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Transcriptome-Based Identification, Characterization, Evolutionary Analysis, and Expression Pattern Analysis of the *WRKY* Gene Family and Salt Stress Response in *Panax ginseng*

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Abstract: WRKY transcription factors are some of the most important transcription factors in planta, and they are involved in biological and abiotic stresses, growth and development, and biochemical processes. The *WRKY* gene family has been found in many higher plants, while little is known about WRKY-encoding genes in ginseng. As a traditional and important economic medicinal herb and health food, ginseng has been a model species for other related medicinal plants. Here, we analyze the WRKY transcript factor gene family in ginseng from a genetics and genomics perspective in the hope that this study can create a new avenue for understanding the role of *PgWRKYs*. By identifying and analyzing all candidate WRKY transcription factor family members based on the transcriptome of ginseng, a total of 118 *PgWRKY* genes was obtained following gene classification, the phylogenetic relationship, conserved domain structure, functional differentiation, and an expression activity analysis. A phylogenetic analysis revealed that the *PgWRKYs* were clustered into three groups: I, II, and III transcription factors. Members in group II were further divided into five subgroups: IIa to IIe. An expression analysis showed a trend of co-expression among most *PgWRKY* genes, forming an interaction network. The coding sequences were WRKYGQK; only two genes were WRKYGKK, and only one gene was WSKYGQK. Moreover, a salt stress treatment analysis of the response of *PgWRKY39-01*, *PgWRKY062*, and *PgWRKY064* genes was investigated using RT-qPCR. After salt stress treatment, the expression of three *PgWRKY* genes was increased, indicating that *PgWRKYs* can participate in regulating the response to salt stresses in ginseng. These comprehensive data provide a reference for elucidating the functions of this transcription factor family in the growth, development, and salt stress response of ginseng.

Keywords: *Panax ginseng*; WRKY transcription factor; functional differentiation; functional genomics; salt stress response

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1. Introduction

WRKY is a crucial transcription factors gene family in planta and is involved in various physiological and biochemical processes, e.g., the plant's defense against attack from pathogens, biotic and abiotic stresses, growth, and development [1–7]. They are named after the highly conserved seven amino acid sequence WRKYGQK at the N-terminus, while at the C-terminus, they present as a zinc-finger structure distinct from other known zinc-finger motifs. The WRKY domain and zinc-finger-like motif possess the DNA-binding domain, which can recognize the W-box sequence (C/T) TGAC (T/C) that is indwelled in the promoter sequence of target genes; by binding to the sequence, they modulate the expression of downstream structural genes or transcription factors of other families [1,8].

The WRKY superfamily is based on the number of WRKY domains and the pattern of the zinc-finger motif that can be divided into three groups: group I, III, and III. WRKY members in group I contain two WRKY domains, and the zinc finger structure is the C_2H_2 type. However, most proteins with one WRKY domain belong to group II and III, but the group II zinc finger structure is also the C_2H_2 type, and group III is the C_2HC type. Members of group II consists of different WRKY domains and motifs that can be further divided into five sub-groups, namely, IIa to IIe [1,9–11]. After identification of the first WRKY transcription factor-encoding gene, *SPF1,* in *Ipomoea batatas* [12], numerous genes for WRKY proteins have since been identified from other plant species, including *Oryza sativa* [13–15], *Arabidopsis thaliana* [3,16], *Nicotiana tabacum* [17], *Hordeum vulgare* [18], *Triticum aestivum* [19], *Glycine max* [20,21], *Zea mays* [22], *Brassica napus* [23], *Solanum tuberosum* [24], *Medicago truncatula* [25], *Solanum lycopersicum* [26], and *Gossypium hirsutum* [27].

