected on all chromosomes. Segments on Chr1 had the most replicated genes (14 genes). Analyses of homologous protein families are important for predicting protein function and establishing relationships among species. Many homologous genes were detected on different chromosomes in *P. sibirica*, suggesting that the AP2/ERF gene family is highly conserved (Fig. 4). Overall, 52 gene duplication events were recorded, involving 46% (52/112) of all PsAP2/ERFs (Table S2). Additionally, 50 genes underwent segmental duplications, accounting for 92% of all syntenic relationships. A cluster of 24 genes encoding PsAP2/ERFs formed by fragment duplication (indicated by colored lines in Fig. 4). The K_a/K_s ratio reflects the selective pressure on genes; specifically, K_a/K_s > 1 indicates positive selection, K_a/K_s < 1 indicates negative selection, and K_a/K_s = 1 indicates neutral evolution. For both duplicate types, the K_a/K_s ratio was significantly lower than 1, consistent with strong purifying selection on the duplicated AP2/ERF genes, thereby limiting functional differentiation (Table S2).

The analysis of covariance between different species allows comparison of similarities and evolutionary processes between genome sequences. Therefore, by analyzing the covariance between different species, we further investigated the evolutionary relationships of AP2/ERF members among plant species, as shown in Fig. 5. The fewest collinear gene pairs were found between *P. salicina* and *P. sibirica*, whereas the most collinear gene pairs were found between *P. sibirica* and *P. avium*.

Cis-acting element analysis of *PsAP2/ERFs*

Cis-acting elements in the *PsAP2/ERF* promoter regions were identified using PlantCARE. Thirteen representative
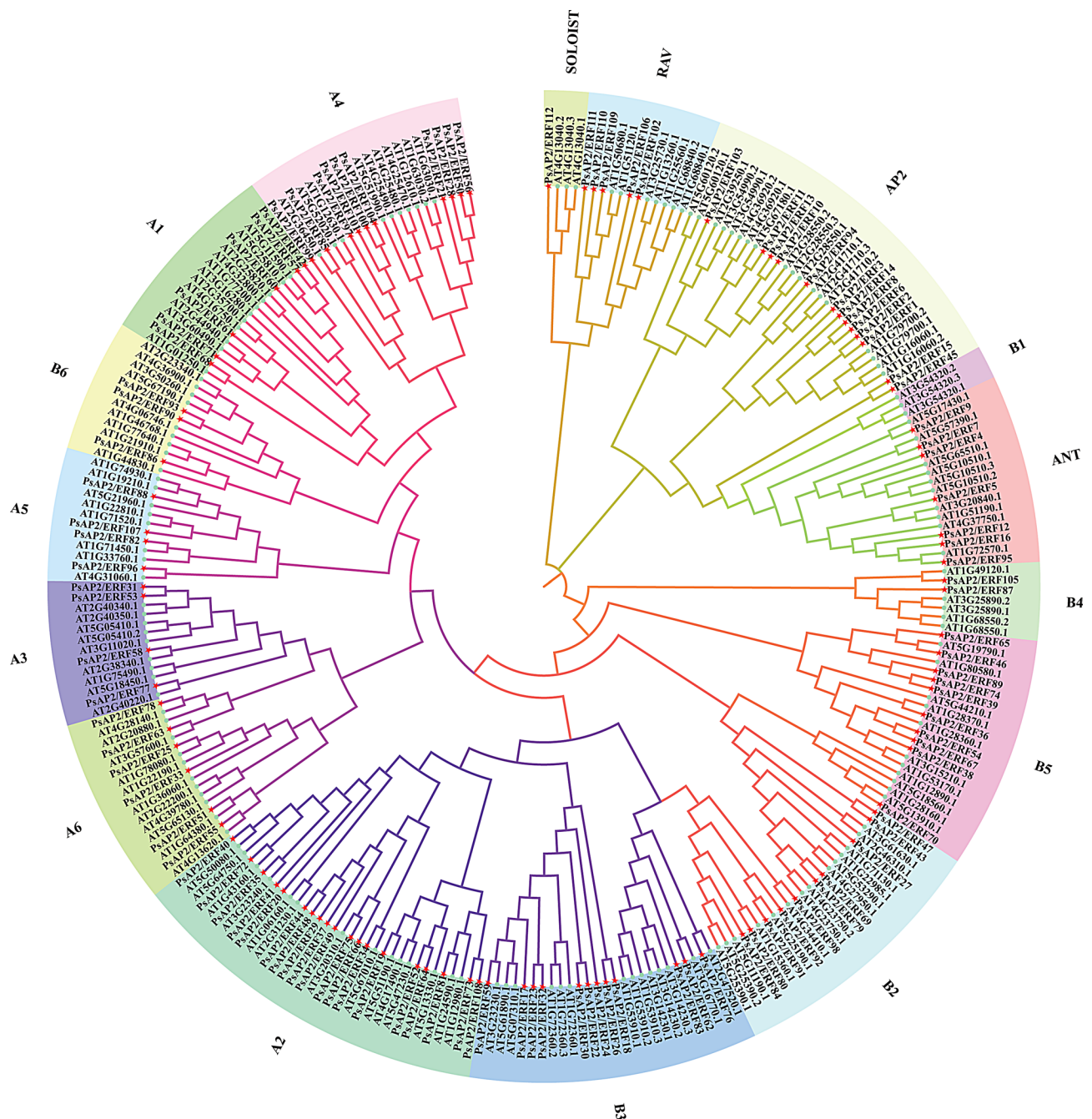
Fig. 1 Based on the full-length amino acid sequence alignment of 112 PsAP2/ERFs with AtAP2/ERFS, a phylogenetic tree was constructed using MEGA11 software and 1000 bootstrap replications were performed to achieve the delineation of the subfamilies of PsAP2/ERFs. Different colors represent different subfamilies. *P. sibirica* and *A. thaliana* are marked by green circles and red pentagrams, respectively. The names of the different subfamilies are labeled in the outermost circle

elements were selected for functional analyses (Table S3). Most were related to the plant maturation process, phytohormone responses, and biotic/abiotic stress responses (Fig. 6). Further, most cis-regulatory elements in the promoter regions of *PsAP2/ERFs* were the CGTCA motif (515) followed by the ABRE motif (459) and the CGTCA motif (133). Additionally, 69 members

had MYB-binding sites involved in drought inducibility (MBS); 52 had auxin-responsive elements (TGA-element), 51 had MYBHv1 binding site (CCAAT-box); 51 had MYB-binding sites involved in flavonoid biosynthetic gene regulation (MBSI); 95 had cis-acting elements involved in abscisic acid responsiveness (ABRE); 57 had cis-regulatory elements involved in low-temperature

Fig. 2 Phylogenetic relationships, conserved motifs, and gene structure of the PsAP2/ERF gene family. (**A**) Neighbor-joining phylogenetic tree of PsAP2/ERF., and labeling of each subfamily name (**B**) PsAP2/ERF conserved motif distribution. Different motifs are represented by boxes of different colors. The higher the order, the higher the frequency of occurrence and the more structurally conserved in PsAP2/ERFs. (**C**) Gene structures of *PsAP2/ERFs*. Green boxes indicate exons, yellow boxes indicate untranslated regions (UTRs), and gray lines indicates introns

responsiveness (LTR). *PsAP2/ERF43* and *PsAP2/ERF44* contained the most cis-elements (44), followed by *PsAP2/ERF7* and *PsAP2/ERF8* (32). These results suggest that *PsAP2/ERFs* have many functions in growth, development, and stress responses.

