

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)

44 **1. Introduction**

45 Plants are subjected to numerous biotic and abiotic stresses all time through their growth and
46 development. Therefore, they have developed a variety of mechanisms by producing secondary
47 signaling molecules (e.g., ethylene and jasmonic acid) and response networks at the molecular,
48 biochemical and physiological levels to perceive the external signals from and response to the stresses
49 [1]. It has been documented that a large number of genes are involved in these processes [2]. Therefore,
50 it is important to decipher the regulatory mechanisms of the defense-related genes involved in the signal
51 transduction pathways and the plant responses to these stresses for enhanced and efficient plant genetic
52 improvement [3]. The APETALA2/Ethylene Responsive Factor (AP2/ERF) transcription factors have
53 been demonstrated to be one of the most important gene families actively functioning in plant response
54 to biotic and abiotic stresses by binding to cis-acting elements of downstream target genes [4].

55 The AP2/ERF family has one or two conserved APETALA2 (AP2) domains (approximately 60 -
56 70 amino acids) [5]. Based on the number and amino acid sequence similarities of the AP2 domains, the
57 AP2/ERF family is divided into the DREB (dehydration responsive element binding), ERF, AP2, RAV
58 (Related to ABI3/VP1) and Soloist subfamilies [6],[7]. Both DREB and ERF subfamilies possess a
59 single AP2 domain, with a specific WLG motif, and could be further subdivided into A1 to A6 and B1
60 to B6 groups, respectively [6]. Alternatively, the DREB and ERF subfamilies were also categorized into
61 I to X, and VI-L and Xb-L groups, respectively [7]. The AP2 subfamily has two tandemly repeated AP2
62 domains, while the RAV subfamily has one AP2 domain and one B3 domain that are commonly found
63 in other transcription factors [8]. The Soloist subfamily also has only one AP2 domain. It was classified
64 into an independent subfamily due to its relatively low sequence homology with the DREB and ERF
65 subfamilies [9]. Although the AP2 domain of the AP2/ERF family is highly conserved, its five

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

80 The AP2/ERF family has been well characterized in the model plants, *A. thaliana* [6],[7] and
81 *Medicago truncatula* [25], several crops, such as rice [7], maize [26], soybean [27], Chinese cabbage
82 [10] and grapevine [28], and *Populus trichocarpa* [29]. However, little is known about the AP2/ERF
83 family in the medicinal herb, *Panax ginseng* (ginseng). *Ginseng* is a perennial of the *Araliaceae* family
84 and has long been cultivated for human medicine in Asia, particularly in China, Korea, and Japan.
85 Ginseng, known as the “king of all herbs” in China, is mainly cultivated in Jilin Province; therefore, it is
86 often known as Jilin ginseng. Ginseng has been widely used as a medicinal herb due to its bioactive
87 components, especially ginsenosides that have been shown to play significant roles in anti-inflammation

88 [30],[31], antitumor [32], and immunomodulation [33]. However, ginseng has been suffering from
89 various biotic and abiotic stresses, which is greatly threatening the ginseng production. Therefore,
90 identification, characterization and utilization of the defense-related genes in ginseng are of significance
91 for ginseng breeding and production. In the present study, we comprehensively studied the AP2/ERF
92 family present in Jilin ginseng in several aspects, including gene identification, protein motif
93 characterization, functional categorization and phylogenetic analysis. Moreover, the expression
94 activities and patterns of AP2/ERF genes were also investigated at different developmental stages, in
95 different tissues, different cultivars, under cold stress and under the methyl jasmonate (MeJA)
96 treatment. The results of these studies have laid the foundation for deeply functional analysis and
97 utilization of the genes of the AP2/ERF family and provided vital information on the molecular
98 mechanism of plant response to biotic and abiotic stresses in ginseng and related plant species.

99 **2. Materials and methods**

100 **2.1 Databases**

101 We previously established a comprehensive transcriptome for Jilin ginseng from 14 tissues (fiber
102 root, leg root, main root epiderm, main root cortex, rhizome, arm root, stem, leaf peduncle, leaflet
103 pedicel, leaf blade, fruit peduncle, fruit pedicel, fruit flesh, and seed), from which 248,993 transcript
104 unigenes (130,557 gene IDs) were assembled [34]. Moreover, we also sequenced and established the
105 databases for the transcriptomes of the roots of 5-, 12-, 18- and 25-year-old plants [34] and the roots of
106 four-year-old plants of 42 genotypes (named from S1 to S42) representing the diversity of Jilin ginseng
107 [35]. In this study, a ginseng line IR826 genome sequence database [36] and another Ginseng Genome
108 Database (<http://ginsengdb.snu.ac.kr/index.php>) reported by Kim *et al.* [37] were also used. In addition,

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

111 **2.2 Identification of *PgERF* genes in ginseng**

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

124 **2.3 Multiple sequence alignment and phylogenetic analysis of *PgERF* genes**

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

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

132 **2.4 Motif prediction of *PgERF* genes**

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

138 **2.5 Expression and functional networks of *PgERF* genes**

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

149 **2.6 Expression activity of *PgERF* genes responding to cold stress**

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

151 250 ml 1/2 Murashige and Skoog (MS) medium in dark culture at 22°C for 30 days. Then, to simulate
152 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,
153 respectively. Afterwards, the ginseng hair roots were harvested and stored in -80°C for the RNA
154 isolation and further quantitative real-time PCR analysis. The total RNA of ginseng hair roots was
155 extracted by TRIzol reagent (Biotek, Beijing, China) according to the manufacturer's instructions,
156 which was further reverse transcribed into cDNA using a PrimeScript™ RT reagent Kit with gDNA
157 Eraser (TaKaRa, Tokyo, Japan), following the manufacturer's instructions. In this study, quantitative
158 real-time PCR (qRT-PCR) of six *PgERF* genes, including *PgERF073*, *PgERF079*, *PgERF110*,
159 *PgERF115*, *PgERF120* and *PgERF128*, was performed. The *PgGADPH* gene was used as the internal
160 reference. The gene-specific primers used in qRT-PCR were designed by Primer Premier Software
161 (version 5) and were listed in Table S1. The qRT-PCR was conducted by an Applied Biosystems 7500
162 Real Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) and SYBR Premix Ex Taq™
163 II (TaKaRa, Tokyo, Japan). The qRT-PCR was performed using the following steps: 30 seconds at
164 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
165 60 seconds at 60°C; 15 seconds at 95 °C. The relative expression levels of these selected genes were
166 calculated using formula $2^{-\Delta\Delta C_t}$ [43], and all the experiments were amplified in triplicate.