Historically, ginseng (*Panax ginseng* C.A. Meyer) is a traditional and important economic medicinal herb and health food in China. It has also been a model species for ginsenoside biosynthesis, synthetic biology, and genomics research medicinal plants [28]. In Nuruzzaman and Xiu's latest study [29,30], the authors identified 48 *WRKY* transcripts based on the methyl jasmonate-treated adventitious root transcriptome, and characterized one of the WRKYs known as *PgWRKY1*; the results showed that *PgWRKY1* might be a multiple stress-inducible gene responding to hormones and salt stresses. In this study, we approach this issue from the perspective of genetics and genomics to the manning of the WRKY gene family's classification, phylogenetic relationship, conserved domain structure, functional differentiation, and evolution, as well as the expression pattern of ginseng. We are hopeful that this study can create a new avenue for understanding the function of the WRKY TFs gene family and its role in biotic and abiotic stress, secondary metabolite biosynthesis, growth, and development.

2. Materials and Methods

2.1. Data Sources and Methods of Analysis

Here, we used the 248,993 transcripts previously generated from the transcriptomes of 14 tissues [28]. Using 248,993 transcripts as a reference, the expression of *PgWRKY* genes in 14 tissues of 4-year-old plants, 4 different aged (5-, 12-, 18-, and 25-year-old) roots, and 4-year-old roots of 42 Jilin ginseng farmers' cultivars was measured using Trinity software. The expression used in this study was measured as transcripts per million (TPM).

2.2. Identification of PgWRKY TFs

Using the highly conserved 7 amino acid sequence, WRKYGQK, as the query sequence, we searched for possible homologs to *WRKY* genes on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast, accessed on 7 October 2019). A tblastn online alignment was also performed with 248,993 transcripts at E-value $\leq 1.0 \times 10^{-6}$, following the extracted and annotated analysis with Perl programming software and Blast2GO software. Proteins present in the WRKY domain were evaluated against the repository of the conserved domain database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi, accessed on 19 October 2019) [31]. Sequences with a partial WRKY domain were omitted from subsequent analyses; the molecular weight and isoelectric point of all *WRKY* genes were also predicted by the ExPASy Proteomics Server (http://expasy.org/, accessed on 26 October 2019).

2.3. Phylogeny and Conserved Domain Protein Sequence Analysis of PgWRKY TFs

PgWRKY genes with complete WRKY domains were extracted and aligned using Jalview version 2.0 [32]. Phylogenetic trees were constructed using MEGA version 7.0 (http://www.megasoftware.net, accessed on 5 November 2019) [33] with a neighbor joining (NJ) method of 2000 bootstrap value, taking into consideration position correction and pairwise deletion. The conserved motifs of *PgWRKY* genes were analyzed using the

MEME online tool (http://meme.sdsc.edu/meme/website/intro.html, accessed on 10 November 2019).

2.4. Prediction of Natural Selection in WRKY Superfamily in Ginseng

The synonymous rate (*Ks*), non-synonymous rate (*Ka*), and *Ka/Ks* ratio were calculated using PAL2NAL version 14 (http://www.bork.embl.de/pal2nal/, accessed on 16 November 2019) [34] to evaluate and analysis the effect of selective pressure on different sets of homologous *WRKY* genes.

2.5. GO (Gene Ontology) Functional Categorization Analysis of PgWRKYs

Blast2GO version 5.0 [35] was used to perform the GO item annotation of *PgWRKY*. The GO functional categorization of 248,893 transcript annotations was used as the background control. At the same time, 248,893 transcript annotations were used for GO enrichment analysis of the *PgWRKY* transcripts. The enrichment significance analysis of the number of *PgWRKY* transcripts in ginseng categorized into each subcategory (level 2) was determined by a chi-square test.

2.6. The Expression Pattern Analysis of PgWRKYs

We constructed three expression databases and extracted the *PgWRKY* gene transcript expression dates, including the expression of *PgWRKY* gene transcripts in the 14 tissues of 4-year-old ginseng (Table S1), the 4 different-aged roots (Table S1), and the 4-year-old ginseng roots of 42 farmers' cultivars (Table S1). The expression of *PgWRKY* gene transcript heatmap analysis was conducted using TBtools version 0.98 [36], and the co-expression networks analysis was constructed and visualized using BioLayout Express ^{3D} version 3.0 [37].