Gene Ontology functional enrichment analysis of PsAP2/ERFs

The analysis of cis-acting elements suggested that PsAP2/ERFs have many potential functions. Gene Ontology (GO) annotation was used to understand the functions of the 112 identified PsAP2/ERFs in a better manner. All TFs were categorized according to protein sequence
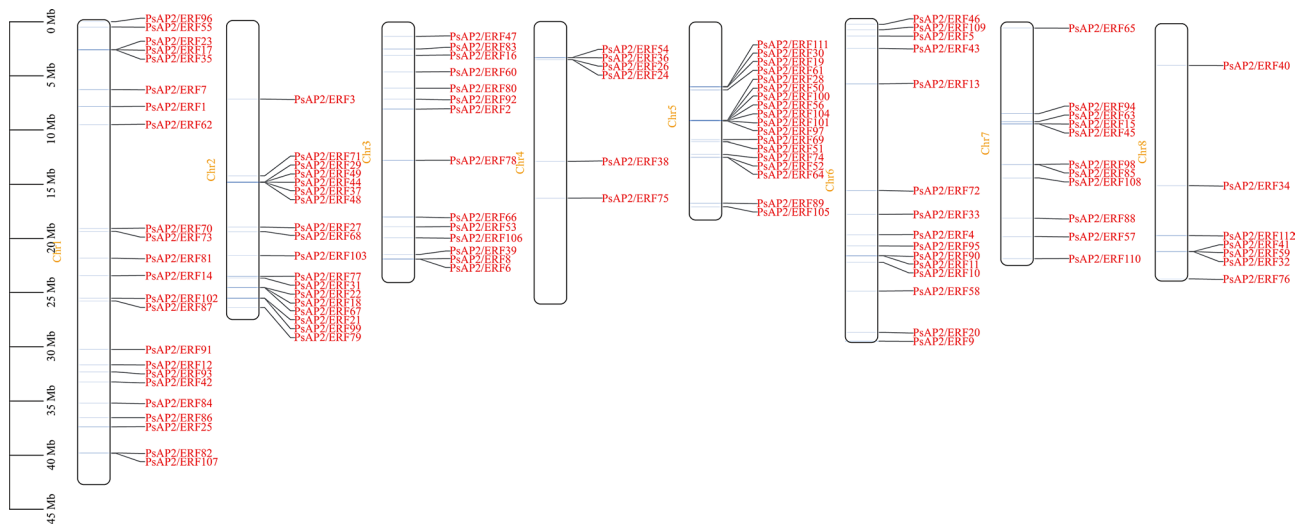
Fig. 3 Chromosomal distribution of PsAP2/ERFs. The gray lines represent the location of each gene on the chromosome

similarity and were classified into 22 functional groups within the three main categories: molecular functions, biological processes, and cellular components. Within the biological process category, most PsAP2/ERFs were involved in the regulation of cellular processes (GO:0009987), biological processes (GO:0050789), biological processes (GO: 0065007), and metabolic processes (GO: 0008152). Among terms in the cellular components category, PsAP2/ERFs were mainly involved in the nucleus (GO:0005634), membrane-bound organelles (GO:0043231), and intracellular organelles (GO:0043229). Furthermore, the molecular functions associated with PsAP2/ERFs were transcription regulator activity (GO:0140110) and nucleus-related (GO:0005634) (Fig. 7, Table S4).

Protein interactions of PsAP2/ERFs

A. thaliana orthologues of the 112 PsAP2/ERFs were used to predict protein interactions (Table S5). Protein interactions were predicted for the vast majority of the PsAP2/ERF family members. Their functions in *P. sibirica* can be inferred from functions in *A. thaliana*. *PsAP2/ERF21* was functionally similar to the cold-inducible gene *AtDREB1D*, and *PsAP2/ERF56* was functionally similar to the stress-resistance gene *AtDREB1B*. The *AtERF1B* homologue *PsAP2/ERF35* showed a number of interactions with other genes (Fig. 8, Table S6).

Differential expression of *PsAP2ERFs* in different cold-resistant clones under low-temperature stress

The AP2/ERF family plays an important role in the response to low-temperature stress. To explore the expression patterns of PsAP2/ERFs under low-temperature stress, we examined transcriptomic data for pistils following cold treatment in different cold-resistant clones

(cold-resistant clone No. 453 and cold-sensitive clone No. 371). Many *PsAP2/ERFs* responded to cold stress. We obtained gene expression data for AP2/ERFs in two different clones for different durations of cold stress. In clone no. 453, the expression levels of genes such as *PsAP2/ERF6* and *PsAP2/ERF23* were noticeably higher than those in clone no. 371 at 15 and 30 min of cold stress, while the expression levels of other genes, such as *PsAP2/ERF21*, *PsAP2/ERF23*, *PsAP2/ERF28*, *PsAP2/ERF34*, *PsAP2/ERF50*, *PsAP2/ERF56*, and *PsAP2/ERF88*, were more than three-fold higher than those of clone no. 371 at 2 h of low-temperature stress. *PsAP2/ERF12*, *PsAP2/ERF62*, *PsAP2/ERF68*, *PsAP2/ERF74*, *PsAP2/ERF87*, *PsAP2/ERF110*, and *PsAP2/ERF112* did not show a rapid increase but exhibited stable expression in the short term. In contrast, the expression levels of *PsAP2/ERF55*, *PsAP2/ERF87*, *PsAP2/ERF102*, *PsAP2/ERF106*, and *PsAP2/ERF107* decreased consistently or were significantly lower than those in clone no. 371, contributing to the negative regulation of frost resistance in *P. sibirica* flowers (Fig. 9).

Expression of *PsAP2/ERFs* in different tissues

The PsAP2/ERF gene family is strongly associated with plant growth and development. To confirm the contribution of these genes, the transcriptome results for 20 *PsAP2/ERF* candidate genes were validated using qRT-PCR. Ten genes were highly expressed in the pistils, and 13 genes were highly expressed in the roots (using levels in petals as a control). In particular, *PsAP2/ERF29* was highly expressed (>60-fold greater, relative to levels in controls) in all tissues except for the pistils. In turn, the expression levels of *PsAP2/ERF18*, *PsAP2/ERF19*, *PsAP2/ERF29*, and *PsAP2/ERF72* were more than 60-fold higher in the roots than in petals. Lastly, *PsAP2/ERF56*
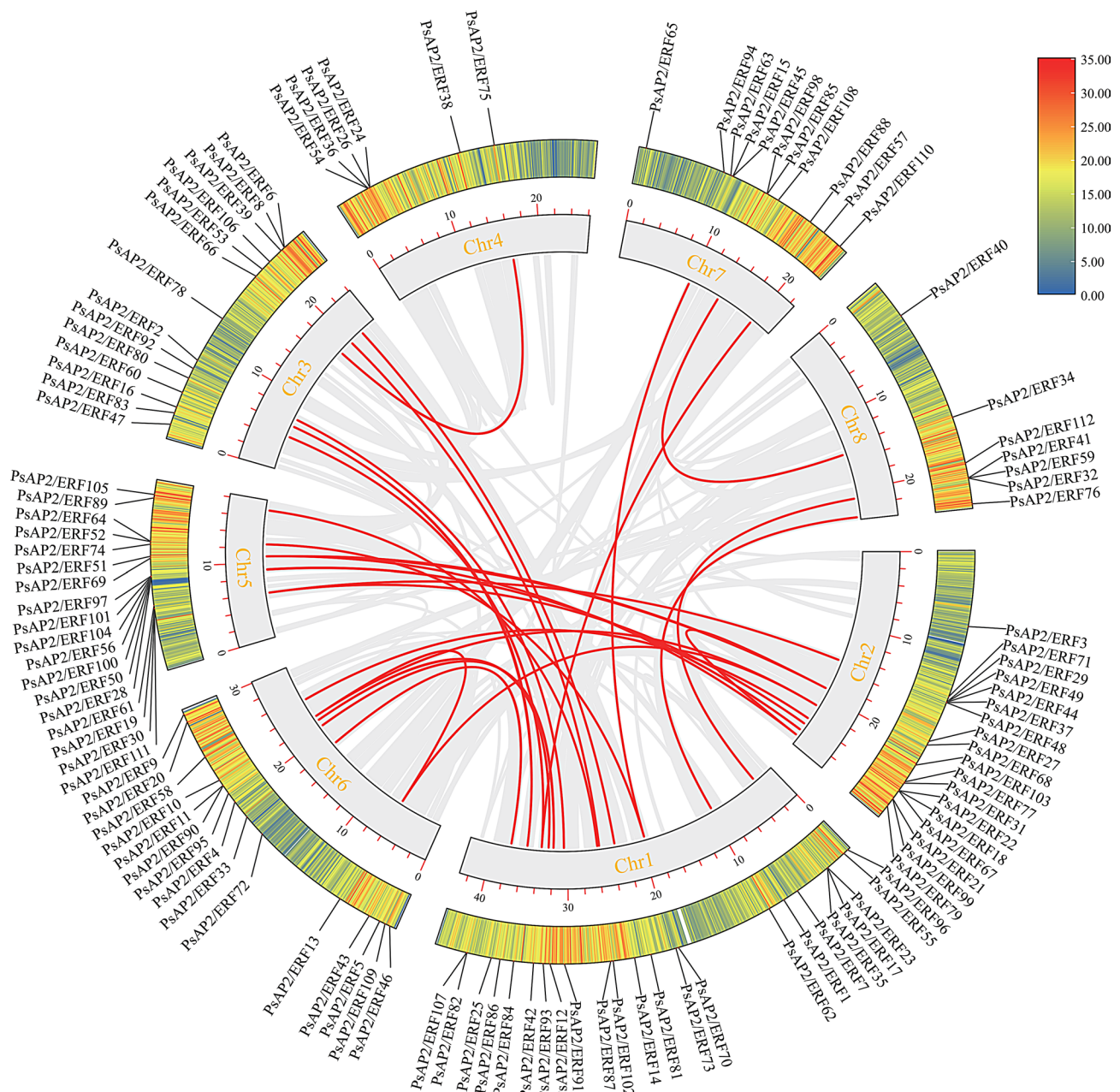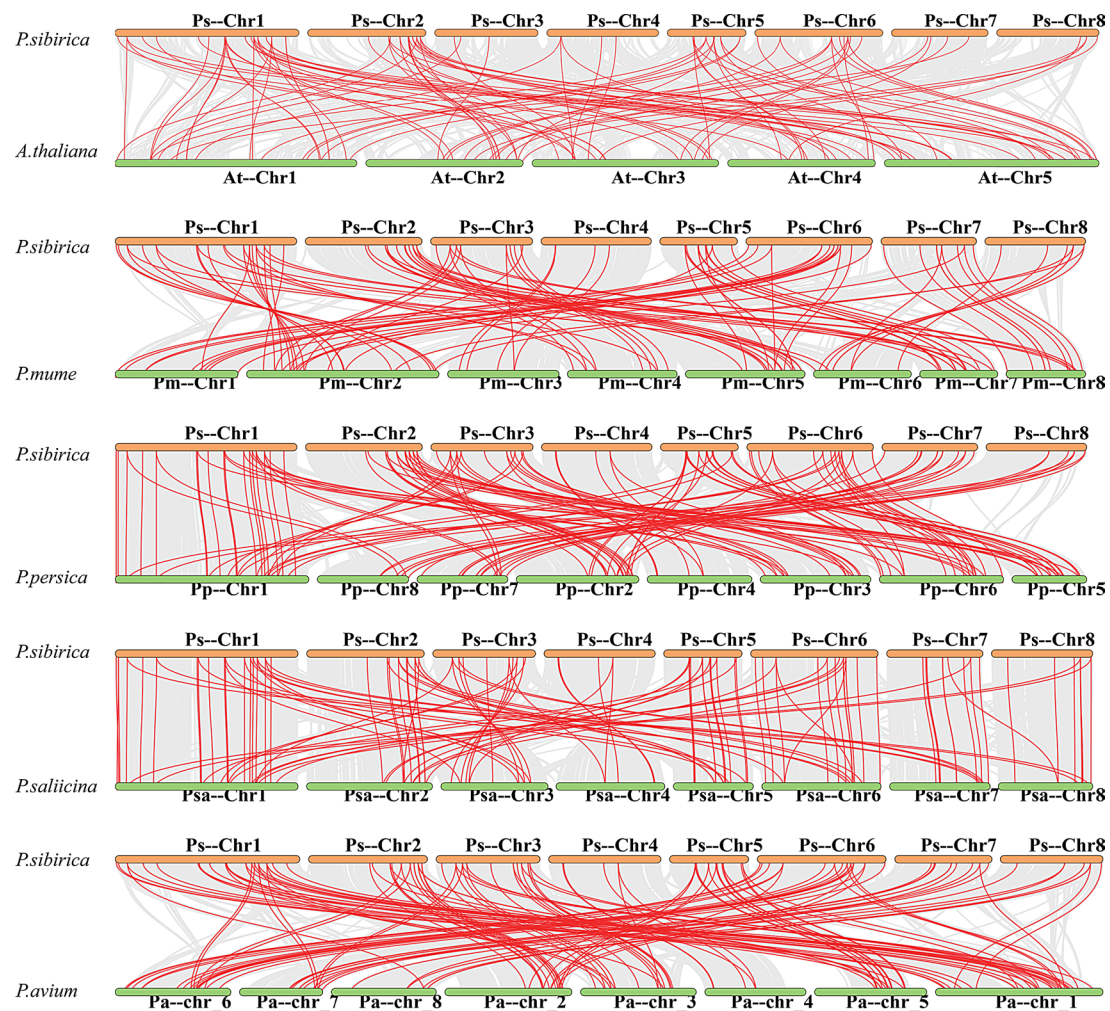
Fig. 4 Synteny analysis of AP2/ERFs in the *P. sibirica* genome. Gray lines represent all syntenic blocks in the *P. sibirica* genome, and red lines indicate segmental duplications, the two genes connected by the red line are homozygous. Gene density is represented by a heat map (inner circle), and the outer circle shows the lengths of chromosomes, the outermost black line segment represents the localization of the gene on the chromosome

