1 **Structural variation, functional differentiation and**
2 **expression characteristics of the AP2/ERF gene family and**
3 **its response to cold stress and methyl jasmonate in *Panax***
4 ***ginseng* C.A. Meyer**

5 **Short title: Structural and functional Analysis of AP2/ERF**
6 **gene family in *Panax ginseng* C.A. Meyer**

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20 **Abstract:**

21 The APETALA2/Ethylene Responsive Factor (AP2/ERF) gene family has been shown to play a
 22 crucial role in plant growth and development, stress responses and secondary metabolite biosynthesis.
 23 Nevertheless, little is known about the gene family in ginseng (*Panax ginseng*), an important traditional
 24 medicinal herb in Asia and North America. Here, we report the systematic analysis of the gene family
 25 present in ginseng using several transcriptomic databases. A total of 189 putative *AP2/ERF* genes,
 26 defined as *PgERF001* through *PgERF189*. The 93 *PgERF* genes that have the complete AP2 domain in
 27 their open reading frames were classified into five subfamilies, DREB, ERF, AP2, RAV and Soloist.
 28 The DREB subfamily and ERF subfamily were further clustered four and six groups, respectively,
 29 compared to the 12 groups of these subfamilies found in Arabidopsis. Gene ontology categorized these
 30 397 transcripts of the 189 *PgERF* genes into eight functional subcategories, suggesting their functional
 31 differentiation and they have been especially enriched for the nucleic acid binding transcription factor
 32 activity subcategory. The expression activity and networks of the 397 *PgERF* transcripts have
 33 substantially diversified across tissues, developmental stages and genotypes. Then, the expression
 34 change of six *PgERF* genes randomly selected from DREB subfamily, i.e., *PgERF073*, *PgERF079*,
 35 *PgERF110*, *PgERF115*, *PgERF120* and *PgERF128* responding to cold stress suggesting that DREB
 36 subfamily genes played an important role in cold resistance of ginseng. Finally, we studied the
 37 responses of the *PgERF* genes to methyl jasmonate (MeJA). 288 (72.5%) of the 397 *PgERF* gene
 38 transcripts responded to the MeJA treatment, with 136 up-regulated and 152 down-regulated, indicating
 39 that most members of the *PgERF* gene family are responsive to MeJA. These results provide resources
 40 and knowledge necessary for family-wide functional analysis of the *PgERF* genes in ginseng and
 41 related species.

42 **Keywords:** Gene family, APETALA2/Ethylene Responsive Factor (AP2/ERF) genes, *Panax ginseng*,
 43 Phylogeny, Functional Differentiation, Co-expression network, cold stress, Methyl Jasmonate (MeJA)

1. Introduction

Plants are subjected to numerous biotic and abiotic stresses all time through their growth and development. Therefore, they have developed a variety of mechanisms by producing secondary signaling molecules (e.g., ethylene and jasmonic acid) and response networks at the molecular, biochemical and physiological levels to perceive the external signals from and response to the stresses [1]. It has been documented that a large number of genes are involved in these processes [2]. Therefore, it is important to decipher the regulatory mechanisms of the defense-related genes involved in the signal transduction pathways and the plant responses to these stresses for enhanced and efficient plant genetic improvement [3]. The APETALA2/Ethylene Responsive Factor (AP2/ERF) transcription factors have been demonstrated to be one of the most important gene families actively functioning in plant response to biotic and abiotic stresses by binding to cis-acting elements of downstream target genes [4].

The AP2/ERF family has one or two conserved APETALA2 (AP2) domains (approximately 60 - 70 amino acids) [5]. Based on the number and amino acid sequence similarities of the AP2 domains, the AP2/ERF family is divided into the DREB (dehydration responsive element binding), ERF, AP2, RAV (Related to ABI3/VP1) and Soloist subfamilies [6],[7]. Both DREB and ERF subfamilies possess a single AP2 domain, with a specific WLG motif, and could be further subdivided into A1 to A6 and B1 to B6 groups, respectively [6]. Alternatively, the DREB and ERF subfamilies were also categorized into I to X, and VI-L and Xb-L groups, respectively [7]. The AP2 subfamily has two tandemly repeated AP2 domains, while the RAV subfamily has one AP2 domain and one B3 domain that are commonly found in other transcription factors [8]. The Soloist subfamily also has only one AP2 domain. It was classified into an independent subfamily due to its relatively low sequence homology with the DREB and ERF subfamilies [9]. Although the AP2 domain of the AP2/ERF family is highly conserved, its five

subfamilies, DREB, ERF, AP2, RAV and Soloist, recognize different DNA cis-acting elements and exhibit substantial functional diversity [10]. Specifically, the members of the AP2 subfamily bind to the GCAC(A/G)N(A/T)TCCC(A/G)ANG(C/T) element and regulate developmental processes of different plant tissues, e.g., embryo, flower, sepal and fruit [11],[12],[13]. The *RAV1* gene of the RAV subfamily was reported to bind to CAACA and CACCTG motifs in *Arabidopsis thaliana* [14]. The roles of the RAV subfamily in plant development and various biotic and abiotic stresses were investigated in several plant species [15],[16],[17]. The only gene of the Soloist subfamily in *Arabidopsis*, *APD1* (*At4g13040*), worked as a positive regulator of disease defense by up-regulating the accumulation of salicylic acid (SA) [18]. The members of the ERF subfamily typically bind to the cis-acting element GCC-box and are involved in the signaling pathways of plant hormone, e.g., ethylene (ET), SA, jasmonic acid (JA) and abscisic acid (ABA), which play an important role in both plant growth and development and response to stresses [19],[20],[21]. On the other hand, the DREB subfamily recognizes the conserved CCGAC motif of the dehydration-responsive element present in stress-responsive genes and is associated with the response of plants to abiotic stresses [22],[23],[24].