2.7. Response of PgWRKY Genes to Salt Stress

The laboratory ginseng adventitious root material was treated with salt stress by adding different concentrations of NaCl (70 mM, 80 mM, 90 mM, and 100 mM) to the B5 medium required for its growth, and the treated adventitious roots were harvested by incubation in the dark at 22 °C for 30 days. Total RNA was extracted from the adventitious roots of ginseng using the TRIzol (SparkZol Reagent, SparkJade Science Co., Ltd., Jinan, China) method. Subsequently, the RNA was reverse transcribed into cDNA according to the instructions of the HiFiScript gDNA Removal cDNA Synthesis Kit (CWBIO, Beijing, China). We used an UltraSYBR One Step RT-qPCR Kit (Low ROX) (CWBIO, Beijing, China) as the reaction system for the RT-qPCR. The *β-Actin 1* gene was used as the internal reference gene, and the reaction was performed in the ABI 7500 Fluorescence Quantitative PCR System. The reaction flow of fluorescence quantitative PCR is as follows: pre-denaturation at 95 °C for 10 min; PCR of 40 cycles at 95 °C for 15 s and 60 °C for 60 s; and melting curve at 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. After three technically and biologically significant replicates, the resulting data were analyzed using the $2^{-\Delta\Delta Ct}$ method to observe the expression of target genes.

3. Results

3.1. Identification and Classification of PgWRKY TFs

A total of 118 out of 214 *WRKY* gene (*PgWRKY001* to *PgWRKY088*) sequences from *Panax ginseng* was identified, which had a complete CDS (coding sequence) and ORF (open reading frame); these are named from *PgWRKY010* to *PgWRKY088* (Table S2) and they all presented the conserved WRKY domain in NCBI CDD. The ORF contained nucleotide sequences from 240 to 2651 bp, and the amino acid residues were between 78 and 643, among which *PgWRKY072* was the shortest one, while *PgWRKY073-32* had the longest sequence. The isoelectric point was between 4.33 (*PgWRKY027-02*) and 9.93 (*PgWRKY70-03*), suggesting a wide range of occupancy in different microcellular environments. Twenty-

seven members showed an acidic pKa range, while others remained at a basic pKa value. The molecular mass of these proteins ranged from 8.689 to 69.874 kDa (Table S3).

3.2. Multiple Sequence Alignment Analysis of PgWRKY Proteins

Among the 118 *PgWRKY* genes, 15 belonged to group I, 91 belonged to group II, and 12 belonged to group III. Group II could be divided into five sub-groups—IIa, IIb, IIc, IId, and IIe—comprising 4, 16, 29, 36, and 6 genes, respectively (Figure 1; Table S3). The WRKYGQK motif was highly conserved among the WRKY proteins; two variable sites of this motif were found in *PgWRKY45* and *PgWRKY10* by multiple sequence alignment; at the 'R' and 'Q' site they changed to WKKYGQK and WRKYGKK, respectively. Another variant, WSKYGQK, was found in *PgWRKY02*, which belonged to group III. The characteristics of the C-terminus were also identified: a $CX_4CX_{22}HXH$ zinc-finger motif, a $CX_4CX_{23}HXH$ motif, and a $CX_5CX_{23}HXH$ motif were found in sub-group I and sub-group II. A G (P) YN box, VTK (R) DNP box, GCQ box, GYR (K), PRS (N) Y box, PRGYYKCS box, and YYRCS box were found in front or behind the WRKY domain.