and *PsAP2/ERF79* were more highly expressed (>15-fold greater, relative to levels in controls) in the pistils. The number of upregulated genes was highest in leaves (i.e., 50% of genes were more highly expressed in leaves than in controls in 20 experimental groups). Additionally, 35% of genes were more highly expressed in leaves than in other tissues. Notably, only *PsAP2/ERF28* was more highly expressed in petals than in other tissues (Fig. 10).

Analysis of *PsAP2/ERF* expression under low-temperature stress

The PsAP2/ERF gene family is important for plant responses to low-temperature stress. To further verify the role of these genes in the response to low temperatures, the expression levels of 20 *PsAP2/ERFs* were evaluated (Fig. 11). These 20 *PsAP2/ERFs* were differentially expressed under low-temperature conditions, with significant variation over time. In particular, 50% of the genes showed elevated expression after 15 min, including

Fig. 5 Synteny analysis of AP2/ERF genes in *P. sibirica* and five representative plant species. Gray lines indicate collinear blocks within the genomes of *P. sibirica* and other plants, while red lines indicate syntenic AP2/ERF gene pairs. More gray lines indicate that the two genomes are more similar, and more red lines indicate that the two species contain more paired genes. The prefixes "At," "Psa," "Pm," "Pp," and "Pa" represent *A. thaliana*, *P. salicina*, *P. mume*, *P. persica*, and *P. avium*, respectively

PsAP2/ERF21 (>20-fold greater than levels in controls), *PsAP2/ERF28* (>10-fold greater), *PsAP2/ERF50* (>10-fold greater), and *PsAP2/ERF18* and *PsAP2/ERF31* (>10-fold greater), after 1 h of low-temperature treatment. Different genes differed in the timing and degree of response to low-temperature stress; most of the genes responded quickly, except for *PsAP2/ERF72*, which did not show significant changes in expression in a short period of time.
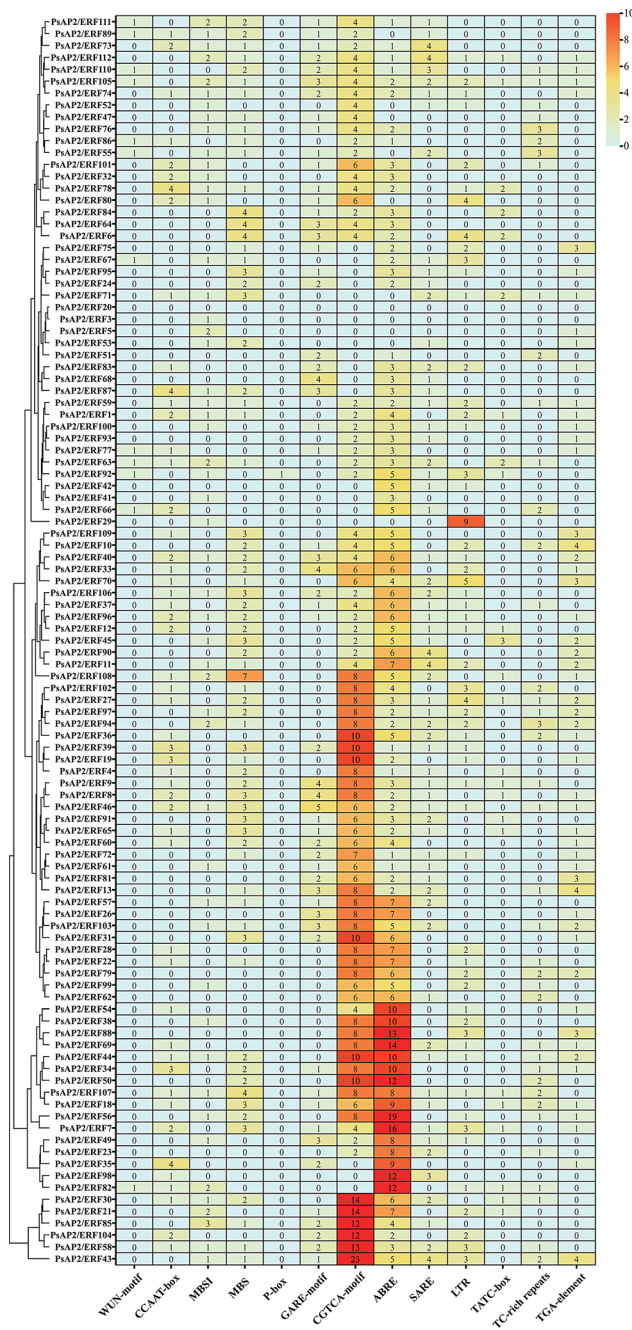
Discussion

The AP2/ERF gene family influences various biological processes, such as plant maturity, stress response, and defense, and has been evaluated in many plants [25]; however, it has not been reported in *P. sibirica*.