167 **3. Results**

168 **3.1 Identification and classification of *PgERF* genes**

169 A total of 397 transcripts that were derived from 189 predicted *PgERF* genes, including those
170 containing a partial or complete AP2/ERF domain, were identified. These *PgERF* genes were defined
171 *PgERF001* to *PgERF189*, with a suffix (e.g., -1) for different transcripts derived from the same gene
172 (Table S2). Then, the sequences of 342 AP2/ERF gene transcripts downloaded from Ginseng Genome
173 Database (<http://ginsengdb.snu.ac.kr/index.php>) were aligned with 397 transcripts identified in this
174 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

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

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

212 **3.2 Phylogenetic analysis of the *PgERF* gene family**

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

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

224 3.3 Motif identification and multiple sequence alignment

225 Next, the 93 predicted *PgERF* genes were subjected to conservative analysis for the conserved
226 motifs of their proteins (Fig. 2; Fig. S3). A total of 25 conserved motifs were identified for the putative
227 proteins of the 93 predicted *PgERF* genes, which were herein designated as Motif 1 through Motif 25.
228 Motif 1 to Motif 6 were located in the AP2/ERF domain and the remaining 19 motifs, including Motif 7
229 through Motif 25, were found outside the AP2/ERF domain. The *PgERF* proteins encoded by gene
230 members of the same subfamily or group contained similar conserved motifs. For example, Motifs 4, 7,
231 15, 16, 17, 21 and 25 were specifically shared by gene members of the AP2 subfamily. Motifs 14 and
232 18 were specifically present within Group I, while Motifs 9 and 20 were only present in the Group III
233 gene members of the ERF subfamily. Motifs 10, 11, 12, 13, and 19 were specific for the DREB
234 subfamily and absent in all four other subfamilies (ERF, AP2, RAV and Soloist subfamilies). These
235 results suggested that most of the 25 motifs were divergent among subfamilies or groups, which might
236 play an important role in their functional divergence [25].

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

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

243 15W, 16V, 18E, 20R, 22P, 39W, 40L, 49A, 52A, 54D and 72N, and 11 conserved amino acid residues,
244 including 4G, 6R, 8R, 11G, 17I, 30R, 42A, 46Y, 47D, 55G and 63F, were identified for the DREB and
245 ERF subfamilies, respectively (Fig. S4 and S5). Besides, all gene members of the DREB subfamily and
246 ERF subfamily obviously contained the two featured conserved elements, including YRG and RAYD,
247 in their AP2 domains.

248 **3.4 Functional categorization of the *PgERF* genes**

249 To estimate the functional differentiation of the *PgERF* family, all 397 transcripts of the 189
250 *PgERF* genes identified in this study were annotated and functionally categorized using the Blast2GO
251 software (Version 4.1.9) [45]. Surprisingly, only 195 (49%) of the *PgERF* gene transcripts could be
252 annotated, while the remaining 202 could not be annotated using the database of the Blast2GO software,
253 suggesting the uniqueness of the *PgERF* genes in ginseng. The annotated *PgERF* gene transcripts were
254 categorized into all three primary gene ontology (GO) categories, molecular function (MF), biological
255 process (BP) and cellular component (CC) (Fig. 3A). Of the 195 *PgERF* transcripts, 186 (95%) were
256 categorized into all the three primary categories, MF, BP and CC. Only one *PgERF* gene transcript,
257 *PgERF069*, had functions in both BP and CC categories, while two *PgERF* transcripts, *PgERF135-1*
258 and *PgERF135-3*, and six *PgERF* transcripts, *PgERF152-3*, *PgERF152-4*, *PgERF152-1*, *PgERF152-2*,
259 *PgERF105-3* and *PgERF087*, were categorized into BP and CC, respectively. At Level 2, these 195
260 transcripts were further categorized into eight subcategories, including nucleic acid binding
261 transcription factor activity, binding, metabolic process, cellular process, developmental process,
262 organelle, cell part and cell (Fig. 3B). Of the eight subcategories, three subcategories, including nucleic
263 acid binding transcription factor activity, binding and cell part subcategories, have been significantly
264 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

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

323 Figure 6. Co-expression network of the *PgERF* gene transcripts in the 4-year-old plant roots of 42
324 genotypes. (A) The co-expression network constructed from 342 of the 397 *PgERF* gene transcripts at P
325 ≤ 0.05 . (B) 17 clusters of the network. (C) Variation in number of nodes in the network of *PgERF*
326 transcripts at different P -values. (D) Variation in number of edges in the network of *PgERF* transcripts
327 at different P -values.

328 **3.6 Expression profiles of the *PgERF* genes in responding to cold stress**

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

331 clarified. To discover the potential functions of *PgERF* genes in resistance cold stress, the expression
332 patterns of six *PgERF* genes randomly selected from DREB subfamily, i.e., *PgERF073*, *PgERF079*,
333 *PgERF110*, *PgERF115*, *PgERF120* and *PgERF128*, in cold-stressed ginseng hair roots were analyzed
334 by qRT-PCR. As shown in Fig.7D and 7E, two members of I group of DREB subfamily, i.e.,
335 *PgERF115* and *PgERF120* were firstly up-regulated but then somewhat different from each other by
336 cold stress. Expression of *PgERF115* gradually rose and the highest change showed at cold stress for
337 24 h (about 13.06 times higher than the untreated hair roots), and after that time point, the expression
338 level of *PgERF115* declined regularly. *PgERF120* respond quickly to cold stress, reaching a 26.88
339 times higher than the untreated hair roots in cold-stressed ginseng hair roots for 6 h and regularly
340 declined to normal level. Similarly, the expression of *PgERF073* and *PgERF110*, two members of II
341 group, were also somewhat different from each other. The expression of *PgERF073* showed similar
342 trends to *PgERF115* while the expression of *PgERF110* showed similar trends to *PgERF120* (Fig.7A
343 and 7C). *PgERF079*, a member from III group of DREB subfamily, responded rapidly and drastically
344 to the cold stress, whose expression were significantly up-regulated by 1057.05, 274.44, 290.81 and
345 173.03 times in cold-stressed for 6 h, 24 h, 48 h and 72 h comparing to the untreated hair roots ($P <$
346 0.01) (Fig.7B). *PgERF128*, which belonged to the IV group of DREB subfamily, exhibited particular
347 trend comparing with the other 5 *PgERF* genes. As shown in Fig.7F, the expression levels of
348 *PgERF128* were rising gradually in ginseng hair roots under cold stress for 6 h, 24 h, 48 h and 72 h. At
349 72 h, the expression of *PgERF128* in cold-stressed ginseng hair roots were significantly up-regulated
350 by 5.68 times than the untreated hair roots ($P < 0.01$).

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

354 **3.6 Expression profiles of the *PgERF* genes in responding to MeJA**

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

367 Figure 8. Expression heatmap of the *PgERF* gene transcripts treated with 200 μ M MeJA for 0, 12,
368 24 and 48 h, respectively.

369 **4. Discussion**

370 The AP2/ERF gene family has been broadly studied in several plant species of economical or
371 biological importance due to its important roles in various biological processes, including growth and
372 development, and responses to environmental stresses based on genome and transcriptome sequences
373 [5]. These species include Arabidopsis [6], rice [7], wheat [46], maize [26], cotton [3], grapevine [28],
374 cucumbers [47] and rubber tree [48]. We have, in this study, comprehensively investigated the
375 AP2/ERF genes in ginseng using several transcriptome databases, including those developed from 14
376 tissues of a four-year-old ginseng plant, the roots of 5-, 8-, 12- and 25-year-old plants and the
377 four-year-old plant roots of 42 diverse genotypes. The *PgERF* gene family in ginseng is also a large

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

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

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

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

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

423 of expressed *PgERF* transcripts also diversified tempo-spatially and across genotypes. The differential
424 expressions of the AP2/ERF genes were previously reported in other plant species, but mainly among
425 tissues [25],[56]. Furthermore, the numbers of the *PgERF* transcripts categorized into each subcategory
426 varied across tissues, developmental stages or genotypes. These variations might be an indication of
427 their functional differentiation. On the other hand, co-expression network analysis revealed that most
428 (>86%) of these *PgERF* transcripts express correlatively and tend to form a co-expression network in
429 different tissues or different genotypes. These results suggest that the *PgERF* genes have functionally
430 differentiated, but they are still somehow functionally collaborative.