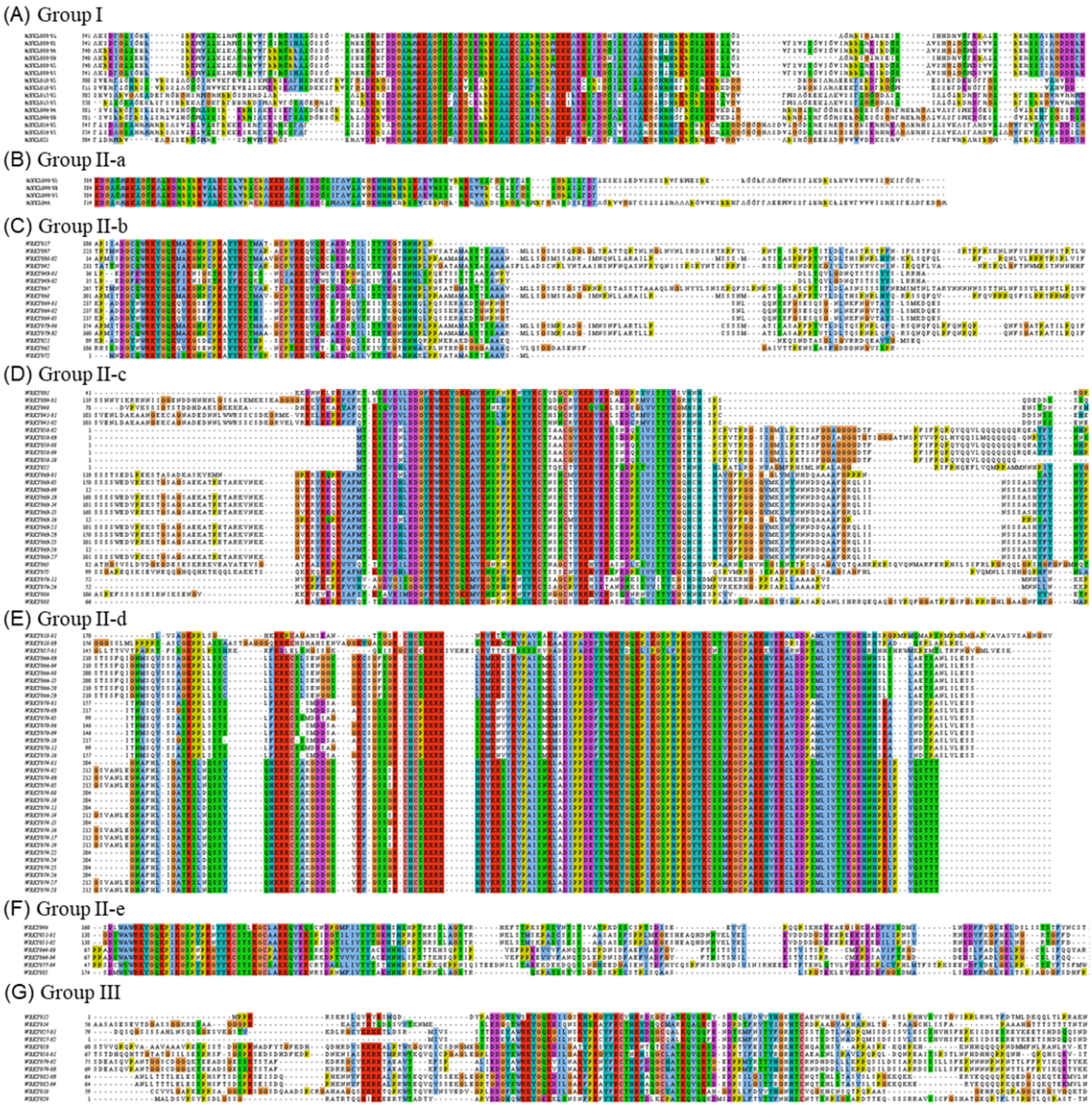

Figure 1. Alignment of conserved motifs of different groups of the *PgWRKY* gene family in *Panax ginseng*. Alignment was performed using the ClustalW program and is displayed using the software Jalview. Residues that were highly conserved within each of the major groups are in color.

3.3. Phylogenetic Analysis of PgWRKYs

A total of 20 motifs was detected (Figure 2A): 5 WRKY DNA-binding domains, 1 Plant-zn-clust domain, and 14 unknown domains (UKD). The distribution of 20 motifs among *PgWRKY* proteins greatly varied (Figure 2B). UKD 02 appeared in all the PgWRKYs. UKD 03 was contained in all the PgWRKYs except group IId; it was also the only one that contained UKD 12. Group IIb was different from the others as it did not show UKD 13. The significant variation between these motifs may be correlated with the diversity function of the *PgWRKYs* gene family. Compared with *Arabidopsis*, *Populus trichocarpa*, and *Oryza sativa*, a new motif known as the 'Leu zipper motif' in *PgWRKYs* was identified. This motif presented in sub-groups IIa, IIb, and IIe and group III of the *PgWRKY* genes; it is a hypothetical structure common to a new class of DNA binding proteins.