The number of AP2/ERF TFs varies greatly among species. For example, *Zingiber officinale* Roscoe has 163 [26], *Triticum aestivum* has 322 [27], *Fagopyrum tataricum* has 134 [28], and *A. thaliana* has 122. The number of AP2/ERF genes is significantly higher in herbaceous plants than in *P. sibirica* but is similar in woody plants, such as *Citrus rootstock* (119) [29], *Chinese jujube* (119) [6], and *P. sibirica* (112). The lower number of AP2/ERF genes in woody plants, in general, could be due to the slower rate of evolution of woody plants.

Analyses of physicochemical properties revealed differences in the relative molecular weight and number of amino acids among PsAP2/ERFs. The majority of AP2/ERF genes were located in the nucleus, and a small number were localized in the mitochondria, chloroplast, cytoplasm, and plasma membrane, suggesting that AP2/ERFs generally function in the nucleus to regulate gene expression (Table S1). Similar results were found in *Triticum durum* [27], *O. sativa* [30], and *oil palm* [31], suggesting that most PsAP2/ERFs are involved in the regulation of target gene expression. Of note, only one gene, *PsAP2/*

Fig. 6 Analysis of cis-acting elements in the promoter regions of *PsAP2/ERFs*. The numbers in the figure represent the number of cis-acting elements contained, from white to red, the darker the color, the higher the number of cis-acting elements contained

exons contribute to functional differentiation between gene families and subfamilies [33]. According to our gene structure analysis, the AP2 subfamily had the most introns within the ANT subfamily (Fig. 2C). It has been shown that intron-less or intron-poor genes in the AP2/ERF gene family are more likely to play a role in drought and salt stress; it is possible that the limited role of the ANT subfamily in the abiotic stress response may be related to its intron number [34]. Members of the ANT subfamily were associated with cell growth in this study, consistent with previous findings in *Brassica rapa* [35]. It can be hypothesized that a larger number of introns increases the diversity of plant cell functions. All AP2/ERF genes contain a complete AP2/ERF structural domain (motif 1) (Fig. 2B). The conserved motifs within the same subfamily were very similar, and these proteins may have similar functions. For example, the RAV subfamily included a unique B3 structural domain (motif 9), and both the ERF and DREB subfamilies contained motif 3. Therefore, motif3 may be important in determining the function of this subfamily.

Gene duplication facilitates gene family expansion, and provides the potential for neofunctionalization [36]. Synteny analysis within gene families can be used to predict homologous genes, and since homologous sequences may have similar functions, it can be used to help predict the function of coding and non-coding regions by aligning them at the nucleotide level of a gene family. In this study, the average K_a/K_s value of PsAP2/ERF gene pairs was less than 1, providing strong evidence for negative selective. The main modes of gene duplication in plants are tandem, segmental, or whole gene duplications, with selection by purging to produce genes with conserved structures [37]. As predicted, 26 groups of duplicated *PsAP2/ERFs* were dispersed in blocks of partial replications, indicating that the expansion of the PsAP2/ERF gene family may be attributed to a large number of duplication events. Furthermore, many duplicated gene-pair blocks were collinear, suggesting that these duplication events were derived from chromosome segmentation or large-scale duplication/triplication events (Fig. 4).

We constructed PsAP2/ERF families in *A. thaliana* and four species of Rosaceae for comparative analyses (Fig. 5). *P. sibirica* shared a number of collinear regions with *P. avium*, *P. mume*, and *P. persica*, consistent with close evolutionary relationships within Rosaceae. Synteny analysis allows us to understand the evolutionary relationships and the degree of genetic similarity between different species. The collinearity between *P. sibirica* and *P. avium* suggests that these two species have a particularly close evolutionary relationship. The correlation between *P. sibirica* and *A. thaliana* was relatively low, and other studies have shown that the correlation between *Ananas comosus* and *A. thaliana* was significantly lower than that
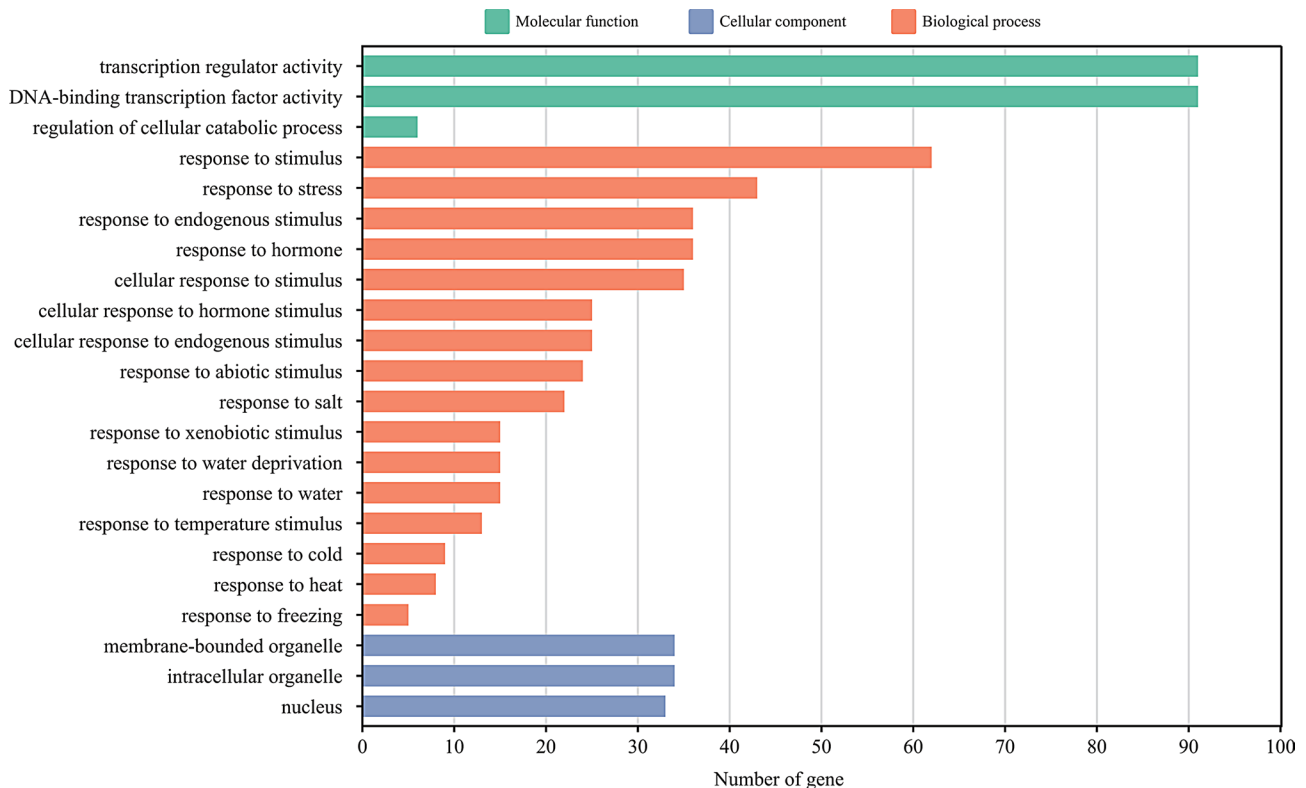
ERF20, was localized in the plasma membrane (Table S1). This gene may be related to plasma membrane synthesis and material transport; however, homologues in other species have not been evaluated. Further studies are needed to determine the role of this gene in the plasma membrane.

Structural variation plays a vital role in evolution [32]. The gain, loss, insertion, or deletion of introns and

Fig. 7 Gene Ontology (GO) annotation analysis of PsAP2/ERFs. PsAP2/ERFs were assigned to terms in three categories: cellular component, molecular function, and biological process. Green squares represent molecular functions, blue squares represent cellular components, and orange squares represent biological processes; the longer the length of a bar, the greater the number of genes associated with a gene function

between *A. comosus* and *Musa acuminata* [38]. Accordingly, we hypothesized that the genetic similarity is high between woody plants of the same genus and between woody plants and herbaceous taxa. Similar results have been reported for the bHLH [39] and WRKY [40] families. Notably, analysis of synteny analysis showed that *P. salicina* and *P. sibirica*, which theoretically may be more similar in terms of gene structure and function, showed lower covariance than *P. sibirica* and *A. thaliana*, a phenomenon that could be explained by the fact that the AP2/ERFs of *P. salicina*, in the face of stressful selection, are more mutant. The phenomenon of gene mutation is increased and members evolve faster, so the genetic similarity with other Rosaceae is reduced.

