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Identification of AP2/ERF Transcription Factors in *Ginkgo biloba* and the Expression Analysis of ERF Gene Family under Adversity Stresses

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Abstract AP2/ERF transcription factors (TF) are widely involved in the biological process of *Ginkgo biloba*, here, the ERF gene family may regulate plant stress responses through mediating secondary metabolism. To understand the regulation mechanisms of ERF gene family during this process, two transcriptome databases of ginkgo leaves under UV-B and drought stress were constructed respectively. The conserved domains, physicochemical properties and gene family homology of AP2/ERF TFs were analyzed using online bioinformatics tools, the expression of ERF gene family under these two adversity stresses were predicted and five ERF genes involved in flavonoid pathway were selected for qRT-PCR validation analysis. The results showed that 61 AP2/ERF TFs were identified from *G. biloba*, and which were classified into 28 GbERF, 23 GbDREB, 5 GbAP2, and 5 GbRAV, according to their conserved domains and motifs. The Amino acid numbers, isoelectric point (pI) and some other physicochemical properties varied greatly between different members. Sequence homology analysis with *Arabidopsis thaliana* and *Glycine max* showed that the members in the same AP2/ERF subfamily were closely related. In *G. biloba*, the GbERF and GbDREB subfamily together constitute the ERF gene family, transcriptome prediction analysis showed that the expression of most members of ERF gene family were increased under UV-B and drought stress. From the validation analysis, we concluded that the changes of the expression levels of these five genes were consistent with the prediction results. This study provided scientific basis for the function analysis of ERF gene family in *G. biloba*.

Keywords *Ginkgo biloba*; AP2/ERF transcription factors; ERF gene family; Adversity stress

Ginkgo (*Ginkgo biloba* L.) is a deciduous tree belonging to *Ginkgoaceae* and *Ginkgo* genus. It is a rare tree species only left in China after the Quaternary Glacial Period. As one of the oldest gymnosperms, *Ginkgo biloba* is called the “Living fossil” of the plant kingdom (Mohanta et al., 2012). Its long life history indicates that *Ginkgo biloba* has a very tenacious vitality, strong adaptability, can withstand low temperature, drought, high temperature, ultraviolet, pests and other environmental pressure. At present, *Ginkgo biloba* has been widely planted all over the world. In addition to being used as ornamental and wood trees, *Ginkgo biloba* is also planted widely in some regions of our country as the important medicinal plant. *Ginkgo biloba* leaves are the main raw materials for extracting flavonoids and terpenolactones in the pharmaceutical industry (Singh et al., 2008). Under natural conditions, the contents of flavonoids and terpenoid lactones in *Ginkgo biloba* leaves are generally low. When the contents are lower than the standard, the purchase price of *Ginkgo biloba* leaves will be directly affected, and the value of related medicinal products will not be improved. Therefore, it is of great practical significance to increase the contents of total flavonoids and terpenoid lactones in *Ginkgo biloba* leaves.

In recent years, with the development of regulation of plant secondary metabolites, transcriptional regulation of metabolism-related genes has become an important topic. Transcription factors such as MYB (Chu et al., 2017), WRKY (Chen et al., 2017), bHLH (Patra et al., 2018), bZIP (Hao et al., 2019) and AP2/ERF (Zhou and Memelink, 2016) were involved in the regulation of metabolic synthesis of flavonoids, terpenoids and alkaloids. Especially under stress conditions, transcription factors are induced in large quantities, which may regulate the synthesis of

secondary metabolites that have protective effects on plants. As one of the common transcription factor families in plants, AP2/ERF gene family is widely involved in plant biological processes, including regulating the growth of roots and leaves, the development of flowers, fruits, seeds and other organs, and the response to UV, high temperature, drought, disease and other stresses (Nakano et al., 2006; Wasternack and Song, 2016). AP2/ERF transcription factor family is very large, its conserved domain is generally composed of 1 or 2 segments of 60~70 amino acid residues. AP2/ERF domains can be divided into 5 subfamilies according to their types and quantities: AP2 (APETALA2), ERF (Ethylene-responsive element binding factors), DREB (dehydration-responsive element binding) protein), RAV (related to ABI3/VP1), and Soloist (Mizoi et al., 2012). The main functions of the five subfamilies in plants are different, and some of the functions also overlap. It is speculated that the ERF and DREB subfamilies are mainly related to plant resistance to stress and synthesis of secondary metabolites. Both of these two subfamilies belong to the ERF gene family and contain only one AP2 conserved domain. The main difference between them lies in the difference between amino acid residues at the 14th and 19th positions in the AP2 domain. The ERF subfamily consists of alanine (A) and aspartic acid (D), while the DREB subfamily consists of valine (V) and glutamate (E) (Sakuma et al., 2002). At present, studies on the regulation of ERF gene family on the synthesis of plant secondary metabolites mainly focus on some medicinal plants, but there are few relevant reports, and further research is needed to understand the function of these transcription factors.

Studies have found that the flavonoid and other active components in *Ginkgo biloba* increased under UV, drought and other stress conditions (Sun et al., 2010; Zhu et al., 2010). In order to explore the response and expression regulation mechanism of AP2/ERF transcription factor in this process, the transcriptomic sequencing of *Ginkgo biloba* leaves under ultraviolet and drought treatment was analyzed, and the AP2/ERF transcription factor was screened out from the obtained Unigenes. The conserved domain, Motif composition and gene expression of the ERF gene family were predicted. Five ERF genes related to flavonoid secondary metabolism were screened for qRT-PCR analysis. This study laid a foundation for further functional analysis of related genes, and provided a theoretical basis for improving the content of active ingredients for traditional Chinese medicine in *Ginkgo biloba* leaves by means of transcriptional regulation.