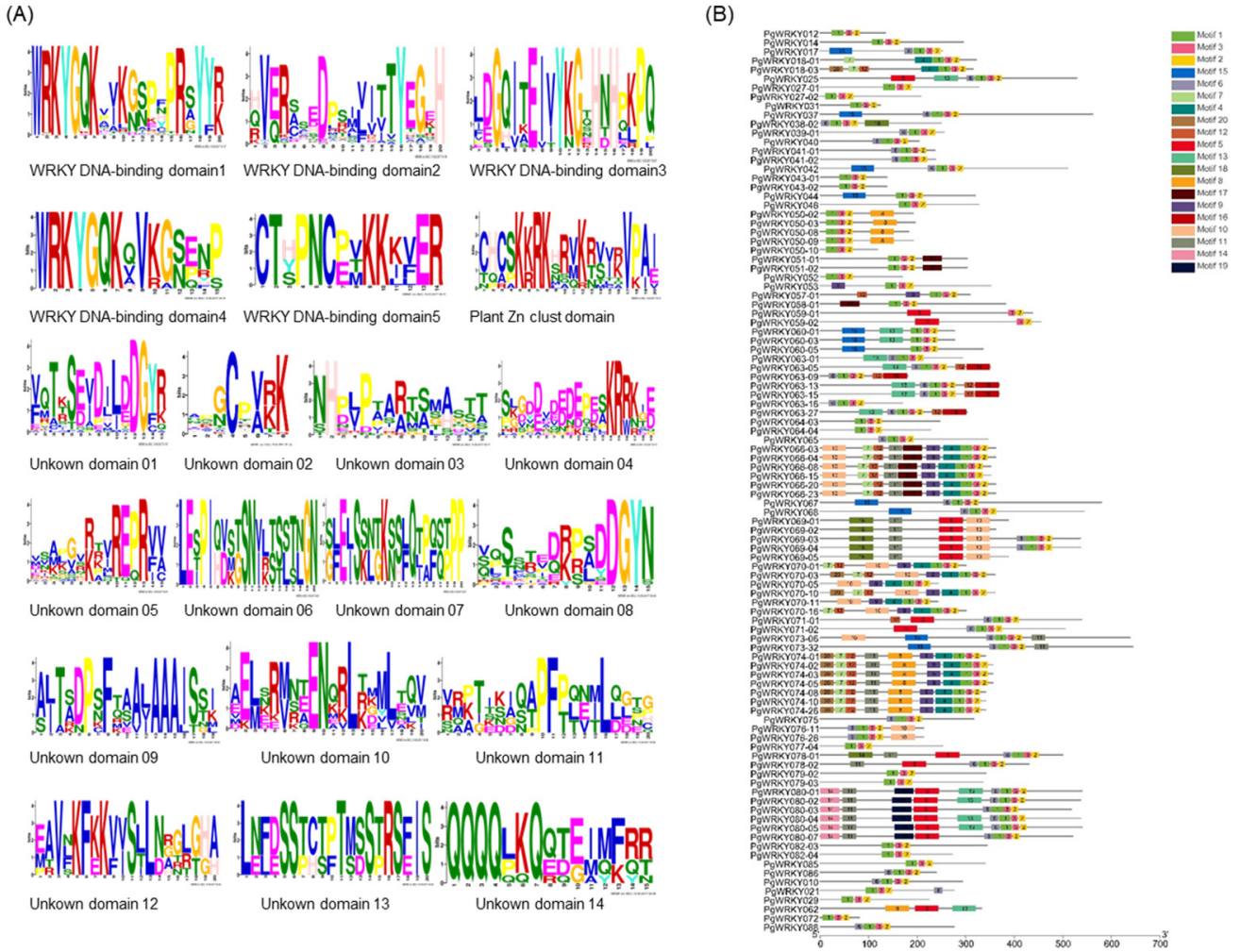

Figure 2. Distribution of 20 predicted conserved motifs in PgWRKY proteins. (**A**) 20 motifs revealed in PgWRKY proteins. (**B**) Conserved 20 motifs of PgWRKY proteins. The conserved motifs of the PgWRKY proteins are indicated by colored boxes. Motifs of PgWRKY proteins were identified by MEME and TBtools program.