TFs play an important role in inducing downstream functional gene expression and signal transduction [41]. Some cis-acting elements associated with the regulation of plant growth under biotic/abiotic stresses were found in *PsAP2/ERFs*, such as TC-rich repeats, MBS, MBSI, and LTR (Fig. 6) (Table S3). Additionally, cis-acting elements related to the phytohormone response were detected, such as the P-box, GARE-motif, and SARE, which sense and transmit signals in response to environmental changes and contribute to homeostasis in plants through a network of hormones [42]. These results suggest that

PsAP2/ERFs are functionally diverse. LTR cis-acting elements were found in the promoters of 56 *PsAP2/ERFs*, including nine in the promoter of *PsAP2/ERF29*. All of these *PsAP2/ERFs* belonged to the ERF subfamily, and were involved in the response to low-temperature stress. Therefore, we hypothesize that the ERF subfamily of AP2/ERF TFs plays a critical role in the plant response to low-temperature stress. In *Poncirus trifoliata*, a yeast one hybrid (Y1H) assays suggested that *PtrERF109* could bind to the GCC-box element in the POD-encoding gene *Prx1* promote to regulate ROS homoeostasis to enhance cold tolerance [43]. The homologous gene of *PtrERF109*, *PsAP2/ERF92*, which has the same functions of transcriptional regulation and response to external stimuli as well as cis-acting elements in response to low-temperature stress, was expressed at a higher level in the transcriptome, and thus it could be hypothesized that the mechanism of action of *PsAP2/ERF92* could be to activate the *Prx1* promoter, and then enhance the freezing resistance of *P. sibirica* by scavenging ROS.

A GO annotation analysis provides insight into gene functions (Fig. 7). In terms of molecular functions, a number of PsAP2/ERFs were associated with regulatory activity and DNA-binding factor activity; in *O. sativa*, some AP2/ERFs contribute to the regulation of plant
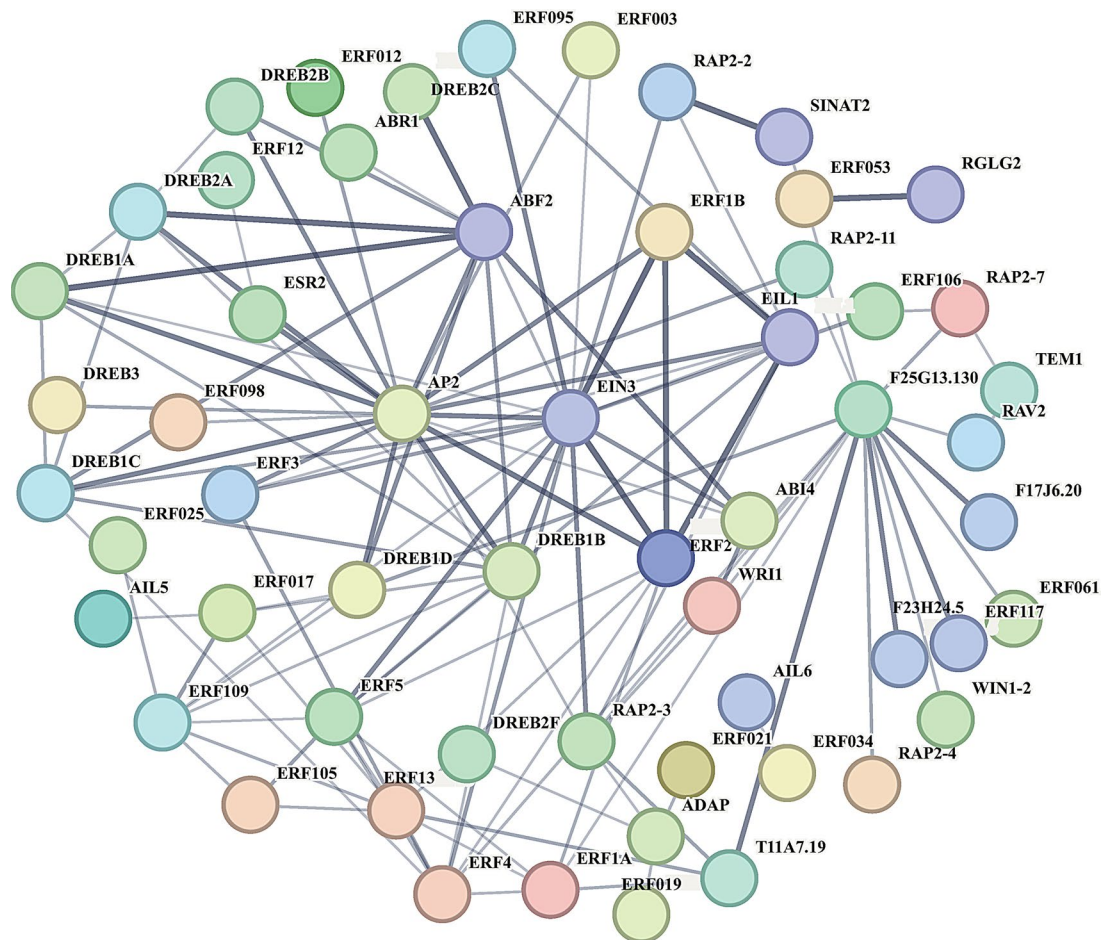
Fig. 8 Protein interaction network of PsAP2/ERFs according to AP2/ERF orthologs in *A. thaliana*. STRING was used to predict the network. Genes connected by gray lines are functionally related. More gray lines indicate more genes interacting with them, and the thickness of the gray lines indicates the tightness of the interaction between the two, with thicker gray lines indicating stronger tightness

cellular metabolism [44]. In terms of biological processes, PsAP2/ERFs showed enrichment for cellular functions related to cold, freezing, salt, and water stress, supporting the roles of PsAP2/ERFs in coping with abiotic stress, especially low-temperature stress. This result was consistent with the results of the cis-acting element analysis (Table S4).

To understand the functions of PsAP2/ERFs further, we performed transcriptomic analyses of cold-resistant clone no. 453 and cold-sensitive clone no. 371 under low-temperature stress (Fig. 9). In this analysis, *PsAP2/ERF6* negatively regulated the response to low temperatures in addition to its homologue *AtWRI4*, which has only been associated with epidermal wax synthesis and not with the response to stress [45]. *PsAP2/ERF31* was homologous to *DREB2C* in *A. thaliana* and was highly expressed in response to low-temperature stress. Indeed, *DREB2C* reportedly shows specific expression in response to temperature stress in many species and can enhance the ability of plants to adapt to temperature changes [46]. The expression of *PsAP2/ERF31* was also somewhat

elevated in the transcriptome and was associated with the response to temperature stimuli in the GO functional enrichment analysis. Therefore, we hypothesized that *PsAP2/ERF31* is involved in the regulation of temperature stress responses. *OsERF096* reduces cold tolerance by inhibiting jasmonic acid (JA)-activated CBF signaling pathway and also targets MYC transcription factor, which activates nutrient storage protein expression to initiate a defense response [4]. Meanwhile *OsERF096* can inhibit the downstream signaling of ICE-activated c -repeat binding factor (CBF) by inhibiting ICE protei [47]. The homologous gene of *OsERF096*, *PsAP2/ERF112*, was highly expressed in the transcriptome and also contained cis-acting elements related to hormones in response to low-temperature stress, and it can be hypothesized that *PsAP2/ERF112* may play an inhibitory role by suppressing the JA-activated CBF signaling pathway in the low-temperature response of *P. sibirica*.