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

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

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

461 **5. Conclusions**

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

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

479 **SUPPLEMENTARY MATERIAL**

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

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486 **AUTHOR CONTRIBUTIONS**

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

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672

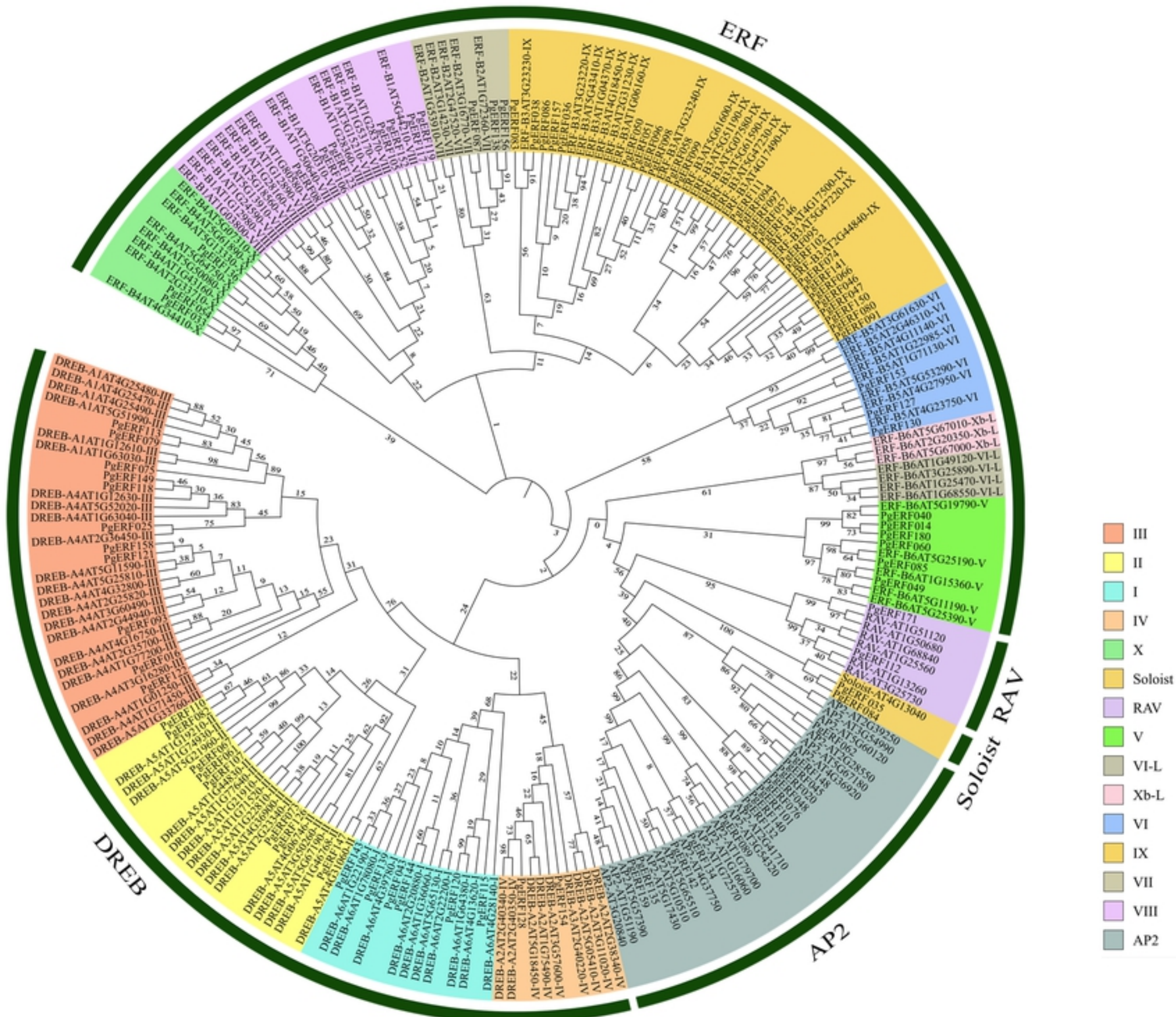


Figure 1

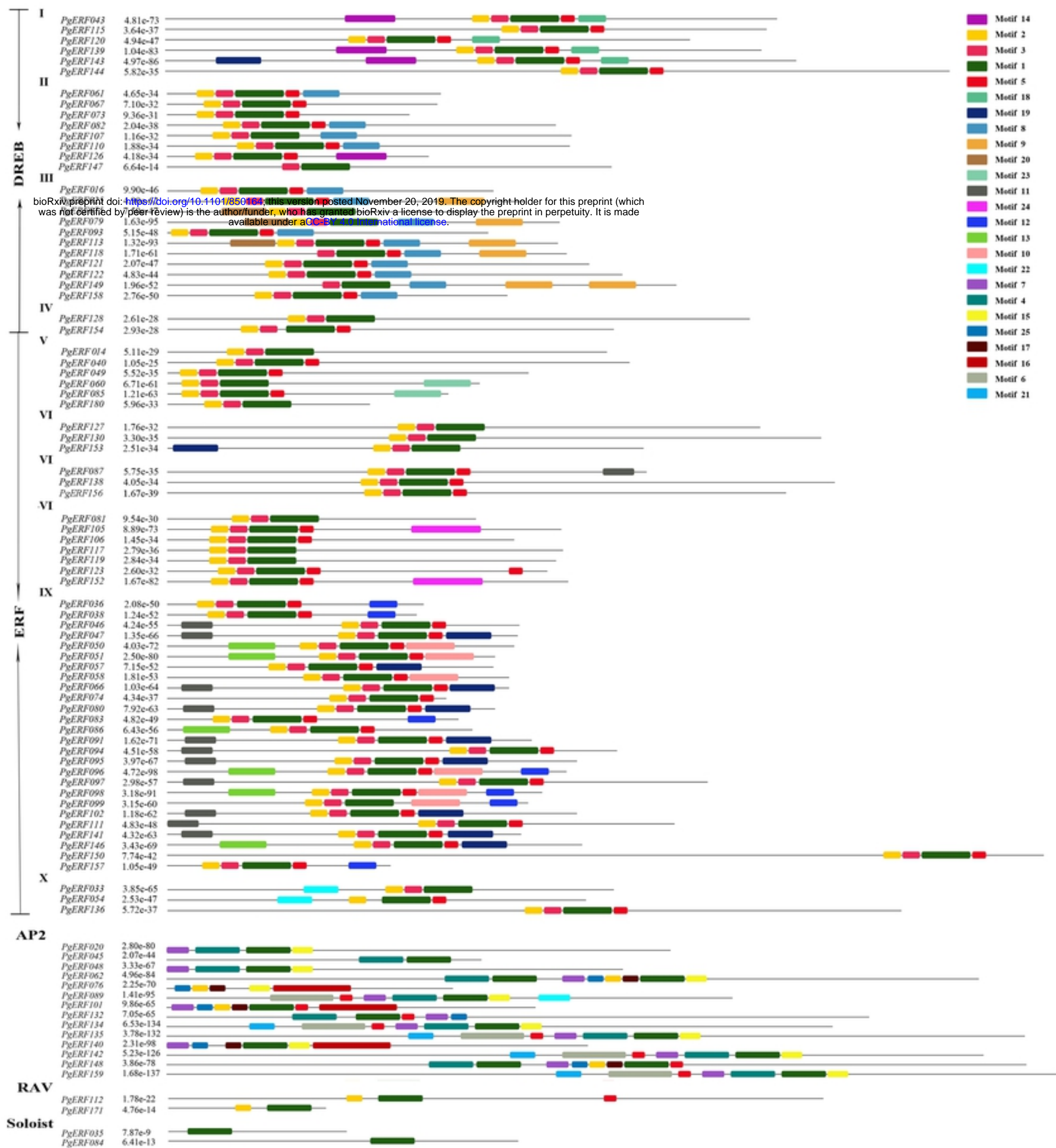


Figure 2

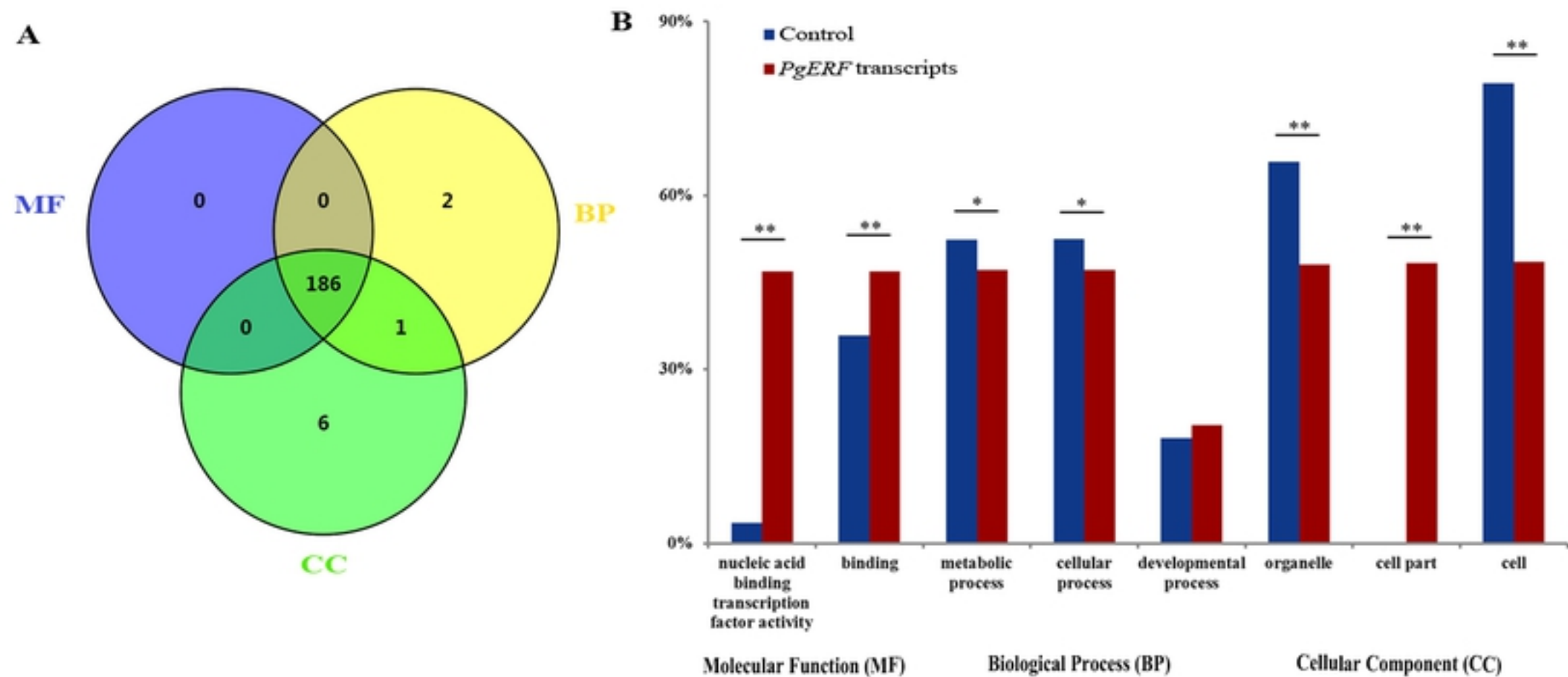


Figure 3

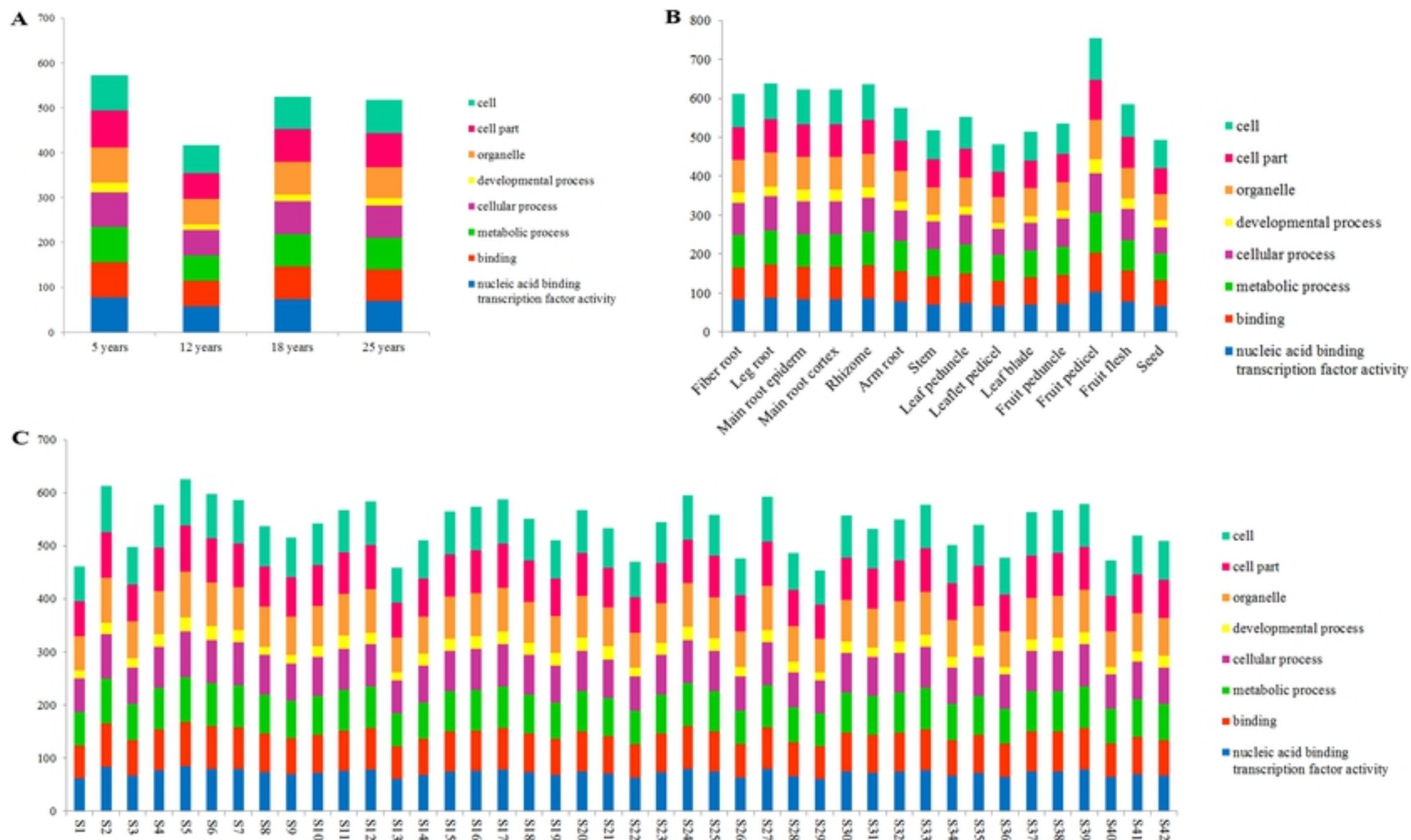


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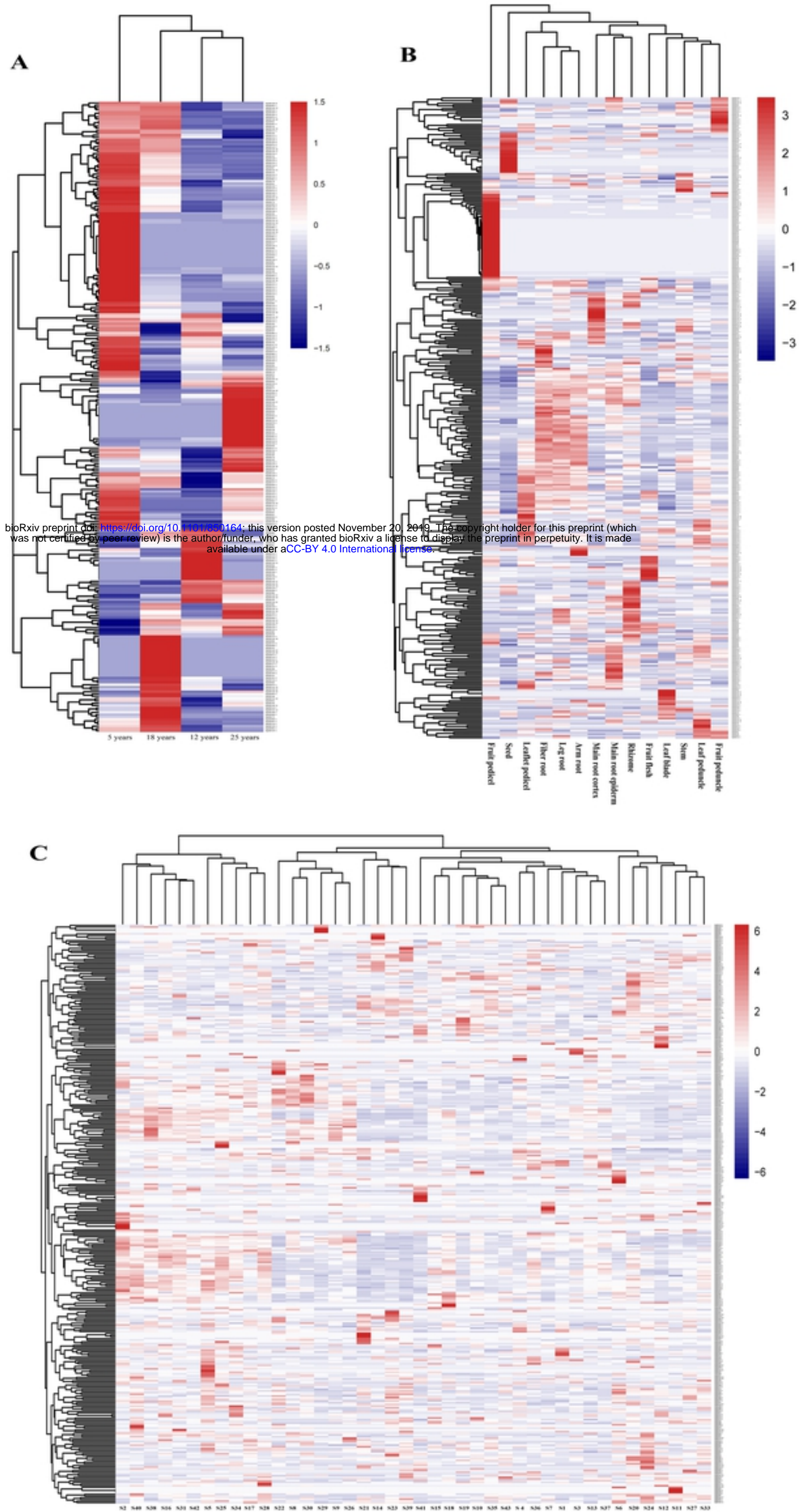