The maximum-likelihood (ML) method was used to construct a phylogenetic relationship tree of the *PgWRKY* gene family. Firstly, 91 sequences of class II and sequences of foreign species were selected to construct an evolutionary tree. Based on the classification of foreign species, 91 sequences were further divided into 5 subclasses: IIa, IIb, IIc, IId, and IIe, including 4, 16, 29, 36, and 6 gene sequences, respectively, as shown in Figure 3A. At the same time, 118 *PgWRKY* gene sequences were selected to construct the family phylogenetic

relationship tree, as shown in Figure 3B, so as to analyze evolutionary relationship differences between different classes in the *PgWRKY* gene family and sequence homology in the same class. We found that sequence homology in the same class was relatively high, and the evolutionary relationship was close, although there were some differences in similarities between different classes showing different branches.

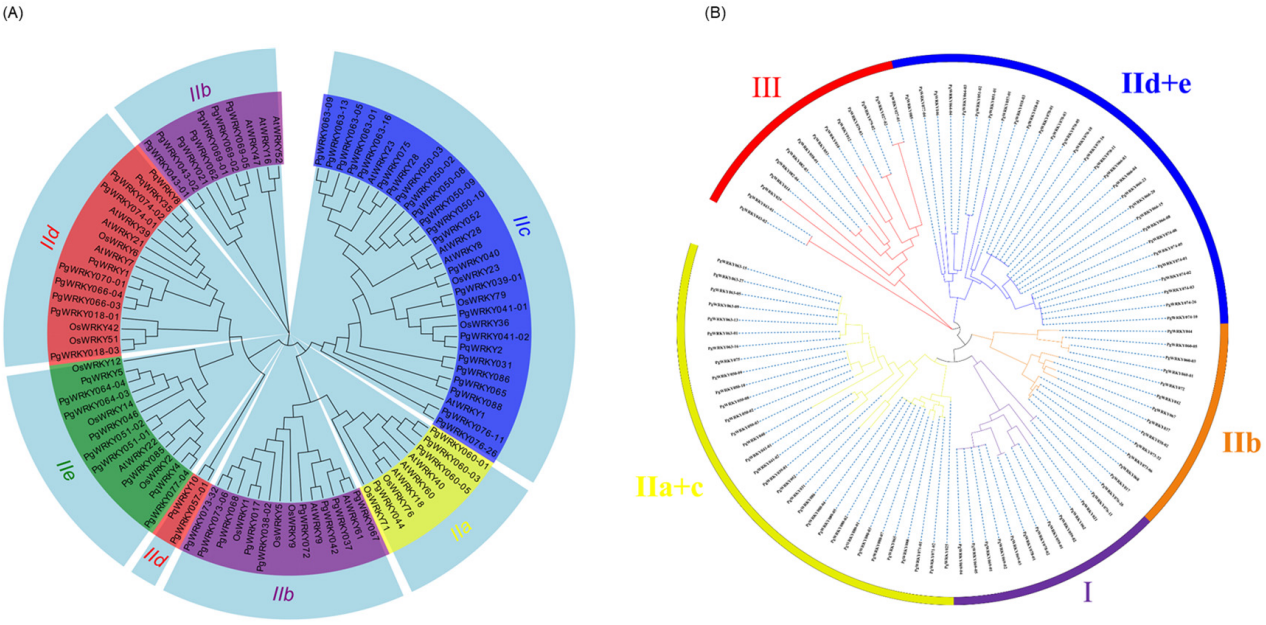

Figure 3. Phylogenetic tree of the *PgWRKY* gene family. (**A**) The total of 91 *PgWRKY* genes in subgroup II that had complete ORFs and were used as representatives to construct the classification and phylogeny with 3 other species 53 *WRKY* genes (Table S3). Yellowish in the inner circle indicates the *WRKY* genes Group II-a; Purple in the inner circle the *WRKY* genes Group II-b; Blue in the inner circle the *WRKY* genes Group II-c; Red in the inner circle the *WRKY* genes Group II-d; Green in the inner circle the *WRKY* genes Group II-e. The total of 53 *WRKY* genes from *Arabidopsis thaliana* (*AtWRKY*), *Oryza sativa* (*OsWRKY*), *Panax quinquefolium* (*PqWRKY*). (**B**) Phylogenetic tree depicting *PgWRKY* gene family evolution in *Panax ginseng*. Purple in the outer circle the *PgWRKY* genes Group I; Yellow in the outer circle the *PgWRKY* genes Group II-a and -c; Orange in the outer circle the *PgWRKY* genes Group II-b; Bule in the outer circle the *PgWRKY* genes Group II-d and -e; Red in the outer circle the *PgWRKY* genes Group III. The numbers for the branches of the tree are bootstrap confidence out of 2000 replications.