Previous studies have demonstrated that *AP2/ERFs* in specific tissues are related to plant growth and development [48]. For example, *AeAP2/ERF61* plays a vital role
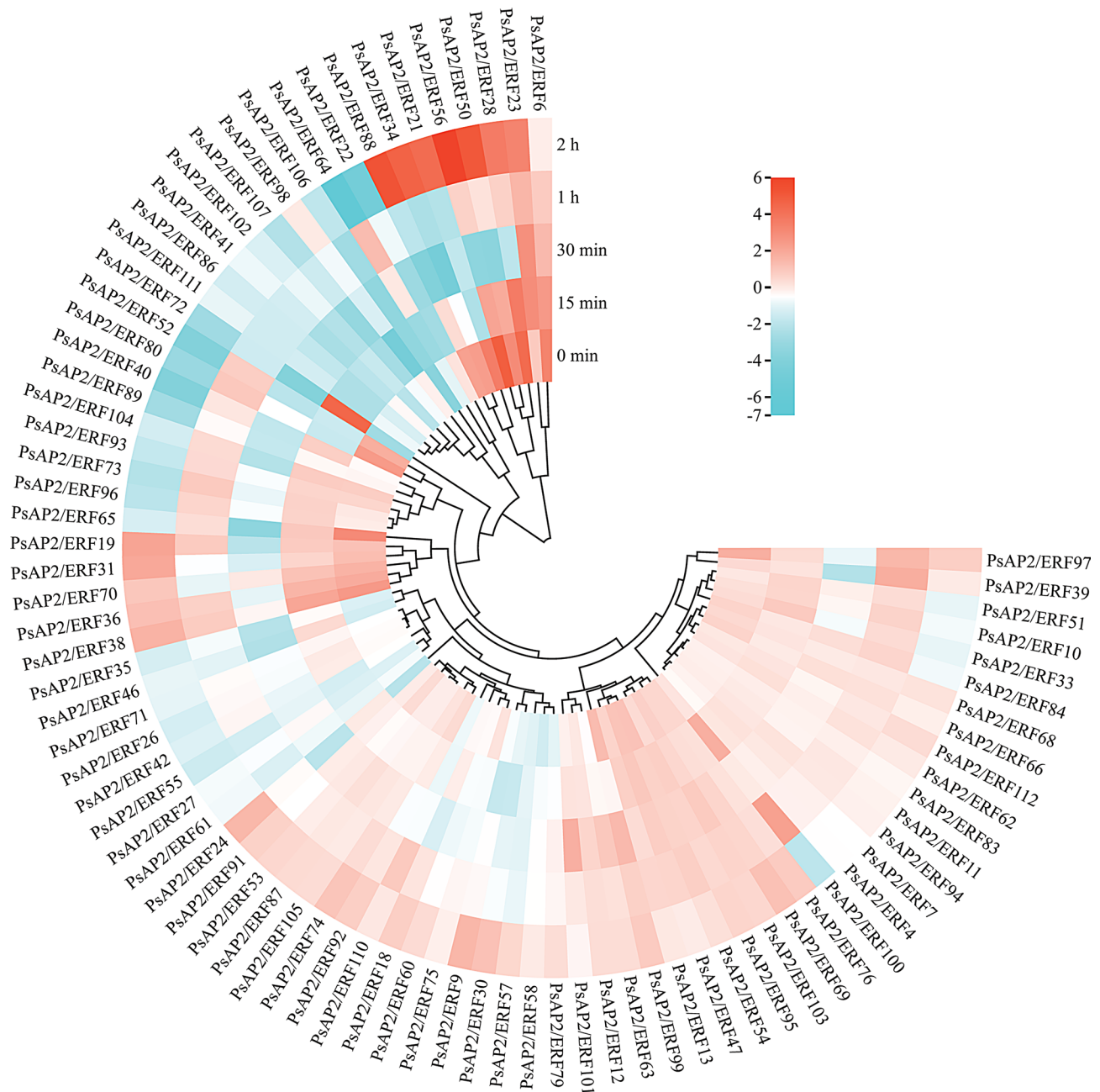
Fig. 9 Transcriptome analysis of *PsAP2/ERFs* for low-temperature stress. The color scale indicates the log2 fold change expression of NO.453 relative to NO.371, with blue (-7) to red (6) indicating low to high expression abundance

in regulating flower development in *Actinidia eriantha* [5]. Notably, *AeAP2/ERF61* is homologous to *PsAP2/ERF83*, closely related to the growth hormone-signaling pathway based on cis-acting elements, GO functional annotations, and high expression in floral organs. Therefore, we hypothesize that *PsAP2/ERF83* is involved in floral organ development (Fig. 10). We further found that *PsAP2/ERF105* is highly expressed in the pistil and contained growth hormone response elements, which might be involved in the regulation of pistil development.

However, the homologous gene *AtCRF9* is a repressor of cytokinin during reproductive development [49]. Therefore, the specific function of PsAP2/ERF105 is unclear and should be explored in subsequent studies.

Elevated *AP2/ERF* expression is directly or indirectly related to freezing tolerance [50]. In this study, among the selected *PsAP2/ERFs*, 95% were upregulated under low-temperature stress, similar to previous results for *Juglans mandshurica* [51], *Brassica napus* [52], and *Rhododendron* [53]. In a study of *Betula platyphylla*,

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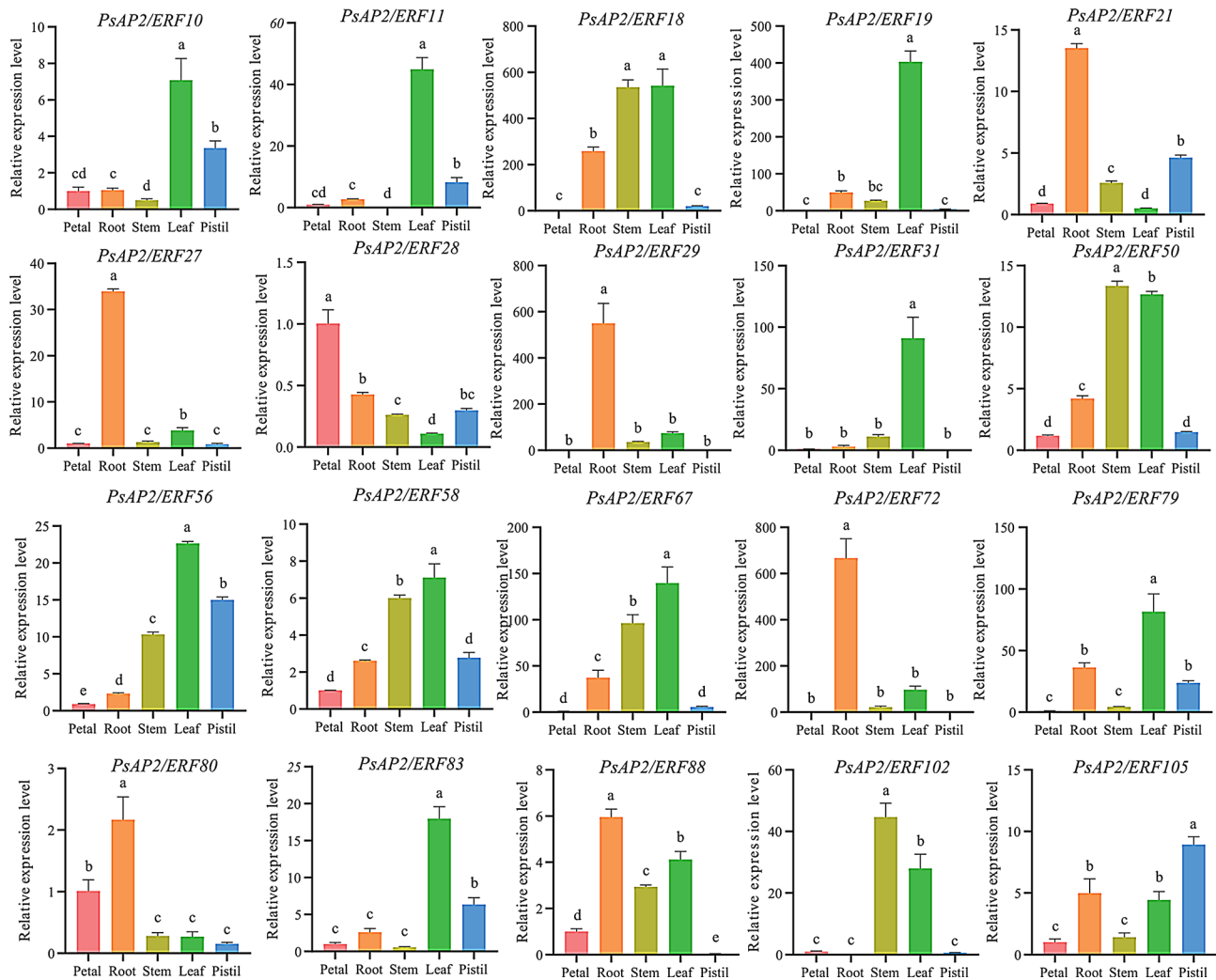
Fig. 10 Expression analysis of 20 *PsAP2/ERFs* using quantitative real-time PCR (qRT-PCR) in different tissues of *P. sibirica*. Expression levels of petals were used as a control. Data are presented as the mean±SD of three biological replicates. Statistical significance ($p < 0.01$) was tested using one-way ANOVA and Tukey's post hoc tests (indicated by lowercase letters). The numbers on the left side of the figure express the multiplicity of expression relative to the control group