1 Results and Analysis

1.1 Identification, classification and physicochemical properties of the AP2/ERF transcription factor family in *Ginkgo biloba*

Eighty-two gene sequences annotated as AP2/ERF transcription factors were screened from *Ginkgo biloba* transcriptome database. Through BlastX preliminary comparison and NCBI-CDD database protein sequence domain analysis, 61 genes with complete domain were identified as *Ginkgo biloba* AP2/ERF transcription factors. According to the characteristics of the domain, these transcription factors can be divided into four subfamilies, namely ERF, DREB, AP2 and RAV. The GbERF family was further divided into B1, B2, B3, B4, B5 and B6 subgroups, while the GbDREB family was divided into A1, A2 and A3 subgroups. See Table 1 for the classification and naming of each family member. The physicochemical properties of different AP2/ERF proteins differ greatly. The AP2 family GbAP2-4 has the largest number of amino acids and the longest protein sequence, which is composed of 987 amino acids. The lowest number of amino acids is GbDREB-A2-2 of the DREB subfamily, which contains only 112 amino acids. pI ranges from 4.73 to 9.92, indicating that these proteins may have different biological functions and need to play a role in the specific cell microenvironment. The fat coefficient represents the thermal stability of the protein. It can be seen from the results of Table 1 that the thermal stability varies greatly among individuals, but the differences among families are not obvious. The mean hydrophobicity coefficients were all negative, indicating that these AP2/ERF proteins were hydrophilic proteins.

Table 1 Physicochemical properties of AP2/ERF TFs in *G. biloba*

Category and naming	Gene ID	Number of residues	Molecular weight (U)	Theoretical pI	Aliphatic index	GRAVY
GbERF-B1-1	Cluster-495.0	208	22713.44	9.17	64.33	-0.642
GbERF-B1-2	Cluster-3580.687	241	26108.89	7.90	64.85	-0.588
GbERF-B1-3	Cluster-859.0	231	24837.83	9.60	64.98	-0.535
GbERF-B1-4	Cluster-3580.9368	300	32736.27	8.72	58.33	-0.725
GbERF-B1-5	Cluster-7711.0	218	23955.30	6.97	52.48	-0.783
GbERF-B1-6	Cluster-3580.24012	249	27957.07	9.11	64.98	-0.894
GbERF-B1-7	Cluster-3580.20282	197	22113.61	9.32	56.04	-0.853
GbERF-B1-8	Cluster-3580.24911	241	26885.04	8.81	56.80	-0.827
GbERF-B2-1	Cluster-3580.4324	239	26666.47	9.44	51.92	-0.990
GbERF-B2-2	Cluster-3580.21045	245	26962.98	9.08	60.20	-0.743
GbERF-B2-3	Cluster-885.0	239	26910.20	6.11	70.59	-0.698
GbERF-B2-4	Cluster-11693.0	259	29121.77	4.78	74.94	-0.508
GbERF-B2-5	Cluster-1584.3	216	23910.00	9.24	65.05	-0.768
GbERF-B2-6	Cluster-3580.9843	317	35019.85	6.36	74.70	-0.530
GbERF-B3-1	Cluster-3580.97	267	29425.98	5.96	44.76	-0.880
GbERF-B3-2	Cluster-2616.0	472	52427.83	5.24	63.28	-0.630
GbERF-B4-1	Cluster-3580.20109	488	54653.81	5.05	62.17	-0.715
GbERF-B4-2	Cluster-3580.21831	488	54651.90	5.17	61.56	-0.660
GbERF-B4-3	Cluster-7750.0	256	29091.00	5.93	71.21	-0.658
GbERF-B4-4	Cluster-11042.1	209	23796.67	5.90	72.82	-0.726
GbERF-B4-5	Cluster-1852.1	359	39705.07	4.82	62.53	-0.629
GbERF-B4-6	Cluster-3580.4524	440	48867.07	5.37	56.18	-0.702
GbERF-B4-7	Cluster-3580.29726	433	48370.64	5.88	52.82	-0.766
GbERF-B4-8	Cluster-3580.20263	455	50392.67	7.53	64.99	-0.604
GbERF-B4-9	Cluster-5011.0	446	49696.70	5.60	64.53	-0.668
GbERF-B5-1	Cluster-14774.1	324	35304.29	9.58	60.28	-0.724
GbERF-B6-1	Cluster-12777.1	227	25890.80	8.54	55.90	-0.892
GbERF-B6-2	Cluster-3580.6215	415	46053.43	7.05	58.60	-0.778
GbDREB-A1-1	Cluster-3580.19365	195	21234.76	7.78	70.67	-0.534
GbDREB-A1-2	Cluster-3580.21598	194	21116.63	8.66	65.05	-0.574
GbDREB-A1-3	Cluster-13465.0	228	25231.27	5.95	77.89	-0.416
GbDREB-A1-4	Cluster-3580.6040	179	20011.48	9.30	66.59	-0.526
GbDREB-A1-5	Cluster-1196.0	199	22139.56	4.81	74.07	-0.479
GbDREB-A1-6	Cluster-3580.733	191	21436.12	6.31	77.17	-0.534
GbDREB-A1-7	Cluster-13943.0	251	27853.10	7.73	67.33	-0.590
GbDREB-A1-8	Cluster-3580.21837	343	36871.40	4.81	62.04	-0.599
GbDREB-A1-9	Cluster-8904.0	344	36912.58	4.73	63.28	-0.553
GbDREB-A2-1	Cluster-3580.29815	441	48991.98	5.85	54.78	-0.779
GbDREB-A2-2	Cluster-3580.18158	112	13085.85	9.43	65.45	-0.685
GbDREB-A3-1	Cluster-3580.13603	210	23374.25	8.81	65.57	-0.640
GbDREB-A3-2	Cluster-14585.0	183	20668.59	9.36	73.66	-0.580
GbDREB-A3-3	Cluster-3580.28258	502	56660.59	5.08	58.86	-0.925
GbDREB-A3-4	Cluster-3580.28259	513	57880.04	5.14	57.80	-0.925
GbDREB-A3-5	Cluster-3580.13883	288	31589.62	5.90	64.31	-0.555
GbDREB-A3-6	Cluster-3580.28807	291	33744.14	6.08	57.01	-0.958
GbDREB-A3-7	Cluster-10894.1	503	56422.22	5.78	56.88	-0.839
GbDREB-A3-8	Cluster-3580.13212	510	55353.60	4.88	61.08	-0.625
GbDREB-A3-9	Cluster-3580.12938	294	32745.76	8.71	71.43	-0.624
GbDREB-A3-11	Cluster-790.0	365	39947.73	7.62	66.36	-0.582
GbDREB-A3-12	Cluster-790.1	369	40375.19	7.62	64.85	-0.590