The AP2/ERF family has been well characterized in the model plants, *A. thaliana* [6],[7] and *Medicago truncatula* [25], several crops, such as rice [7], maize [26], soybean [27], Chinese cabbage [10] and grapevine [28], and *Populus trichocarpa* [29]. However, little is known about the AP2/ERF family in the medicinal herb, *Panax ginseng* (ginseng). *Ginseng* is a perennial of the *Araliaceae* family and has long been cultivated for human medicine in Asia, particularly in China, Korea, and Japan. Ginseng, known as the “king of all herbs” in China, is mainly cultivated in Jilin Province; therefore, it is often known as Jilin ginseng. Ginseng has been widely used as a medicinal herb due to its bioactive components, especially ginsenosides that have been shown to play significant roles in anti-inflammation

[30],[31], antitumor [32], and immunomodulation [33]. However, ginseng has been suffering from various biotic and abiotic stresses, which is greatly threatening the ginseng production. Therefore, identification, characterization and utilization of the defense-related genes in ginseng are of significance for ginseng breeding and production. In the present study, we comprehensively studied the AP2/ERF family present in Jilin ginseng in several aspects, including gene identification, protein motif characterization, functional categorization and phylogenetic analysis. Moreover, the expression activities and patterns of AP2/ERF genes were also investigated at different developmental stages, in different tissues, different cultivars, under cold stress and under the methyl jasmonate (MeJA) treatment. The results of these studies have laid the foundation for deeply functional analysis and utilization of the genes of the AP2/ERF family and provided vital information on the molecular mechanism of plant response to biotic and abiotic stresses in ginseng and related plant species.

2. Materials and methods

2.1 Databases

We previously established a comprehensive transcriptome for Jilin ginseng from 14 tissues (fiber root, leg root, main root epiderm, main root cortex, rhizome, arm root, stem, leaf peduncle, leaflet pedicel, leaf blade, fruit peduncle, fruit pedicel, fruit flesh, and seed), from which 248,993 transcript unigenes (130,557 gene IDs) were assembled [34]. Moreover, we also sequenced and established the databases for the transcriptomes of the roots of 5-, 12-, 18- and 25-year-old plants [34] and the roots of four-year-old plants of 42 genotypes (named from S1 to S42) representing the diversity of Jilin ginseng [35]. In this study, a ginseng line IR826 genome sequence database [36] and another Ginseng Genome Database (<http://ginsengdb.snu.ac.kr/index.php>) reported by Kim *et al.* [37] were also used. In addition,

a transcriptome database of the adventitious roots of ginseng cv. Cheongsun treated with 200 μ M MeJA for 0, 12, 24 and 48 h, respectively [38] was also consulted.

2.2 Identification of *PgERF* genes in ginseng

To identify the genes of the AP2/ERF family in ginseng, the Hidden Markov Model (HMM) profile of the AP2/ERF domain (Pfam: PF00847) and the protein sequences of the AP2/ERF genes downloaded from NCBI (<http://blast.ncbi.nlm.nih.gov/Blast>) were used to query the 248,993 Jilin ginseng transcript unigenes [34] by TBLASTN at E-value $\leq 1e-6$. The obtained sequences were then used as a query to search for homologs in the ginseng line IR826 genome database [36]. Furthermore, TBLASTN were performed again to search the 248,993 transcript unigenes [34] using the homologs as query with E-value $\leq 1e-6$ to maximize identification of the AP2/ERF family genes in ginseng. After merged all these aforementioned results, the identified genes were defined as *PgERF* for the AP2/ERF genes in ginseng and extracted by a Perl programming software. Finally, the predicted *PgERF* genes were analyzed by the conserved domain database (CDD) (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and the ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) at NCBI.

2.3 Multiple sequence alignment and phylogenetic analysis of *PgERF* genes

The encoded AP2 domain of the *PgERF* genes was aligned using the ClustalW program [39] and an unrooted phylogenetic tree was first constructed from the genes to have a preliminary phylogenetic tree of the *PgERF* genes. Then, another unrooted phylogenetic tree was constructed using 93 of the predicted *PgERF* genes representing the AP2/ERF family in ginseng and 147 *AtERF* genes previously identified and annotated in Arabidopsis [7]. These two phylogenetic trees were both constructed using

MEGA 5.0 by the Neighbor-Joining method with 1,000 bootstrap replications, using the Poisson correction model and the pairwise deletion [40].

2.4 Motif prediction of *PgERF* genes

The putative protein sequences of the above 93 *PgERF* genes used for construction of the AP2/ERF family phylogenetic tree were subjected to the online software, MEME (multiple EM for motif elicitation, V5.0.3) (<http://meme-suite.org/tools/meme>) [41] to identify the conserved motifs of these predicted *PgERF* genes in ginseng. Motif length was set to 6 - 50 amino acids and the maximum number of motifs was set to 25, while other parameters were set as default.