3.4. Ka/Ks Analysis of Natural Selection in PgWRKYs

Eight paralogous pairs were identified in different groups of *PgWRKYs* based on phylogenetic relationships, gene structures, and conserved motifs (Table 1). The nonsynonymous and synonymous substitution rates (Ka/Ks) were calculated to verify Darwinian position selection. The results showed that all of the Ka/Ks ratios were lower than 1.0; moreover, the Ka/Ks were from 0.0898 to 0.807 at the *PgWRKY* paralogous pairs (Table 2), indicating that the *PgWRKY* family had undergone purifying selection pressure. All of these results indicated that the *PgWRKY* genes in the eight species above had been undergoing a strong purifying selection.

Table 1. The number of *PgWRKY* genes in ginseng.

Group Type	Group I	Group IIa + IIb	Group IIc	Group IId + IIe	Group III	Total
PgWRKY	15	20	29	42	12	118
DcWRKY	17	20	18	29	11	95
AtWRKY	13	11	18	16	14	72
OsWRKY	15	12	15	18	36	96
CsWRKY	10	8	16	15	6	55
BdWRKY	17	9	21	16	23	86
PtWRKY	50	14	13	17	10	104
PqWRKY	23	4	8	5	5	55
BnWRKY	121	45	55	58	51	330
GaWRKY	17	22	30	28	12	109
LjWRKY	12	13	13	14	7	61
MaWRKY	10	11	9	13	8	51

Table 2. Divergence between paralogous *PgWRKY* gene pairs.

Gene 1	Gene 2	Group	Ka	Ks	Ka/Ks
PgWRKY059-01	*PgWRKY071-01*	I	0.3429	2.9142	0.118
PgWRKY057-01	*PgWRKY066-03*	IId	1.5078	3.0856	0.489
PgWRKY031	*PgWRKY075*	IIc	2.5019	3.0983	0.807
PgWRKY079-02	*PgWRKY053*	III	0.6561	2.9124	0.225
PgWRKY064-04	*PgWRKY051-01*	IIe	0.8981	3.0321	0.296
PgWRKY060-01	*PgWRKY044*	IIa	0.1556	1.7321	0.0898
PgWRKY069-02	*PgWRKY043-01*	IIb	2.6637	3.8128	0.698

3.5. GO (Gene Ontology) Functional Categorization Analysis of PgWRKYs

Of the 118 *PgWRKYs*, 65 (55.09%) genes were categorized into the three categories; 5 (4.24%) (*PgWRKY039-01*, *PgWRKY050-10*, *PgWRKY063-01*, *PgWRKY038-02*, and *PgWRKY053*) genes had functions in two of the three categories, while 30 (25.42%) were individual, functional, and category-specific in molecular function (MF) (Figure 4A; Table S4). A further 18 (15.25) genes were missing GO function annotations.