Continuing Table 1

Category and naming	Gene ID	Number of residues	Molecular weight (U)	Theoretical pI	Aliphatic index	GRAVY
GbRAV-1	Cluster-3580.9020	682	75721.32	6.83	66.38	-0.694
GbRAV-2	Cluster-3580.21507	658	72878.02	6.94	64.65	-0.713
GbRAV-3	Cluster-3580.5228	728	80797.68	7.87	62.87	-0.644
GbRAV-4	Cluster-3580.26803	696	77251.51	7.02	62.67	-0.672
GbRAV-5	Cluster-3580.25351	623	68607.21	7.94	66.98	-0.588
GbAP2-1	Cluster-5409.0	790	86877.51	6.01	62.39	-0.745
GbAP2-2	Cluster-3580.16796	657	72014.87	5.91	62.22	-0.557
GbAP2-3	Cluster-3580.16244	675	74108.58	6.07	61.88	-0.583
GbAP2-4	Cluster-6037.0	987	110602.65	6.57	61.27	-0.935
GbAP2-5	Cluster-3580.2960	628	71075.59	9.92	60.00	-0.919

1.2 Homology analysis of AP2/ERF family in *Ginkgo biloba*

The phylogenetic tree was constructed by combining *Ginkgo biloba* AP2/ERF transcription factors with similar members in *Arabidopsis thaliana* and *Glycine max*. According to the analysis results, the results of branch clustering are basically consistent with those of family classification, and the relatives of members of the same subfamily are close (Figure 1). Among them, B1~B4 in GbERF subfamily is divided into four subclaves, but the homology is high. It comes from the same big clade as B5, and is the most closely related to B6. Gberf-b6-1 and Gberf-B6-2 have high homology with GBAP2-2 and GBAP2-3 in GbAP2. The A1-A3 members of GbDREB subfamily all come from the same branch and are closely related. Five members of the GbRAV family had a juxtaposes of 100 with members of the RAV family in *Arabidopsis thaliana* and *Glycine max*, indicating high homology. Gbap2-1 in the GbAP2 family is distantly related to the other members and is a single clade.

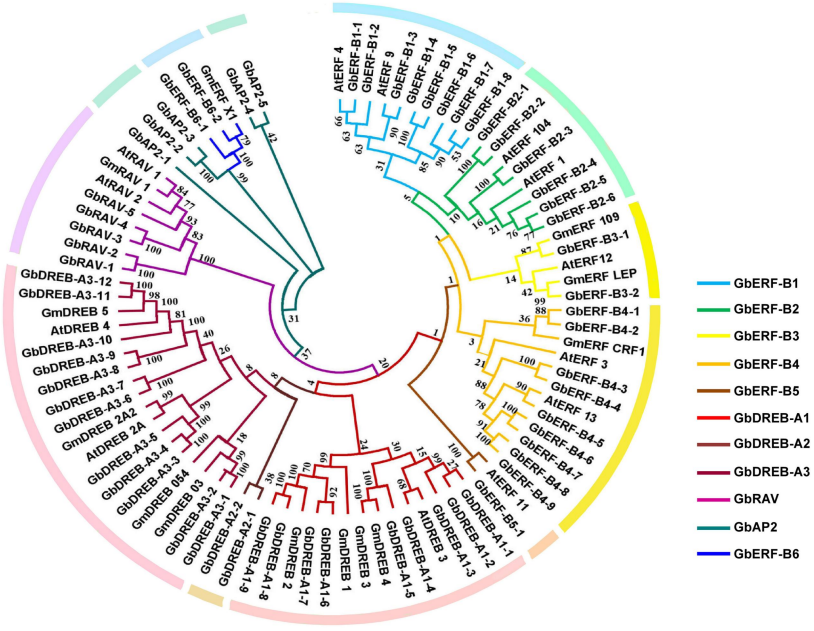

Figure 1 Phylogenetic tree representing relationship among AP2/ERF family in *G. biloba*, *Arabidopsis thaliana* and *Glycine max*

1.3 Conserved domain analysis of ERF gene family in *Ginkgo biloba*

A total of 51 *Ginkgo biloba* ERF gene family members were identified, including 28 GbERF and 23 GbDREB subfamilies. Both of these two subfamilies contain only one AP2 conserved domain, which is composed of YRG elements and RAYD elements. Through comparison and analysis of protein sequences, amino acid residues at positions 14 and 19 of the conserved domain of GbERF subfamily are alanine and aspartic acid (Figure 2A). The 14th amino acid residue of the GbDREB subfamily is valine, but the 19th amino acid will change and may be other amino acid residues in addition to glutamic acid (Figure 2B).