2.5 Expression and functional networks of *PgERF* genes

The expression profiles of all putative transcripts of *PgERF* genes identified above were extracted by a Perl programming software from the above four transcriptome databases: (1) the 14 tissues of a Jilin ginseng four-year-old plant, (2) the roots of Jilin ginseng 5-, 12-, 18- and 25-year-old plants, (3) the four-year-old roots in 42 Jilin ginseng genotypes and (4) the ginseng cv. Cheongsun adventitious roots treated with 200 μ M MeJA for 0, 12, 24 and 48 h, respectively. The expression profiles of the putative transcripts of *PgERF* genes were measured as transcripts per million (TPM) and visualized by expression heatmap using the R programming language and software (<http://www.r-project.org/>, V3.3.3). Finally, the co-expression networks of these *PgERF* gene transcripts were constructed and analyzed among different tissues and different genotypes of Jilin ginseng using the BioLayout Express^{3D} software (Version 3.2) [42].

2.6 Expression activity of *PgERF* genes responding to cold stress

Equivalent ginseng hair roots (1 gram) were freshly cut from mature hair roots and cultured with

250 ml 1/2 Murashige and Skoog (MS) medium in dark culture at 22°C for 30 days. Then, to simulate the cold stress treatments, the 30-days-old hair roots were placed in 4°C for 6 h, 24 h, 48 h and 72 h, respectively. Afterwards, the ginseng hair roots were harvested and stored in -80°C for the RNA isolation and further quantitative real-time PCR analysis. The total RNA of ginseng hair roots was extracted by TRIzol reagent (Bioteke, Beijing, China) according to the manufacturer's instructions, which was further reverse transcribed into cDNA using a PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Tokyo, Japan), following the manufacturer's instructions. In this study, quantitative real-time PCR (qRT-PCR) of six *PgERF* genes, including *PgERF073*, *PgERF079*, *PgERF110*, *PgERF115*, *PgERF120* and *PgERF128*, was performed. The *PgGADPH* gene was used as the internal reference. The gene-specific primers used in qRT-PCR were designed by Primer Premier Software (version 5) and were listed in Table S1. The qRT-PCR was conducted by an Applied Biosystems 7500 Real Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) and SYBR Premix Ex Taq™ II (TaKaRa, Tokyo, Japan). The qRT-PCR was performed using the following steps: 30 seconds at 95°C; forty cycles of 5 seconds at 95°C and 34 seconds at 60°C; one cycle of 15 seconds at 95°C and 60 seconds at 60°C; 15 seconds at 95°C. The relative expression levels of these selected genes were calculated using formula $2^{-\Delta\Delta C_t}$ [43], and all the experiments were amplified in triplicate.

3. Results

3.1 Identification and classification of *PgERF* genes

A total of 397 transcripts that were derived from 189 predicted *PgERF* genes, including those containing a partial or complete AP2/ERF domain, were identified. These *PgERF* genes were defined *PgERF001* to *PgERF189*, with a suffix (e.g., -1) for different transcripts derived from the same gene (Table S2). Then, the sequences of 342 AP2/ERF gene transcripts downloaded from Ginseng Genome Database (<http://ginsengdb.snu.ac.kr/index.php>) were aligned with 397 transcripts identified in this study with identity $\geq 95\%$, alignment length ≥ 200 bp (about AP2 maximum domain length). As a result,

175 138 (73%) *PgERF* genes which contained 302 transcripts (88%) identified in this study were similar to
 176 266 (78%) transcripts of the AP2/ERF gene from Ginseng Genome Database, which were supposed to
 177 the same genes (Fig.S1 and Table S3). However, the other 51 (27%) *PgERF* genes, whose sequences
 178 were quite different from Korean ginseng AP2/ERF genes, were assigned as newly discovered
 179 AP2/ERF genes in ginseng (Fig.S1 and Table S3). The *PgERF* gene transcripts identified in this study
 180 had nucleotide sequences ranging from 203 bp to 2,897 bp, with an average length of 1,216 bp. Of
 181 these 397 *PgERF* gene transcripts, 176, derived from 96 *PgERF* genes, had partial AP2 domains, or
 182 complete AP2 domains but being outside of open reading frames (ORFs). The remaining 221 *PgERF*
 183 transcripts, derived from 93 *PgERF* genes, had complete AP2 domains within ORFs. Therefore, these
 184 221 *PgERF* transcripts were further analyzed. The 221 *PgERF* gene transcripts encode putative
 185 proteins with a length varying from 96 to 561 amino acids, with an average length of 256 amino acids
 186 (Table S4). Analysis using the ExPASy Server showed that these putative proteins had an isoelectric
 187 point between 4.43 (*PgERF025*) and 11.12 (*PgERF180*) and a molecular mass ranging from 11.00 kDa
 188 (*PgERF140*) to 62.83 kDa (*PgERF159*) (Table S4).