BpERF13 enhanced cold tolerance by binding to LTRE-COREATCOR15 or MYBCORE cis-elements in the promoter region of target *CBF* genes to regulate expression and reduce reactive oxygen species [54]. Y1H analysis and ChIP-seq validation demonstrated that *BpERF13* could directly bind to the promoters of the *CBF3* and *CBF4* and up-regulate their expression levels under low-temperature stress, leading to an increase in cold hardiness of the transgenic lines. Thus, it was demonstrated that *CBF* genes are not only activated by *ICE1*, but they can also be activated by ERF transcription factors such as *BpERF13* [54]. Further, the expression of the *BpERF13* homologue *PsAP2/ERF67* increased rapidly after low-temperature stress in *P. sibirica*. Therefore, it is hypothesized that *PsAP/ERF67* also enhances plant freezing tolerance by regulating CBF gene expression. In turn, *AtERF012* is involved in the response to abiotic stress, such as low

temperature stress [55]. The homologous gene *PsAP2/ERF88* contained many cis-acting elements involved in defense and stress responsiveness and was highly expressed in the transcriptome; consequently, *PsAP2/ERF88* may also be involved in response to cold stress. In particular, *PsAP2/ERF21* was closely related to freezing stress based on analyses of cis-acting elements, GO functional annotation, transcriptome sequencing, and qRT-PCR (Fig. 11). In *P. persica*, *PpRAP2.12* activated *PpVIN2* expression and reduced tolerance to cold stress, and its homologous gene, *PsAP2/ERF83*, was expressed in both the transcriptome and experiments, but at low levels, and it is hypothesized that it may also negatively regulate plant cold tolerance by regulating downstream sucrose cleavage-related genes [56]. The *PmCBF03* gene promoted the accumulation of soluble proteins in transgenic *A. thaliana* and increased the expression levels of
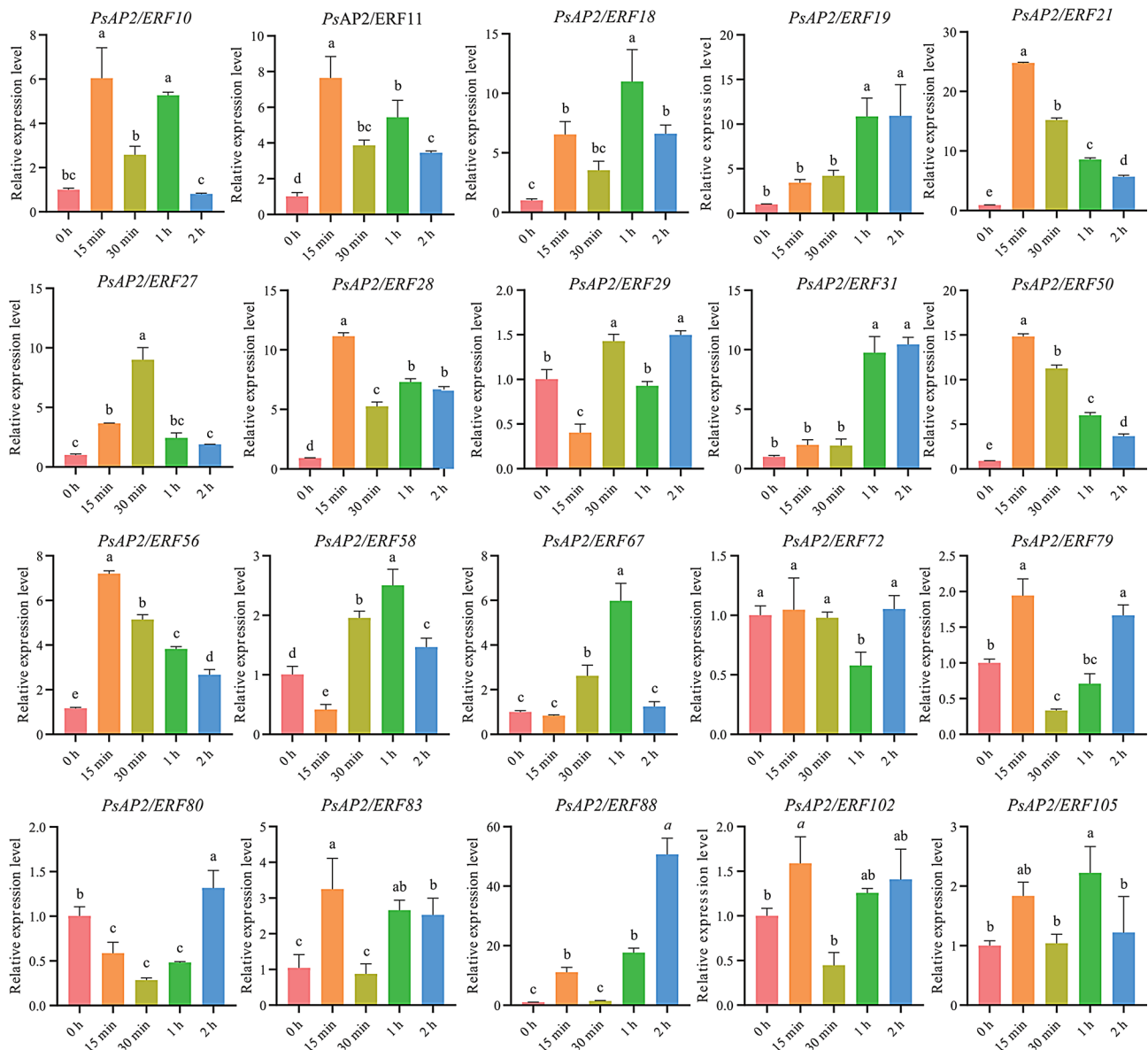
Fig. 11 Quantitative real-time PCR (qRT-PCR) was used to analyze the expression of 20 *PsAP2/ERFs* under different times of low-temperature stress in *P. sibirica* pistils. The expression level at 0 h was used as a control. Data are presented the mean ± SD of three biological replicates. Statistical significance (*p* < 0.01) was tested using one-way ANOVA and Tukey's post hoc tests (indicated by lowercase letters). The numbers on the left side of the figure express the multiplicity of expression relative to the control group

antioxidant-related genes in transgenic plants, as verified by double-luciferase analysis with Y1H in Japanese apricot (*Prunus mume*) [24]. Its homologous gene, *PsAP2/ERF28*, was also associated with low-temperature stress, and it was hypothesized that it might also affect antioxidant-related genes to improve the cold resistance of plants. It was also verified that *EjCBF3*, the homologous gene of *PsAP2/ERF28* in *Eriobotrya japonica*, has the function of enhancing plant cold resistance by increasing the activity of antioxidant enzymes [57]. *AtCBF2*, a homolog of *PsAP2/ERF21*, is important for cold resistance in plants [58]. *AtCBF2* responds to cold stress

through the ICE-CBF-COR response pathway, which enables cold sensors located in the plasma membrane to sense cold stress. The influx of Ca^{2+} ions triggers a calcium downstream effect, and the ICE protein binds to the canonical MYC cis-element (CANNTG) in the *CBF3/DREB1A* promoter, leading to the induction of *CBF/DREB1* regulation, which is sequentially triggered by MPK3/6 activity triggers the MAPK cascade and direct repression of *ICE2* and/or activation of the CBF gene via CAMTA3. This enhances *CBF/DREB1A* gene expression and cold tolerance [59]. In *Solanum tuberosum, StCBF1*, a homologue of *PsAP2/ERF21*, and *StCBF4*, a homologue

of *PsAP2/ERF28*, were both shown to be involved in the ICE-CBF-COR signaling pathway, which was validated transgenically, and confirmed to be active in *A. thaliana* by enhancing antioxidant defense systems to enhance cold resistance [60]. Therefore *PsAP2/ERF28* and *PsAP2/ERF21* will also be an important candidate gene for us to study the function and transcriptional regulatory mechanism of cold resistance of *PsAP2/ERFs* afterwards.

Conclusions

In summary, 112 PsAP2/ERFs were identified through a genome-wide survey and were analyzed using bioinformatics approaches to reveal their physicochemical properties as well as phylogenetic and colinear relationships. In addition, we analyzed the expression levels of 20 PsAP2/ERFs in different tissues and in response to low-temperature stress to provide insights into their cold-induced expression patterns and general characteristics. *PsAP2/ERF21*, *PsAP2/ERF28*, *PsAP2/ERF56*, *PsAP2/ERF67*, *PsAP2/ERF83*, and *PsAP2/ERF88* which may play key roles in low-temperature stress in *P. sibirica*, were finally screened out, and the functional validation of these genes will be carried out subsequently. In particular, *PsAP2/ERF21*, which showed significant ability to respond to low-temperature stress in function prediction, transcriptome data and qRT-PCR, can be a key target for subsequent research. *PsAP2/ERF28* may be involved in a variety of regulatory mechanisms related to the response to low-temperature stress and is an important gene for future studies of low-temperature signaling. *PsAP2/ERF67* enhances cold resistance by up-regulating the expression of CBF genes and reactive oxygen species scavenging genes, and may be an important upstream target gene, which is important for the subsequent study of the PsAP2/ERFs regulatory network. *PsAP2/ERF83* may negatively regulate the low-temperature response by regulating soluble sugars and is an important candidate gene for studying the pathway of soluble sugar response to low-temperature stress. This study lays a solid foundation for further studies of the functions of AP2/ERFs in cold resistance in plants and their molecular regulatory mechanisms.