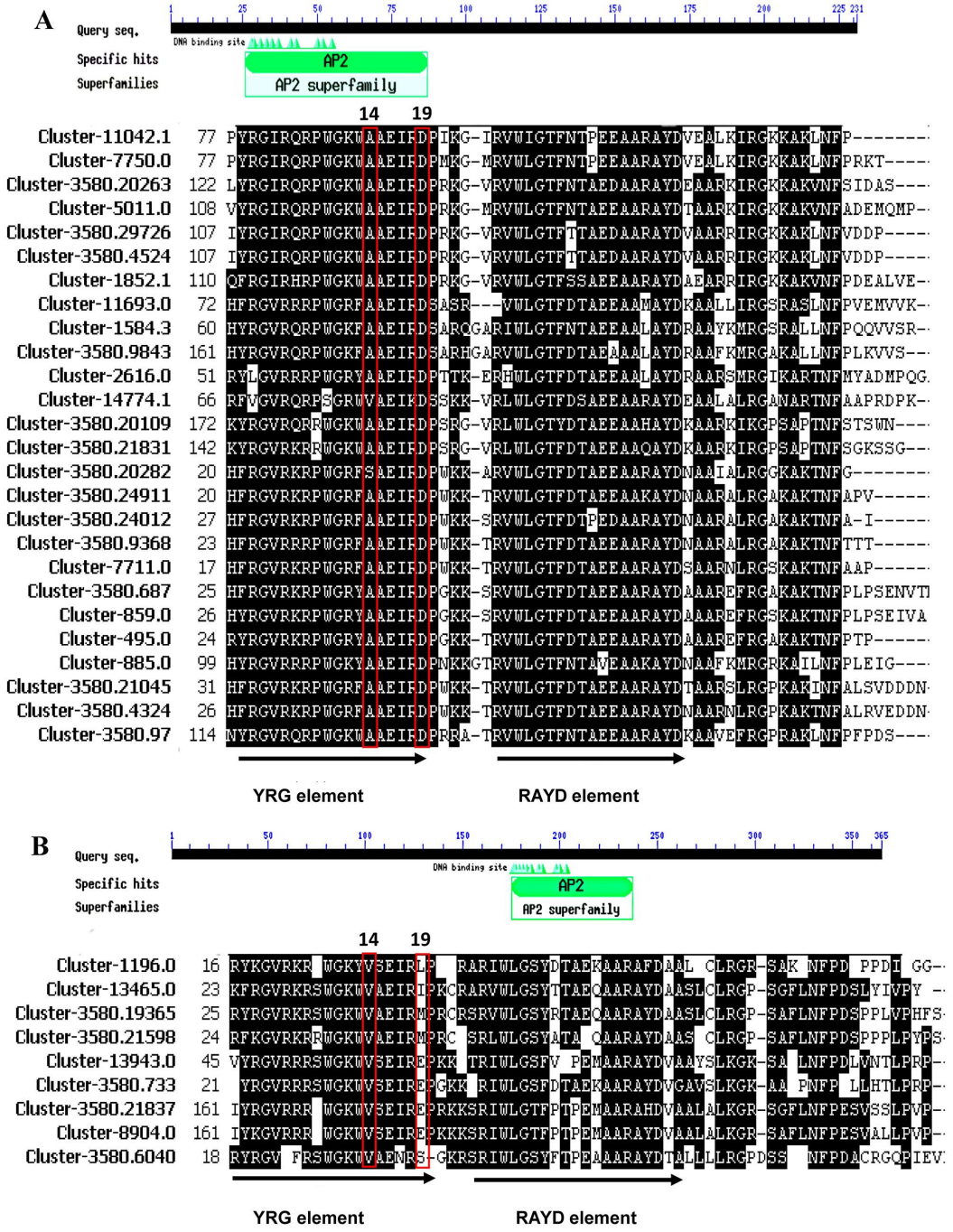

Figure 2 Characteristics analysis of conserved domains of ERF gene family in *G. biloba* (the left were Genes ID in transcriptome database)

Note: A: ERF subfamily (GbERF); B: DREB subfamily (GbDREB)

1.4 Protein motif and classification of ERF gene family in *Ginkgo biloba*

Motif prediction analysis of ERF gene family proteins in *Ginkgo biloba* using MEME database (Figure 3), the results showed that in addition to RAYD motif (motif 1) and YRG motif (motif 2) in conserved AP2 domains (Figure 4), the ERF gene of *Ginkgo biloba* contains several other types of motif structures. The Motif composition of each member in the same subgroup is similar, but there are differences between different subgroups. As shown in Figure 3, the A3 subgroup of the GbDREB subfamily contains at least 9 Motif structures, including Motif 1, Motif 2, Motif 4, Motif 5, Motif 9, Motif 10, Motif 11, Motif 12 and Motif 15. Subgroup A1 contains conserved Motif 1, Motif 2 and Motif 7, Motif 8 and Motif 9. Different Motif components indicate that these gene proteins may have different functions in *Ginkgo biloba*.