189 Sakuma et al. [6] classified that the Arabidopsis AP2/ERF family into five subfamily, ERF, DREB,
 190 AP2, RAV and Soloist. We also classified the 93 predicted *PgERF* genes whose transcripts had
 191 complete AP2 domains within ORFs into these five subfamilies, according to the structures and the
 192 number of AP2/ERF domains. These five *PgERF* subfamilies, ERF, DREB, AP2, RAV and Soloist,
 193 contained 27, 48, 14, 2 and 2 genes, respectively (Fig. 1). Specifically, two genes, *PgERF035* and
 194 *PgERF084*, were classified into the Soloist subfamily due to their low homology with the remaining
 195 AP2/ERF genes and their high homology with the Arabidopsis Soloist subfamily gene, *AT4G13040*.
 196 The *PgERF112* gene was the only one that codes one AP2/ERF domain with one B3 domain; therefore,
 197 it was classified into the RAV subfamily. Moreover, *PgERF171* was also classified into the RAV

subfamily, even though it does not contain the B3 domain, because it has a high homology with the Arabidopsis RAV subfamily genes, *ATIG51120* and *ATIG50680*. As seven *PgERF* genes, *PgERF062*, *PgERF089*, *PgERF134*, *PgERF135*, *PgERF142*, *PgERF148* and *PgERF159*, contain two repeated AP2/ERF domains, they were classified into the AP2 subfamily. In addition, another seven *PgERF* genes, including *PgERF020*, *PgERF045*, *PgERF048*, *PgERF076*, *PgERF101*, *PgERF132* and *PgERF140*, were also classified into the AP2 subfamily as they have high sequence similarity with the members of the AP2 subfamily, even though they do not contain two repeated AP2/ERF domains. Of the 75 remaining *PgERF* genes, 48 and 27 were classified into the ERF and DREB subfamilies, respectively (Fig. 1). Furthermore, the DREB and ERF subfamilies of ginseng were each further divided into six groups, A1 through A6 and B1 through B6, respectively, or these two subfamilies were divided into 12 groups, from I to X, VI-L and Xb-L, based on Nakano et al. [7] (Fig. 1).

Figure 1. Phylogenetic tree of the AP2/ERF gene family present in ginseng and Arabidopsis. The amino acid sequences of the AP2 domain were aligned using Clustal W and the phylogenetic tree was constructed using neighbor-joining method.

3.2 Phylogenetic analysis of the *PgERF* gene family

To determine the phylogeny of the *PgERF* gene family, the 93 predicted *PgERF* genes whose transcripts had complete AP2 domains were also used. An unrooted phylogenetic tree was constructed from the 93 predicted *PgERF* genes for the *PgERF* gene family. The result showed that the *PgERF* gene family was apparently classified into five clades, corresponding to ERF, DREB, AP2, RAV and Soloist subfamilies (Fig. S2). Then, we constructed an unrooted phylogenetic tree of the *PgERF* gene family using 147 Arabidopsis annotated *AtERF* genes as controls based on their conserved AP2 domains. The resultant phylogenetic tree clustered the 93 *PgERF* genes and 147 *AtERF* genes into 15 distinct clades, of which 13 was corresponding to the I, II, III, IV, V, VI, VII, VIII, IX and X groups of

the DREB and ERF subfamilies, and AP2, RAV and Soloist subfamilies of the *PgERF* family, and two, corresponding to Xb-L and VI-L groups of the DREB and ERF subfamilies, had only *AtERF* genes (Fig. 2). This result was consistent with the classification of the AP2/ERF family.

3.3 Motif identification and multiple sequence alignment

Next, the 93 predicted *PgERF* genes were subjected to conservative analysis for the conserved motifs of their proteins (Fig. 2; Fig. S3). A total of 25 conserved motifs were identified for the putative proteins of the 93 predicted *PgERF* genes, which were herein designated as Motif 1 through Motif 25. Motif 1 to Motif 6 were located in the AP2/ERF domain and the remaining 19 motifs, including Motif 7 through Motif 25, were found outside the AP2/ERF domain. The *PgERF* proteins encoded by gene members of the same subfamily or group contained similar conserved motifs. For example, Motifs 4, 7, 15, 16, 17, 21 and 25 were specifically shared by gene members of the AP2 subfamily. Motifs 14 and 18 were specifically present within Group I, while Motifs 9 and 20 were only present in the Group III gene members of the ERF subfamily. Motifs 10, 11, 12, 13, and 19 were specific for the DREB subfamily and absent in all four other subfamilies (ERF, AP2, RAV and Soloist subfamilies). These results suggested that most of the 25 motifs were divergent among subfamilies or groups, which might play an important role in their functional divergence [25].

Figure 2. Distribution of conserved motifs among the gene members of the *PgERF* family. Each motif is represented by a colored box. Box length corresponds to the motif length.

As the featured sequences within the specific domains of transcription factors are critical to their functions [44], the conserved amino acid residues of the AP2 domains were identified for the genes of both DREB and ERF subfamilies. By aligning the amino acid sequences of the AP2 domains of these two subfamilies in ginseng and Arabidopsis, 14 conserved amino acid residues, including 4G, 6R, 8R,

15W, 16V, 18E, 20R, 22P, 39W, 40L, 49A, 52A, 54D and 72N, and 11 conserved amino acid residues, including 4G, 6R, 8R, 11G, 17I, 30R, 42A, 46Y, 47D, 55G and 63F, were identified for the DREB and ERF subfamilies, respectively (Fig. S4 and S5). Besides, all gene members of the DREB subfamily and ERF subfamily obviously contained the two featured conserved elements, including YRG and RAYD, in their AP2 domains.