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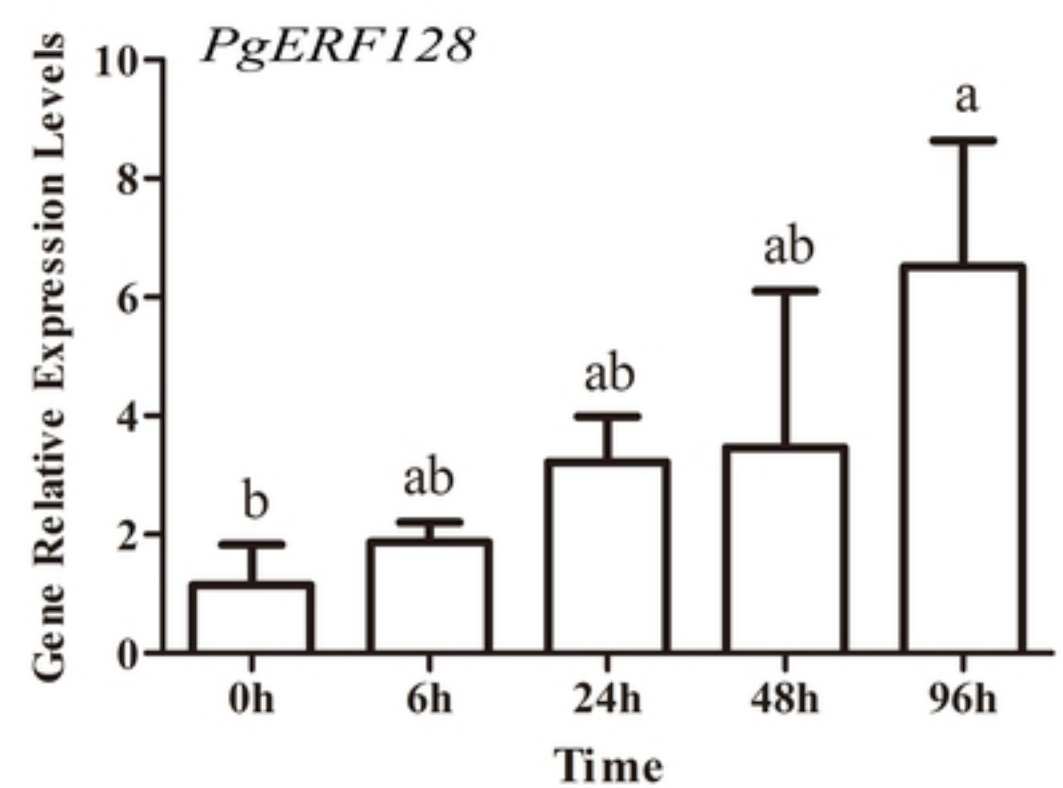
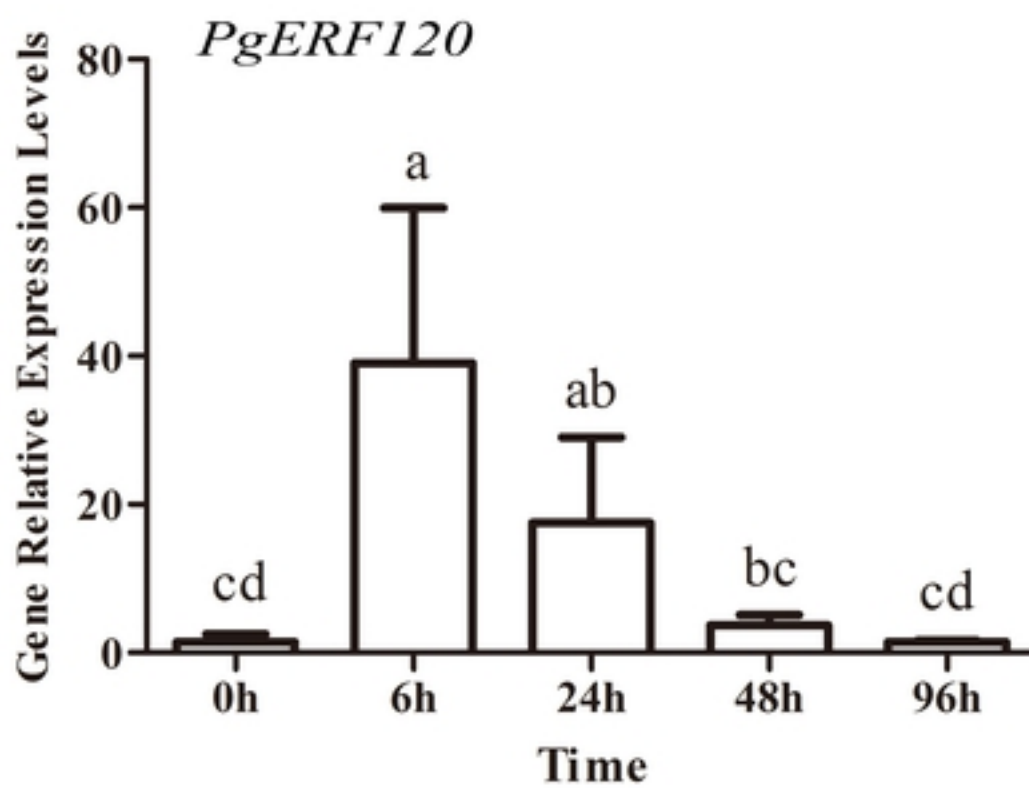