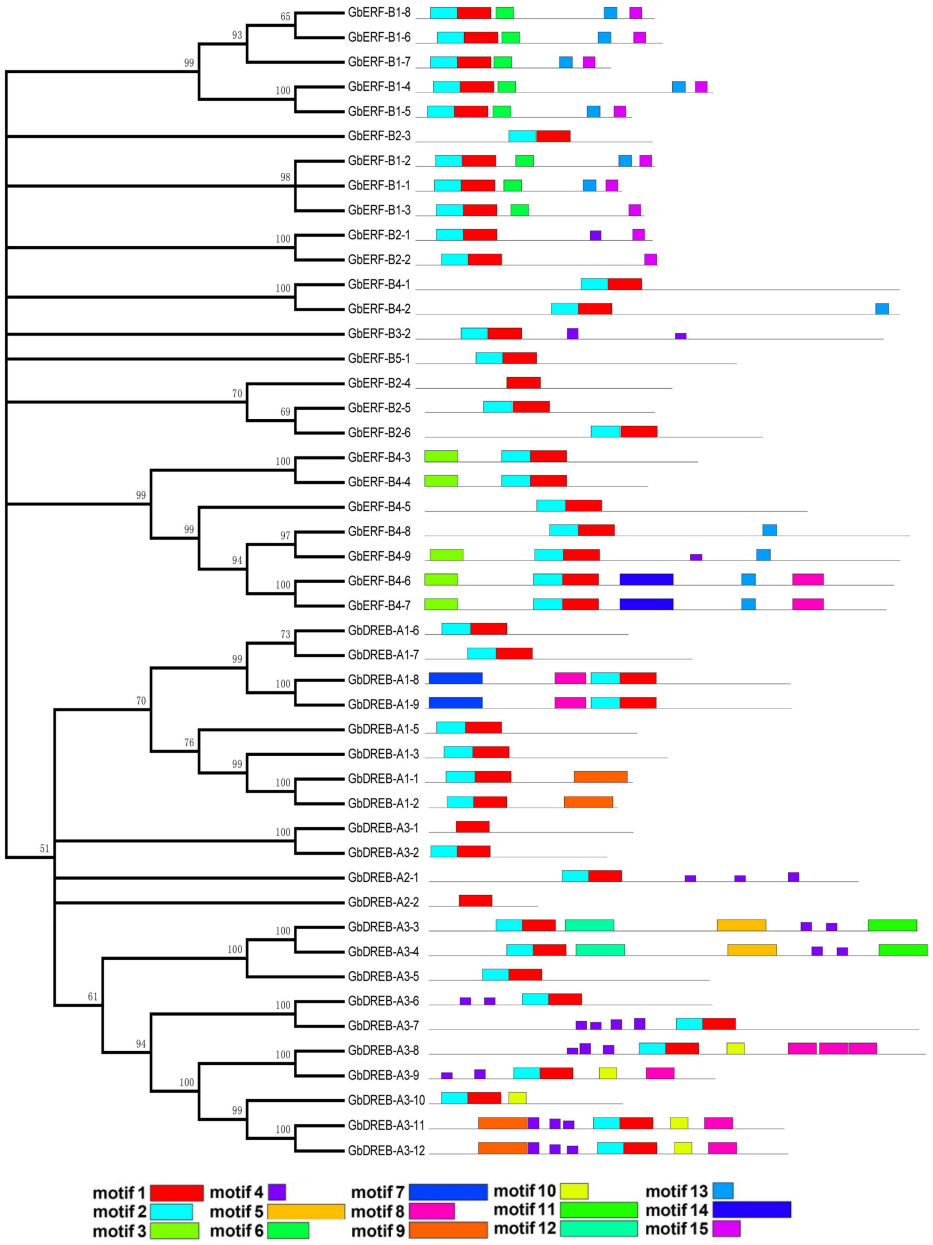

Figure 3 The conserved motifs of ERF gene family in *G. biloba*

Figure 4 The motifs of AP2 domain

1.5 Expression analysis of ERF gene family in *Ginkgo biloba* under stress

In this study, transcriptomic sequencing was used to analyze the differential expression of ERF gene family in *Ginkgo biloba* under UV and drought stress. Under ultraviolet conditions, 18 genes in the GbERF subfamily were induced to express, and the expression levels of Gberf-B4-2 decreased and Gberf-B4-6 and Gberf-B5-1 did not change significantly, while the expression levels of other family members increased. The expressions of

GbERF-B1-2, GbERF-B1-4, GbERF-B1-6, GbERF-B1-7, GbERF-B1-8, GbERF-B2-3, GbERF-B4-3 and GbERF-B4-7 were significantly increased. This subfamily was also induced by drought stress, and the expression levels of 15 genes increased, with the most significant increases in GbERF-B2-6, GbERF-B4-1, GbERF-B4-2, GbERF-B4-3 and GbERF-B4-4 (Figure 5A). The GbDREB subfamily plays an important role in drought stress. Thus, 19 genes were induced to express under drought conditions, Nine genes, including GbDREB-A1-1, GbDREB-A1-3, GbDREB-A1-6, GbDREB-A2-1, GbDREB-A2-2, GbDREB-A3-6, gbdreb-A3-6, gbdreb-A3-6, GbDREB-A3-9, GbDREB-A3-10, GbDREB-A3-12, were significant express. Compared with the control group, the expression levels of 15 genes of GbDREB subfamily increased under ultraviolet treatment, while the expression levels of another 5 genes did not change significantly. The expression levels of GbDREB-A1-1, GbDREB-A1-4, GbDREB-A3-7 and GbDREB-A3-8 were significantly increased (Figure 5B).