3.4 Functional categorization of the *PgERF* genes

To estimate the functional differentiation of the *PgERF* family, all 397 transcripts of the 189 *PgERF* genes identified in this study were annotated and functionally categorized using the Blast2GO software (Version 4.1.9) [45]. Surprisingly, only 195 (49%) of the *PgERF* gene transcripts could be annotated, while the remaining 202 could not be annotated using the database of the Blast2GO software, suggesting the uniqueness of the *PgERF* genes in ginseng. The annotated *PgERF* gene transcripts were categorized into all three primary gene ontology (GO) categories, molecular function (MF), biological process (BP) and cellular component (CC) (Fig. 3A). Of the 195 *PgERF* transcripts, 186 (95%) were categorized into all the three primary categories, MF, BP and CC. Only one *PgERF* gene transcript, *PgERF069*, had functions in both BP and CC categories, while two *PgERF* transcripts, *PgERF135-1* and *PgERF135-3*, and six *PgERF* transcripts, *PgERF152-3*, *PgERF152-4*, *PgERF152-1*, *PgERF152-2*, *PgERF105-3* and *PgERF087*, were categorized into BP and CC, respectively. At Level 2, these 195 transcripts were further categorized into eight subcategories, including nucleic acid binding transcription factor activity, binding, metabolic process, cellular process, developmental process, organelle, cell part and cell (Fig. 3B). Of the eight subcategories, three subcategories, including nucleic acid binding transcription factor activity, binding and cell part subcategories, have been significantly enriched, which is consistent with the transcription regulation functions of the *ERF* genes, while the

265 abundances of the remaining five subcategories are either not changed or significantly reduced relative
266 to the whole genome background control.

267 Figure 3. Functional categorization of the AP2/ERF gene family in ginseng. (A) Venn diagram of
268 the *PgERF* gene transcripts categorized into three primary categories, biological process (BP),
269 molecular function (MF) and cellular component (CC). (B) The subcategories of the *PgERF* gene
270 transcripts (Level 2). The enrichment of the *PgERF* gene transcripts in each subcategory was calculated
271 using all the gene transcripts of ginseng as the background control. A single asterisk “*” indicates the
272 significant difference of the number of *PgERF* gene transcripts categorized into the subcategory from
273 that of all the gene transcripts of ginseng at $P \leq 0.05$, while double asterisks “**” indicate the difference
274 at a significance level of $P \leq 0.01$.

275 Furthermore, the *PgERF* transcripts expressed in the roots of 5-, 12-, 18- and 25-year-old plants,
276 14 tissues of the 4-year-old plant, and the roots of 4-year-old plants of 42 genotypes were further
277 categorized (Fig. 4). The *PgERF* transcripts expressed in differently-aged plant roots, different tissues
278 and the roots of different genotypes were all categorized into these eight subcategories, suggesting that
279 the functions of *PgERF* transcripts were consistent among developmental stages, tissues or genotypes.
280 Nevertheless, a substantial variation of the categorization in the numbers of the *PgERF* transcripts
281 categorized into these eight categories (Level 2) was observed across developmental stages, tissues or
282 genotypes.

283 Figure 4. Variation in functional categories of the *PgERF* gene transcripts. (A) Variation in
284 functional categories among the roots of differently aged plants. (B) Variation in functional categories
285 among 14 tissues of a 4-year-old plant. (C) Variation in functional categories among the 4-year-old

286 plant roots of 42 genotypes.

287 **3.5 Expression profiles and networks of the *PgERF* genes**

288 To profile the activation patterns of *PgERF* genes, the expressions of all 397 transcripts were
 289 quantified in 5-, 12-, 18- and 25-year-old plant roots, 14 4-year-old plant tissues and the roots of
 290 4-year-old plants of 42 genotypes. The expressions of the transcripts varied dramatically across
 291 developmental stages, tissues and genotypes, from silenced (0.0 TPM) to 586.3, 666.0 and 1159.2
 292 TPM, respectively. Of the 397 *PgERF* transcripts, 136 (34.3%), 98 (24.7%) and 83 (20.9%) expressed
 293 in all 5-, 12-, 18- and 25-year-old plant roots, all 14 4-year-old plant tissues and the roots of 4-year-old
 294 plants of all 42 genotypes, respectively (Tables S5-S7), while 53 (13.4%), 39 (9.8%) and 14 (3.5%) of
 295 the 397 transcripts were development-, tissue- and genotype-specific, respectively. Nevertheless, the
 296 expression of a transcript varied dramatically across developmental stages, tissues and genotypes.

297 Moreover, we constructed the heatmaps of the *PgERF* genes expressed at different
 298 developmental stages of roots, in different tissues, and across different genotypes to find out whether
 299 the expressions of the genes were co-regulated. The results showed that although the expression
 300 co-regulation was observed for some of the genes at a developmental stage, a single tissue or a
 301 genotype and across developmental stages, it was not apparent across tissues or genotypes (Fig. 5). For
 302 instance, *PgERF140-12*, *PgERF046*, *PgERF089-3*, *PgERF093-3*, *PgERF108-1*, *PgERF184*,
 303 *PgERF118-2* and *PgERF170* were apparently co-regulated at a developmental stage and across
 304 developmental stages of roots (Fig. 5A).