Materials and methods

Identification and characterization of PsAP2/ERFs

Genome data for *P. sibirica* were downloaded from the Rosaceae genome database (https://www.rosaceae.org/) and *A. thaliana* AP2/ERF protein sequences were acquired from The Arabidopsis Information Resource (TAIR) database (https://www.arabidopsis.org/). PsAP2/ERFs were searched using the Hidden Markov Model (HMM) for the AP2/ERF structural domain (PF00847). Further analyses using Pfam [61] and NCBI-CDD

confirmed that all candidate AP2/ERF proteins contained a complete AP2/ERF domain.

The physicochemical properties of all identified PsAP2/ERFs, including the molecular weight (MW), theoretical isoelectric point (pI), and instability index (II), were analyzed using ExPASy (https://www.expasy.org/). The subcellular localization of PsAP2/ERFs was predicted using WoLF PSORT [62].

Phylogenetic analysis and multiple sequence alignment of AP2/ERFs

The AP2/ERF gene families of *P. sibirica*, *A. thaliana*, and *P. trichocarpa* were compared using the ClustalW tool in MEGA 11 [63]. A phylogenetic tree was constructed using the adjacency method and default parameter values, with 1000 bootstrap replicates [40]. The phylogenetic tree was visualized using the ITOLS website (http://itol.embl.de). *P. trichocarpa* genomic data were downloaded from the DOE Joint Genome Institute website (http://genome.jgi-psf.org/Poptr1/Poptr1.download.html). PsAP2/ERF genes were classified based on a previously reported classification of the AtAP2/ERF and PtAP2/ERF families [64].

Phylogenetic analysis, conserved motifs, and gene structure analysis of PsAP2/ERFs

A phylogenetic analysis of PsAP2/ERFs was performed using TBtools and the MEME v5.1.1 online program to predict the conserved motifs [65]. Specifically, the number of motifs was set to 10, and the motif results in XML format obtained from MEME were visualized using TBtools [66]. The deoxyribonucleic acid (DNA) and coding sequences (CDS) of *PsAP2/ERFs* were screened from the whole-genome sequence and gene annotation file for *P. sibirica*. TBtools was used to analyze and visualize the exon-intron structures of *PsAP2/ERFs*.

Chromosomal localization, gene duplication, and covariance analysis of PsAP2/ERFs

The *P. sibirica* genomic data annotation file was downloaded from the Rosaceae genome database to obtain locations of all genes and chromosome length information. Chromosome length and chromosomal localization were evaluated and visualized using TBtools [67].

The K_a/K_s Calculator tool was used to calculate the synonymous (K_s) and non-synonymous mutation frequencies (K_a) and the ratio of non-synonymous to synonymous mutation rates (K_a/K_s) of AP2/ERF genes in *P. sibirica*.

TBtools was used to analyze *A. thaliana*, *P. salicina*, *P. mume*, *P. persica*, and *Prunus avium* in comparison with *P. sibirica*. Then, TBtools was utilized for synteny analysis, and a collinearity analysis of gene repeats in *P. sibirica* with the Advanced Circos function.

Promoter cis-acting element and GO annotation analyses of PsAP2/ERFs

The region 2000 bp upstream of the CDS of *PsAP2/ERFs* was extracted using TBtools software (GTF/GFF3 sequence extractor). The cis-elements in the promoter regions were analyzed using the PlantCARE database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Finally, TBtools was used to visualize the cis-elements. GO annotations for PsAP2/ERFs were obtained using eggNOG (http://eggnog5.embl.de/) and visualized using ChiPlot (https://www.chiplot.online/) [68].

Protein-protein interaction network of PsAP2/ERFs

All PsAP2/ERF sequences were submitted to STRING (version 11.0, http://string-db.org) [69], and *A. thaliana* was selected as the reference organism. BLAST was used to construct protein interaction networks based on the highest-scoring homologues in *A. thaliana*.

Transcriptome analysis

Transcriptome data, including data for two 7-year-old *P. sibirica* clones differing in the frost resistance of floral organs (cold-tolerant 'NO. 453' and cold-sensitive 'NO. 371' as the control group) from the Liaoning National Long-term Research Base for Siberian Apricot Germplasm Conservation and Breeding) and pistils under cold treatment (-4 °C for 0 h, 15 min, 30 min, 1 h, and 2 h) were downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/, GSE204685). AP2/ERF genes were screened and analyzed (Table S7). The ChiPlot website was used to construct a heat map for visualization [68].

RNA isolation and qRT-PCR validation

Experimental materials were selected from 7-year-old *P. sibirica* clone 'Shanxing5' (improved forest tree varieties in Liaoning Province, S-SV-PS-002-2021, Nomenclator: Shengjun Dong and Quangang Liu) from the Liaoning National Long-term Research Base for Siberian Apricot Germplasm Conservation and Breeding (longitude 119°44′48.829″E, latitude 41°8′0.820″N). Healthy plants in good condition with no pest and disease infestation were selected, and petals (control), stems, leaves, roots, and pistils were collected as experimental materials with three biological replicates per sample.

Additionally, whole healthy plants free of pests and diseases and in good condition were placed in an artificial cold chamber at -4 °C, and sampled after 0 h (control), 15 min, 30 min, 1 h, and 2 h. Three biological replicates were created for each sample and stress treatment was performed [40].

Primers were designed using Primer Premier 5.0, and 18 S rRNA was used as the reference gene (Table S8) [39]. The synthesis was entrusted to GENEWIZ (Suzhou, China), and 18 S rRNA served as a reference gene. Total RNA was extracted from the samples using an RNAprep Pure Plant Kit (Tian gen, Beijing, China), and cDNA was obtained using a FastKing RT Kit (Tiangen), according to the manufacturer's instructions. The qRT-PCR was performed on a StepOne Real-Time PCR System (Applied Biosystems) using 2X Universal SYBR Green Fast qPCR Mix. The PCR program consisted of 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 95 °C for 15 s, with a final step at 95 °C for 15 s. The experiment included three biological repetitions and relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method [39]. Statistical analyses were performed using SPSS (version 26.0) [70]. Histograms were generated using GraphPad Prism 8.4.5. Comparisons were performed using Duncan's multiple range test ($p < 0.05$, $n = 3$) (in the figures, different letters represent significant differences).

Abbreviations

MEME	Multiple Em for Motif Elicitation
HMM	Hidden Markov model
K_a	Non-synonymous substitution rate
K_s	Synonymous substitution rate
K_a/K_s	Ratio of the non-synonymous to synonymous substitution rate
At	Arabidopsis thaliana
Ps	Prunus sibirica
TF	Transcription factor
UTR	Untranslated region

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05601-8.

Supplementary Material 1: **Fig. S1** Phylogenetic analysis of the AP2/ERF structural domains of *P. trichocarpa* and *P. sibirica*

Supplementary Material 2: **Fig. S2** Multiple sequence alignments of conserved domains in PsAP2/ERF transcription factors

Supplementary Material 3: **Fig. S3** Conserved motifs of PsAP2/ERFs

Supplementary Material 4: **Table S1** Detailed information for all identified PsAP2/ERFs

Supplementary Material 5: **Table S2** K_a/K_s analysis of segmental and tandem gene duplications of *PsAP2/ERFs*

Supplementary Material 6: **Table S3** Cis-acting elements in promoters of *PsAP2/ERFs*

Supplementary Material 7: **Table S4** GO functional enrichment analysis of *PsAP2/ERFs*

Supplementary Material 8: **Table S5** Analysis of protein interactions of PsAP2/ERFs

Supplementary Material 9: **Table S6** Protein interaction analysis of PsAP2/ERFs

Supplementary Material 10: **Table S7** Transcriptome data for *PsAP2/ERFs*

Supplementary Material 11: **Table S8** Primer sequences for qRT-PCR

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Data availability
The AP2/ERF domain HMM (Hidden Markov Model) profile PF00847 was extracted from the Pfam protein family database (https://www.ebi.ac.uk/interpro/entry/pfam/PF00847/). P. sibirica genome data were downloaded from the Rosaceae genome database (https://www.rosaceae.org/Analysis/10254124). AtAP2/ERF sequences were obtained from the Arabidopsis database (https://www.arabidopsis.org/). P. trichocarpa genomic data were downloaded from the DOE Joint Genome Institute web site (http://genome.jgi-psf.org/Poptr1/Poptr1.download.html). The datasets analyzed in this study are included in the published article and supplementary files.

Declarations

Ethics approval and consent to participate
Plant material used in the study complies with relevant institutional, national, and international guidelines and legislation. Plant material was not obtained for a wild species and permission for the use of the plant material was not required.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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