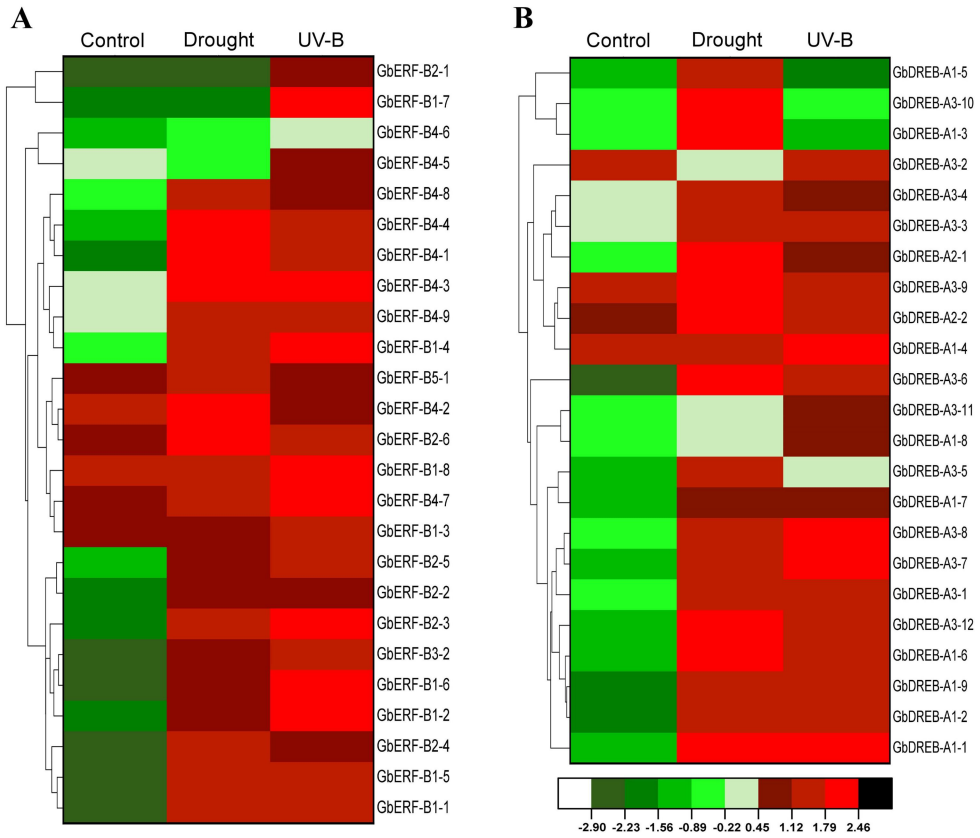

Figure 5 The expression analysis of ERF gene family in *G. biloba* under drought and UV-B stresses

Note: A: The expression pattern of GbERF subfamily; B: The expression pattern of GbDREB subfamily

1.6 Expression verification analysis of five ERF family genes in *Ginkgo biloba*

Five ERF genes related to the regulation of secondary metabolism such as flavonoids in *Ginkgo biloba* were screened and their expressions were verified under two stress treatments. The results showed that the expression levels of GbERF-B1-2, GbERF-B1-8, GbERF-B2-5, GbERF-B4-3 and GbDREB-A1-3 increased in different degrees under drought and ultraviolet stress conditions. On the 10th day of drought stress, the expression levels of GbERF-B4-3 and GbDREB-A1-3 increased significantly, about 3 times that of the control group. On the 15th and 20th day, the expression levels of GbERF-B1-8, GbERF-B4-3 and GbDREB-A1-3 were increased by 3 to 5 times (Figure 6A). The expression of GbERF-B2-5 induced by ultraviolet treatment was the most significant, and the expression of Gberf-B2-5 increased about 10 times after 24 h and 48 h irradiation, followed by GbERF-B1-8, GbERF-B4-3, GbERF-B1-2 and GbDREB-A1-3. The gene expression levels were 2~3 times that of the control group (Figure 6B). The results of five gene expression verification analysis were consistent with the results of transcriptome detection.

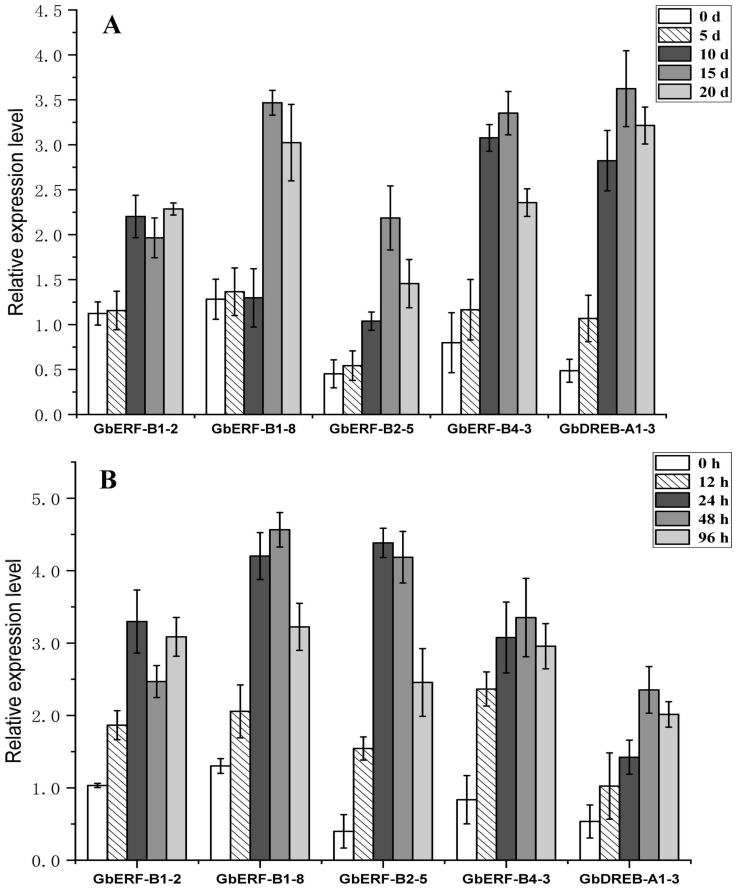

Figure 6 The validation of 5 ERF genes expression profile in *G. biloba* under drought and UV-B stresses
Note: A: Drought; B: UV-B