305 Figure 5. Expression heatmaps of the *PgERF* gene transcripts at different developmental stages,
 306 in different tissues and across genotypes. (A) In the roots of different year-old plants. (B) In the 14
 307 tissues of a 4-year-old plant. (C) In the 4-year-old plant roots of 42 genotypes.

308 To determine the functional relationships of the *PgERF* genes, the co-expression network of the

PgERF transcripts were constructed for 14 tissues of a four-year-old plant and the four-year-old plant roots of 42 genotypes, respectively. Of the 397 *PgERF* gene transcripts, 364 (91.7%) formed a co-expression network ($P \leq 0.05$) in the 14 tissues of the four-year-old plant (Fig. S6A). The network consisted of 364 gene transcript nodes, 5,303 co-expression edges and 17 closer co-expression clusters (Fig. S6A and B). Nevertheless, the tendency of this network formation had no substantial difference from that of the network formed from randomly selected ginseng gene transcripts (Fig. S6C and D). In the four-year-old plant roots of different genotypes, 341 (85.9%) of the 397 *PgERF* gene transcripts formed a co-expression network ($P \leq 0.05$), consisting of 341 gene transcript nodes, 5,606 co-expression edges and 24 clusters (Fig. 6A and B). The tendency of this network formation was stronger in terms of number of nodes and number of edges than that of the network formed from randomly selected ginseng gene transcripts (Fig. 6C and D). Together, analysis of these networks revealed that the gene members of the *PgERF* gene family were functionally quite independent, even though some of them formed a co-expression network, because the tendency of the network formation was similar to that of randomly-selected unknown genes.

Figure 6. Co-expression network of the *PgERF* gene transcripts in the 4-year-old plant roots of 42 genotypes. (A) The co-expression network constructed from 342 of the 397 *PgERF* gene transcripts at $P \leq 0.05$. (B) 17 clusters of the network. (C) Variation in number of nodes in the network of *PgERF* transcripts at different P -values. (D) Variation in number of edges in the network of *PgERF* transcripts at different P -values.

3.6 Expression profiles of the *PgERF* genes in responding to cold stress

As a perennial herb, ginseng is frequently suffering from various environmental stresses. However, to date, the molecular mechanisms of the stress tolerance in ginseng were not clearly

clarified. To discover the potential functions of *PgERF* genes in resistance cold stress, the expression patterns of six *PgERF* genes randomly selected from DREB subfamily, i.e., *PgERF073*, *PgERF079*, *PgERF110*, *PgERF115*, *PgERF120* and *PgERF128*, in cold-stressed ginseng hair roots were analyzed by qRT-PCR. As shown in Fig.7D and 7E, two members of I group of DREB subfamily, i.e., *PgERF115* and *PgERF120* were firstly up-regulated but then somewhat different from each other by cold stress. Expression of *PgERF115* gradually rose and the highest change showed at cold stress for 24 h (about 13.06 times higher than the untreated hair roots), and after that time point, the expression level of *PgERF115* declined regularly. *PgERF120* respond quickly to cold stress, reaching a 26.88 times higher than the untreated hair roots in cold-stressed ginseng hair roots for 6 h and regularly declined to normal level. Similarly, the expression of *PgERF073* and *PgERF110*, two members of II group, were also somewhat different from each other. The expression of *PgERF073* showed similar trends to *PgERF115* while the expression of *PgERF110* showed similar trends to *PgERF120* (Fig.7A and 7C). *PgERF079*, a member from III group of DREB subfamily, responded rapidly and drastically to the cold stress, whose expression were significantly up-regulated by 1057.05, 274.44, 290.81 and 173.03 times in cold-stressed for 6 h, 24 h, 48 h and 72 h comparing to the untreated hair roots ($P < 0.01$) (Fig.7B). *PgERF128*, which belonged to the IV group of DREB subfamily, exhibited particular trend comparing with the other 5 *PgERF* genes. As shown in Fig.7F, the expression levels of *PgERF128* were rising gradually in ginseng hair roots under cold stress for 6 h, 24 h, 48 h and 72 h. At 72 h, the expression of *PgERF128* in cold-stressed ginseng hair roots were significantly up-regulated by 5.68 times than the untreated hair roots ($P < 0.01$).

Figure 7. Expression levels of *PgERF* genes in ginseng hair roots after 6, 24, 48 h and 96 h of cold stress treatment. The values were given as mean $\pm$ SD of triplicate samples. Different letters represent significant differences between the treatment means ($p < 0.05$, LSD).