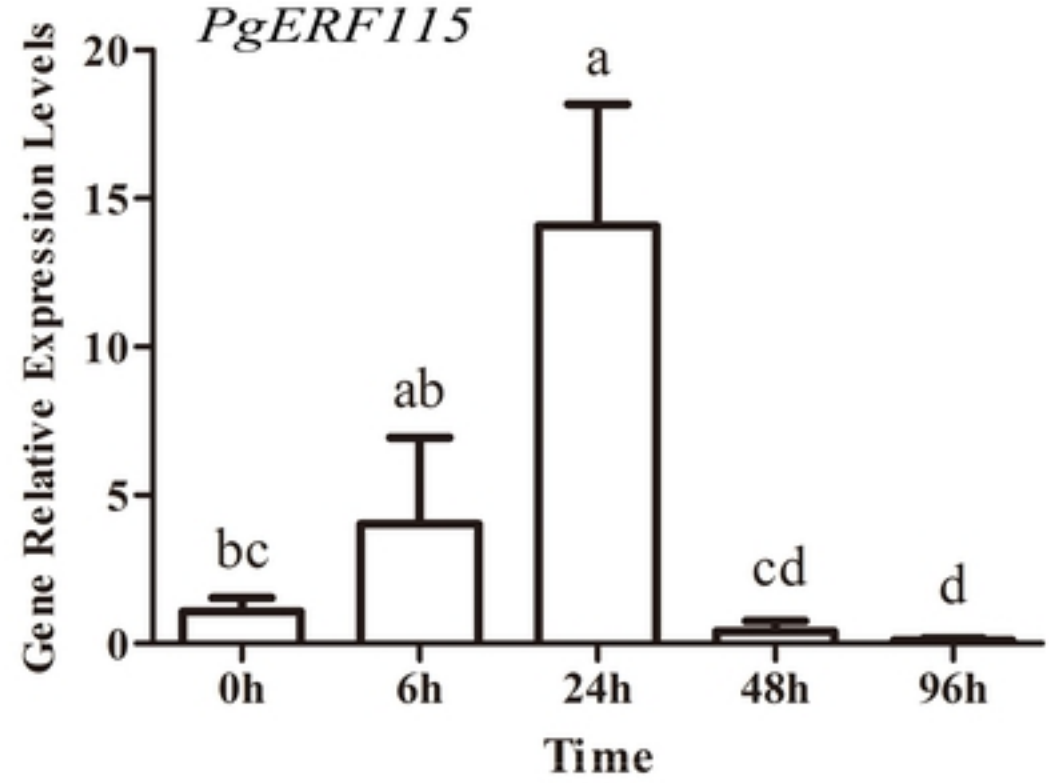
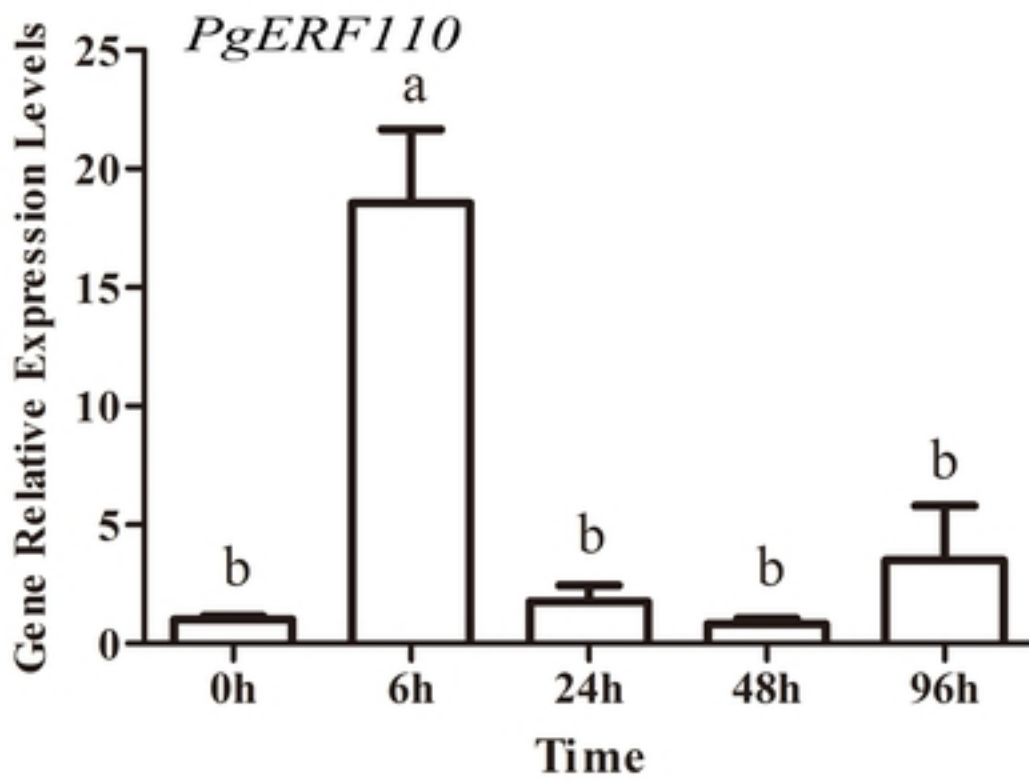
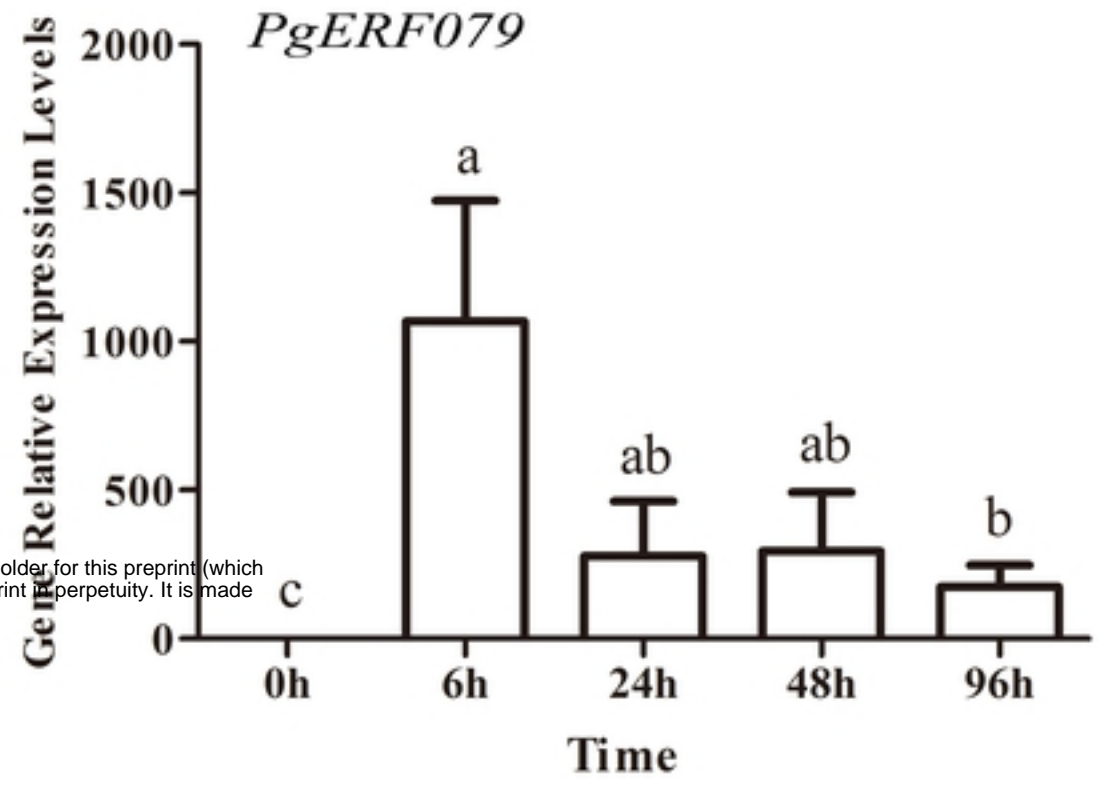
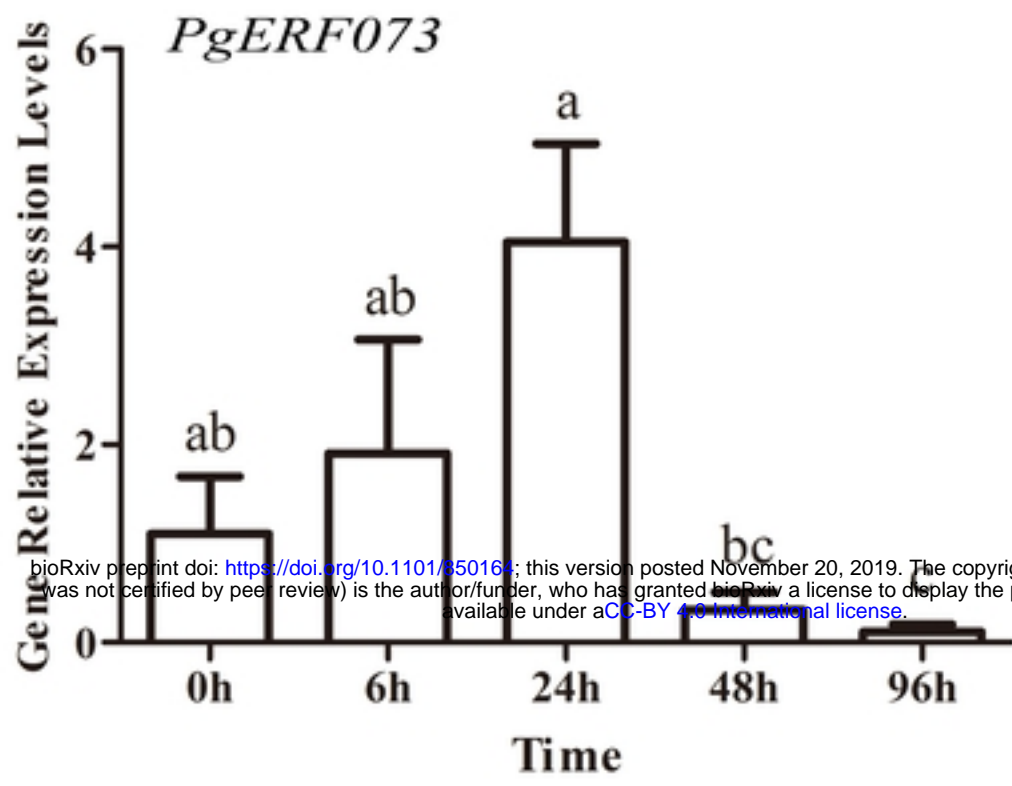