2 Discussion

Recent studies have found that transcription factors play a crucial role in plant secondary metabolism. By regulating the expression of multiple enzyme genes in secondary metabolic pathways such as flavonoids, they can effectively turn on or off secondary metabolic flow, thus regulating the formation of specific secondary metabolites (Ramakrishna and Ravishankar, 2011). Stress generally induces a large number of transcription factors, and at the same time, some protective secondary metabolites accumulate specifically in plants, indicating that plant response to environmental stress is correlated with the level of accumulation of secondary metabolites. Transcription factors may help plants resist environmental stress by regulating secondary metabolism (Meraj et al., 2020). The regulation mechanism of transcription factors on the synthesis of secondary metabolites in medicinal plants has been a hot topic in recent years. Under natural conditions, the content of active ingredients in medicinal plants is generally very low, but under adverse stress conditions, the content will change to a certain extent. Exploring the regulatory role of transcription factors in this process will help us understand the root causes of physiological changes at the level of gene expression.

In this study, a total of 61 transcription factors of *Ginkgo biloba* AP2/ERF were identified and divided into 4 subfamilies, namely ERF, DREB, AP2 and RAV, according to the characteristics of their conformal domains, among which 28 were GbERF subfamilies and 23 were GbDREB subfamilies. Both of these two subfamilies belonged to the ERF gene family. It plays a major regulatory role in stress response and secondary metabolism of *Ginkgo biloba*. ERF gene family has been reported in other medicinal plants. For example, Zhang et al. (2020) treated ginseng advents with MeJA and found that through the expression and correlation analysis of key genes for ginsenoside biosynthesis and ERF family genes, ERF003, ERF118 and ERF012 may regulate ginsenoside synthesis by inhibiting DDS expression and promoting CYP716A53v2 expression. The expression of ERF1B was significantly negatively correlated with CYP716A47, suggesting that ERF1B is likely to inhibit the expression of

CYP716A47 and participate in the regulation of ginsenoside synthesis. Li et al. (2020) cloned the *AsERF1* gene from the rare medicinal material Baimuxiang (*Aquilaria sinensis* (Lour.) Spreng.), and found that salt, drought and low temperature and heavy metal stress could induce the expression of *AsERF1*, among which drought stress had the most significant effect on the expression level of *AsERF1*. *AsERF1* gene may play a regulatory role in defense response and formation of Baimuxiang (*Aquilaria sinensis* (Lour.) Spreng.) and Chenxiang (*Aquilaria* spp.). Sun et al. (2019) identified the transcription factor *SmERF115* by analyzing the transcriptome data of Danshen (*Salvia miltiorrhiza Bge*.) induced by MeJA. The content of salvianolic acid increased and the content of tanshinone decreased in hairy root with *SmERF115* overexpression. Silencing *SmERF115* decreased salvianolic acid levels but increased tanshinone content. *SmERF115* was a positive regulator of salvianolic acid biosynthesis by yeast single hybridization and double luciferase assay. In addition, ERF gene family may also be involved in taxol (Dai et al., 2009), artemisinin (Xiang et al., 2019), nicotine (Hayashi et al., 2020) and vinblastine (Leslie and Memelink, 2010) and other alkaloid metabolism regulation. When *Ginkgo biloba* faces UV, drought and other stresses, the content of flavonoids in *Ginkgo biloba*, as a natural antioxidant, will be increased appropriately, which can help plants resist drought, UV and other environmental damage. There are few studies on the regulation of ERF gene family on the synthesis of secondary metabolites such as flavonoids in *Ginkgo biloba*. In this study, *Ginkgo biloba* under these two kinds of stress was used to build transcriptome database, identify the AP2/ERF transcription factors and conduct a comprehensive analysis of the expression of ERF gene family, in order to carry out functional analysis of transcription factors that play a key regulatory role.

Verification analysis of the expression of five ERF genes in *Ginkgo biloba* showed that the expression levels of GbERF-B1-8, GbERF-B4-3 and GbDREB-A1-3 increased significantly on the 15th and 20th days of drought stress, and the expression levels of GbERF-B1-2 and GbERF-B2-5 also increased appropriately. Uv treatment had the most significant effect on the expression of GbERF-B2-5. The expression levels of transcription factors increased significantly after 24, 48 and 96 h ultraviolet irradiation, indicating that these transcription factors may play a positive role in the secondary metabolism of *Ginkgo biloba*. Corresponding analysis of the content changes of flavonoids and other secondary metabolites in *Ginkgo biloba* under these two stress treatment conditions and their relationship with the expression of key metabolic enzyme genes will provide the possibility to reveal the regulation mechanism of ERF gene family. Further study of transcription factor AP2/ERF is helpful to the application of molecular regulation mechanism in metabolic engineering of medicinal plants such as *Ginkgo biloba*, or to improve the content of active ingredients of medicinal plants such as *Ginkgo biloba* by appropriately increasing environmental pressure, so as to achieve the purpose of improving the quality of medicinal materials.