3.6 Expression profiles of the *PgERF* genes in responding to MeJA

MeJA is a plant hormone and a kind of elicitors and has been widely used in regulation of genes involved in ginsenoside biosynthesis in ginseng [38]. Therefore, we further analyzed the expressions of the *PgERF* genes in the adventitious roots of ginseng treated with MeJA for 0, 12, 24 and 48 h, respectively. The expressions of the *PgERF* gene transcripts in the control and MeJA-treated adventitious roots varied from silent (0 TPM) to 197.731 TPM (Table S8). Of the 397 *PgERF* gene transcripts profiled, 173 (43.6%) expressed and 109 (27.5%) silenced in the control and all treated adventitious roots, and the remaining 115 (29.0%) either expressed or silenced in these adventitious roots. The expressions of the 288 *PgERF* gene transcripts expressed the adventitious roots were visualized by the expression heatmap (Fig. 8). Overall, all the 288 *PgERF* gene transcripts responded to the MeJA treatment, with 136 of them up-regulated and 152 down-regulated by MeJA. Among the three treatment times, 12 h, 24 h and 48 h, the responses of these *PgERF* gene transcripts to MeJA varied from time to time.

Figure 8. Expression heatmap of the *PgERF* gene transcripts treated with 200 μ M MeJA for 0, 12, 24 and 48 h, respectively.

4. Discussion

The AP2/ERF gene family has been broadly studied in several plant species of economical or biological importance due to its important roles in various biological processes, including growth and development, and responses to environmental stresses based on genome and transcriptome sequences [5]. These species include Arabidopsis [6], rice [7], wheat [46], maize [26], cotton [3], grapevine [28], cucumbers [47] and rubber tree [48]. We have, in this study, comprehensively investigated the AP2/ERF genes in ginseng using several transcriptome databases, including those developed from 14 tissues of a four-year-old ginseng plant, the roots of 5-, 8-, 12- and 25-year-old plants and the four-year-old plant roots of 42 diverse genotypes. The *PgERF* gene family in ginseng is also a large

gene family, consisting of 189 or more gene members. This result is in consistence with those identified in other plant species such as Arabidopsis [6], rice [7] and grapevine [28]. Although the family size is non-comparable with those identified in the other species listed above due to the dramatic variation of gene family size within a plant species [49] and the difference of the databases used for these analyses, the *PgERF* gene family is unambiguously classified into five subfamilies, ERF, DREB, AP2, RAV and Soloist, as were those identified in Arabidopsis [6], rice [7] and grapevine [28]. These results indicate the *PgERF* gene family has a similar functional differentiation pattern as those in the three latter species.

It has been consensus that the conserved motifs of the AP2/ERF transcription factor are crucial to the function of transcription factors, such as nuclear localization and transcriptional activity [7]. The DNA binding domain of AP2/ERF transcription factors, i.e., AP2 domain, was highly conserved in plant species [50],[51]. The AP2 domain of the *PgERF* genes was also found to be highly conserved. This study has identified 14 and 11 completely conserved amino acid residues through all gene members of the DREB and ERF subfamilies, respectively, in both ginseng and Arabidopsis (Figs. S3 and S4). In Arabidopsis, the two conservative elements, YRG and RAYD, were shown to be critical to the binding of AP2/ERF transcription factors to the promoter regions of the target genes and modulate their expression [29]. The conservative YRG and RAYD elements identified in the AP2 domain of the DREB and ERF subfamilies in ginseng may suggest their necessity for similar functions of the *PgERF* genes. Nevertheless, subtle variation exists among the amino acid sequences of *PgERF* transcription factors, which has led to the separation of the DREB subfamily from the ERF subfamily. The difference between the DREB and ERF subfamilies might result in their functional divergence in ginseng. Moreover, the “EIR” in the AP2 domain was found to be shared by all gene members of the ERF

subfamily and the vast majority of the gene members of the DREB subfamily in both ginseng and Arabidopsis, while the “EVR” exists only in Group III of the DREB subfamily in both ginseng and Arabidopsis and only in Group II (*PgERF061*) of the DREB subfamily in ginseng. It has been reported that the sequence similarity of the conserved motifs that exist outside of the DNA binding domain was low [6],[27],[43]. In ginseng, 19 conserved motifs, except for Motif 1 to Motif 6, were identified outside the AP2 domain. The vast majority of these 19 motifs were found to be divergent across subfamilies or even subfamily groups in ginseng. The subfamily/group-specific distribution pattern of these motifs might have led to the functional divergence between subfamilies or groups of the *PgERF* transcription factors.

Because different transcripts alternatively spliced from the same gene may have different functions [52], the 397 *PgERF* transcripts, instead of the 189 *PgERF* genes, were annotated and functionally categorized in this study. The *PgERF* transcripts were categorized into eight subcategories at Level 2. Although this result suggested a substantial functional differentiation of the *PgERF* genes, the differentiation was much smaller than those observed in the *PgNBS* gene family [35], *PgRLK* gene family [53] and *PgCYP* gene family [54] in ginseng. Interestingly, of the eight Level 2 subcategories, only two, especially those in the nucleic acid binding transcription factor activity subcategory, were significantly up-enriched, which is consistent with the roles of the *PgERF* genes as transcription factors by binding to the promoters of target genes. While the functions of the AP2/ERF genes have been shown to play important roles in plant growth and development, response to stresses and signal pathway in the model plants such as Arabidopsis and rice [5],[55], further research is needed to determine the functions of the *PgERF* genes in ginseng.