Figure 7

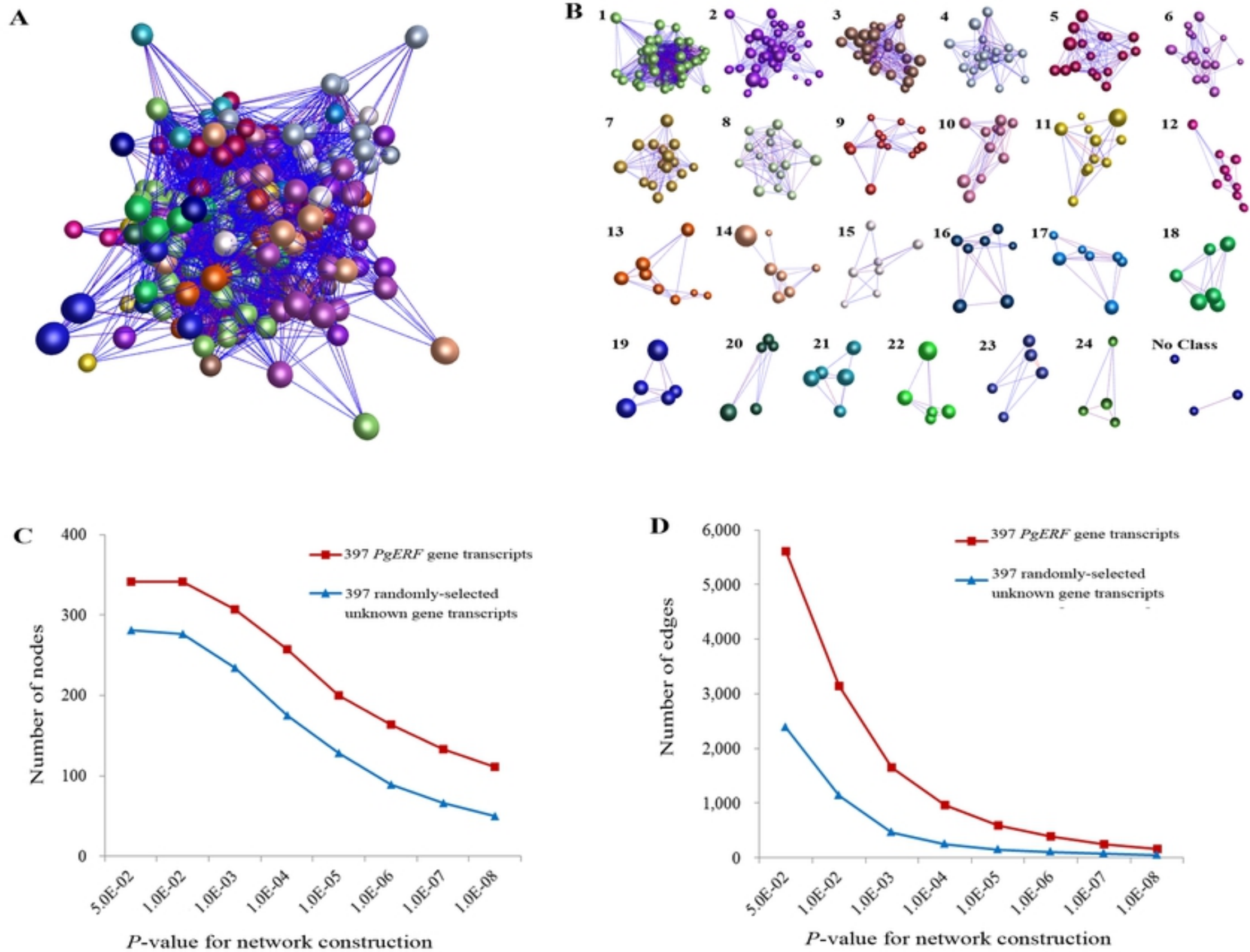


Figure 6

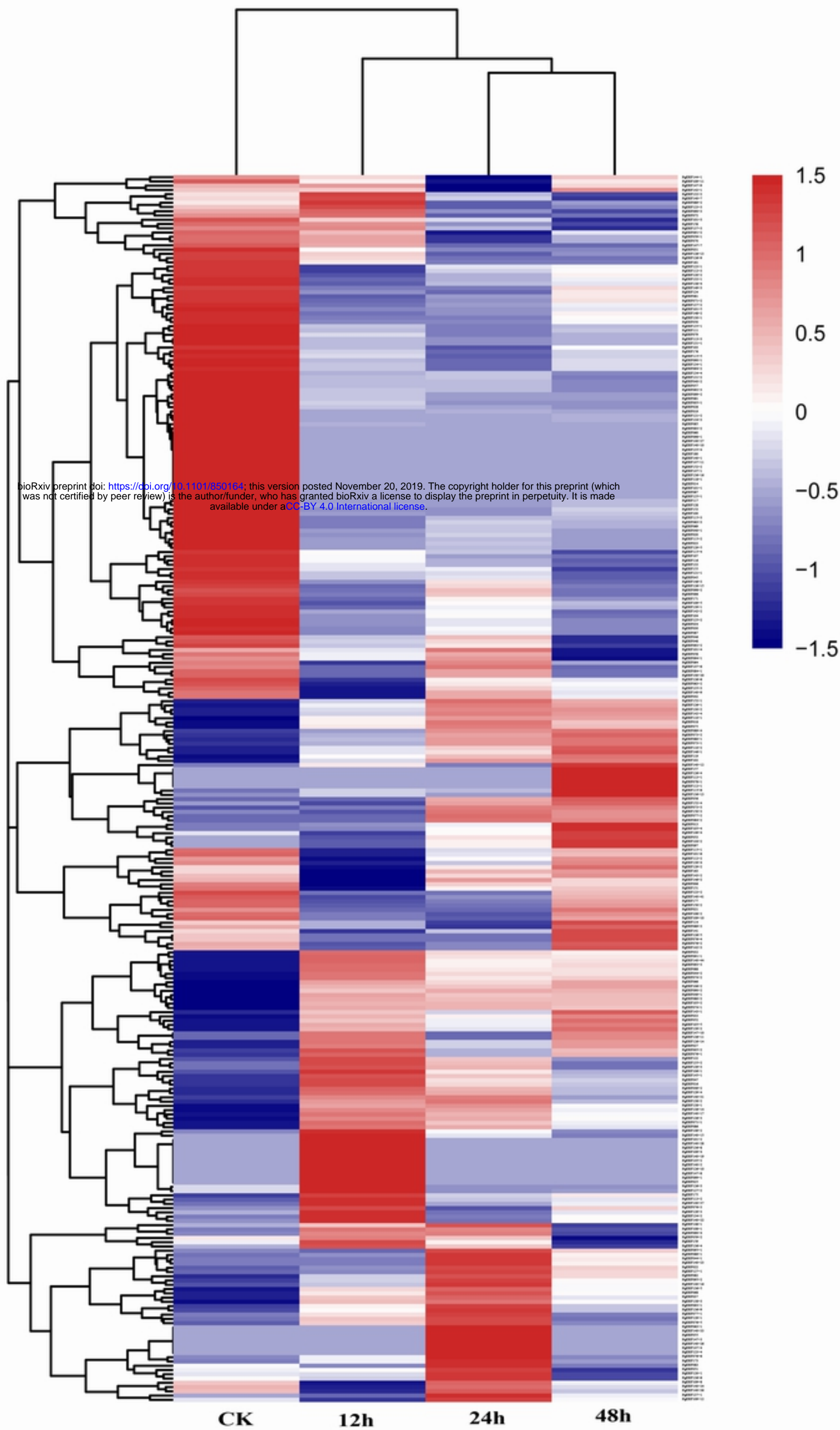


Figure 8