3 Materials and Methods

3.1 Materials of test

Three-year-old *Ginkgo biloba* seedlings growing in the nursery base of Wuhan Polytechnic University were used as experimental materials. Sixty seedlings with the same growth were selected and divided into three groups. One group was used as control, and the other two groups were treated with UV and drought respectively. Ultraviolet treatment was to place *Ginkgo biloba* seedlings in a closed culture chamber without other light sources, and only 1 500 J/m^2 ultraviolet light was provided. *Ginkgo biloba* leaves were collected after 12, 24, 48 and 96 h of irradiation. The drought treatment lasted for 20 days, and leaves were collected at the 5th, 10th, 15th and 20th days, respectively. The leaves of *Ginkgo biloba* were rapidly frozen with liquid nitrogen, and then the leaves collected at different time points under the two treatment conditions were mixed in equal amounts to extract total RNA, which was sent together with the total RNA of the control group to Novgene Biotech Co., Ltd. for transcriptome sequencing. Three cDNA libraries were constructed by this sequencing, namely ultraviolet treatment group, drought treatment group and control group. The sequencing was completed by Illumina HiSeq 2500 platform, and the original data were filtered, the splices and low-quality data were removed, and the transcripts were spliced by Trinity software to obtain Unigenes.

3.2 Identification, classification, physicochemical properties and phylogenetic analysis of the AP2/ERF transcription factor family of *Ginkgo biloba*

According to the annotation results of Unigenes in transcriptomic database, all AP2/ERF transcription factor sequences were screened out. After preliminary comparison by BlastX on NCBI, conserved domains were identified in PFAM and NCBI-CDD databases, and the sequences without complete AP2/ERF domains were excluded. *Arabidopsis thaliana* AP2/ERF transcription factor protein sequences from *Arabidopsis thaliana* genome database TAIR 9.0 (http://www.arabidopsis.org/index.jsp) to download, AP2/ERF protein sequences for other species can be downloaded from NCBI (http://www.ncbi.nlm.nih.gov/). Vector NTI was used to compare the AP2/ERF conserved domains of *Ginkgo biloba* with those of *Arabidopsis thaliana* and *Glycine max*. The classification of the AP2/ERF gene families in *Ginkgo biloba* was clarified. The physicochemical properties of the families were analyzed using Expasy online platform. At the same time, MEGA 5.0 and Clustal X software were used to construct phylogenetic tree between the identified *Ginkgo biloba* AP2/ERF gene family members and similar members in *Arabidopsis thaliana* and *Glycine max*. Neighbor-joining (NJ) method was adopted and Bootstrap was repeated 1 000 times.

3.3 Analysis of conserved domain and protein motif of ERF gene family in *Ginkgo biloba*

Multiple sequences of GbERF and GbDREB subfamilies of ERF gene family in *Ginkgo biloba* were compared in Vector NTI software to analyze the characteristics of conserved domain and amino acid composition of key sites of these two subfamilies. MEME online analysis software (http://meme-suite.org/tools/meme) was used to identify the conserved domain motif of the ERF gene family of *Ginkgo biloba*. A maximum of 15 models were set for analysis, with amino acid numbers ranging from 10 to 100.

3.4 Expression and verification analysis of ERF gene family in *Ginkgo biloba* under stress

Transcriptome sequencing was used to detect the expression of ERF gene family in *Ginkgo biloba* under ultraviolet and drought conditions. *Ginkgo biloba* leaf RNA was extracted at 5, 10, 15, 20 days after drought treatment and 12, 24, 48, 96 hours after ultraviolet irradiation, and reversetranscribed into cDNA. According to the instructions of SYBR Green qRT Mix (Takara, Dalian) kit, 5 ERF family genes were verified by real-time fluorescent quantitative PCR. The reaction procedure is: 95℃ for 30 S, 40 cycles (95℃ for 5 S, 60℃ for 15 S). With GAPDH (L26924) as the internal reference gene, three biological replicates were set for each sample (Table 2).

Table 2 The primers for qRT-PCR

Name of genes	Primer sequences
GbERF-B1-2	F: CTTGGTAGTGCTACTATTCGGT
	R: CCTGGTTCTAACTCTAGATTGC
GbERF-B1-8	F: CGTCGTACCGTAATTACAGAGAT
	R: GACGAGGTTCTGAGATGCCACG
GbERF-B2-5	F: TATGTTTGATCGCCACCTCCA
	R: CTAGATCTGTGTTGAACAACTC
GbERF-B4-3	F: GAACCAACTTAGTACCGCATC
	R: CTCTCGAAGTTGTAGTATTC
GbDREB-A1-3	F: CCAGAGATTCTGAATCACTC
	R: TCGGATACTGCGACGGAGATA
GAPDH	F: GGTGCCAAAAAGGTGGTCAT
	R: CAACAACGAACATGGGAGCAT

3.5 Data statistics and analysis

SPSS 21.0 was used for data statistics and analysis, and Origin 9.0 software was used for plotting.

Authors' contributions

YHH was the executor of the experimental design and experimental research of this study, who completed the data analysis and the writing of the first draft of the paper. LLL and CH participated in experimental design and analysis of experimental results. XX participated in part of the experiments. CSY was the proposer and the person in charge of the project, directing the experimental design, paper writing and modification. All authors read and approved the final manuscript.

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