Companioned with their functional differentiation, the expressions of 397 *PgERF* transcripts dramatically varied in a tissue, at a development stage or in a genotype. Moreover, the type and number

of expressed *PgERF* transcripts also diversified tempo-spatially and across genotypes. The differential expressions of the AP2/ERF genes were previously reported in other plant species, but mainly among tissues [25],[56]. Furthermore, the numbers of the *PgERF* transcripts categorized into each subcategory varied across tissues, developmental stages or genotypes. These variations might be an indication of their functional differentiation. On the other hand, co-expression network analysis revealed that most (>86%) of these *PgERF* transcripts express correlatively and tend to form a co-expression network in different tissues or different genotypes. These results suggest that the *PgERF* genes have functionally differentiated, but they are still somehow functionally collaborative.

As a perennial herb, ginseng frequently suffers from different kinds of environmental stresses. It was reported that members of AP2/ERF superfamily, especially DREB subfamily, played an essential role in response to biotic and abiotic stresses [16],[17],[57]. To tap the potential AP2/ERF genes of DREB subfamily resisting to cold stress in ginseng, the expression of six genes randomly selected from DREB subfamily under cold stress were analyzed using qRT-PCR. The expression of *PgERF079*, one gene from III group or A1 group of DREB subfamily, was dramatically changed (up to 1057.05 times with a brief period of cold-stressed for 6 h), suggesting it played an extremely important role in freezing tolerance. In fact, A-1 group was considered to be the major regulator of cold-stress responses as overexpressing any one of the three cold-inducible DREB1s, DREB1A/CBF3 (AT4G25480), DREB1B/CBF1 (AT4G25490) and DREB1C/CBF2 (AT4G25470), significantly improved freezing tolerance in Arabidopsis [57],[58],[59]. Besides, the expression level of the other five genes from other groups of DREB subfamily, i.e., *PgERF073*, *PgERF110*, *PgERF115*, *PgERF120* and *PgERF128*, also showed significant changes ($p<0.01$) in cold-stressed ginseng hair roots. Therefore, it speculates that besides A1 group, the other groups of DREB subfamily may also be effective in freezing tolerance in

ginseng, either directly or indirectly. Herein, the results of these cold-inducible genes would provide some valuable information for the functional studies of *PgERF* genes in ginseng in the future.

It has been reported that some genes of the AP2/ERF family are involved in response to hormone signals in plants [60],[61],[62]. It was showed that MeJA, as one of the signaling molecules, was rapidly synthesized in plants, when subjected to various biotic and abiotic stresses, and then, induced defense-related responses to the stresses and regulate plant growth and development [63]. MeJA has been also used as an effective elicitor, since it can stimulate the biosynthesis of plant secondary metabolites [64],[65],[66]. The biosynthesis and accumulation of ginsenosides, a cluster of important secondary metabolites and the most valuable bioactive components in ginseng, were also reported to be induced by MeJA [67],[68]. This study showed that the addition of exogenous MeJA to adventitious roots dramatically changed the expression of a majority of the *PgERF* gene transcripts. The expressions of some of the transcripts were up-regulated while those of the other down-regulation or inhibited by MeJA, relative to the control not treated by MeJA. Given the demonstrated functions of MeJA in plant responses to biotic and abiotic stresses, growth and development and secondary metabolite biosynthesis in other plant species [57],[60],[61],[62],[63],[64],[65],[66], the *PgERF* genes may also be involved in these processes, including the biosynthesis of ginsenosides.

5. Conclusions

The present study, for the first time, reports identification and systematic characterization of the AP2/ERF family present in ginseng, i.e., the *PgERF* gene family. A total of 189 *PgERF* genes that were actively expressed in 14 tissues of a four-year-old ginseng plant were identified and these genes were alternatively sliced into 397 transcripts. These *PgERF* genes were also classified into five subfamilies

(DREB, ERF, AP2, RAV and Soloist) as those previously identified in Arabidopsis. As expected, the conserved motifs that characterize the AP2/ERF family and several conserved domains were identified among the members of the *PgERF* gene family. Nevertheless, the transcripts of the *PgERF* genes were apparently categorized into eight subcategories by GO, especially into the subcategory for nucleic acid binding transcription factor activity, which indicates their functional differentiation. Along with their functional differentiation, the expressions of the *PgERF* genes, including the type, number and expression level of their transcripts, have also substantially diversified tempo-spatially and across genotypes. In spite of these differentiations, most of the *PgERF* genes remain to co-express and form a co-expression network, suggesting that most of the genes in the *PgERF* gene family remain functionally correlated. These *PgERF* genes and findings provide resources and knowledge valuable for family-wide functional analysis of the *PgERF* genes and determination of their roles in plant responses to biotic and abiotic stresses, growth and development, and biosynthesis of secondary metabolites, especially ginsenosides, in *P. ginseng* and related species.

SUPPLEMENTARY MATERIAL

Supplemental information is available with the online version of this manuscript.

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AUTHOR CONTRIBUTIONS

MPZ and YW planned and designed this study; JC and QZ performed the bioinformatic analysis; JC wrote the manuscript; LL, YZ, PC, HL, RL, YH, CS, KW, JL, MZ and YFW prepared the tables and figures. MPZ revised the manuscript. All the authors read and approved the final version of the manuscript.

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Figure 1

Figure 2

Figure 3

Figure 4

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Figure 5

Figure 7

Figure 6

Figure 8