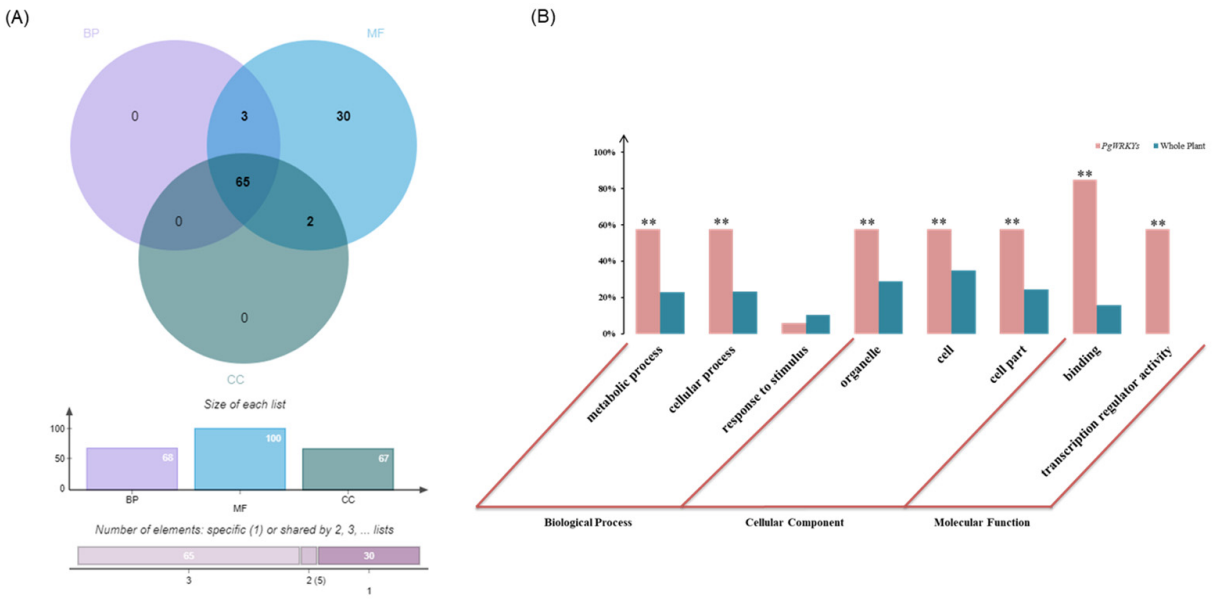

Figure 4. Functional categorization and GO term enrichment of the *PgWRKY* gene transcripts. (**A**) Venn diagram of the *PgWRKY* gene transcripts among the biological process (BP), molecular function (MF) and cellular component (CC) functional categories. (**B**) Subcategories (Level 2) into which the *PgWRKY* transcripts are categorized and GO enrichments. The GO terms of the transcripts expressed in 14 tissues of the four-year-old of ginseng used for identification of the *PgWRKY* genes as the background control for the enrichment analysis. "**" as significant at $p \leq 0.01$; "No asterisk" as not significant at $p \geq 0.05$.

In the GO enrichment analysis (Figure 4B), there were five GO terms in relation to biological processes (BP), including metabolic processes, cellular processes, and response to stimuli. This indicates their putative role in biological and abiotic stresses, growth and development, and biochemical processes. There were three GO terms in relation to cellular components (CC), including organelle, cell, and cell part, suggesting their role in the regulation of transcription machinery. There were also two GO terms in relation to molecular function (MF); binding and transcription regulator activity terms were significantly highlighted. Of these eight subcategories, all except response to stimuli were enriched in a number of the *PgWRKY* genes ($p \leq 0.01$).

All the *PgWRKYs* were categorized into eight subcategories, while the expression pattern of the *PgWRKYs* in 14 tissues of 4-year-old ginseng (Figure 5A), 4-year-old ginseng roots of 42 ginseng farmers' cultivars (Figure 5B), and 4 different-aged (5, 12, 18, and 25-year-old) roots (Figure 5C) in each subcategory (level 2) varied dramatically. These *PgWRKYs* were also categorized into the above mentioned eight subcategories, but the number of *PgWRKYs* categorized into eight subcategories varied substantially across different tissues, developmental ages, and farmers' cultivars. These results showed the functional differentiation of the *WRKY* gene family in ginseng and also confirmed *PgWRKY* genes' functional diversity across different tissues, developmental ages, and farmers' cultivars.

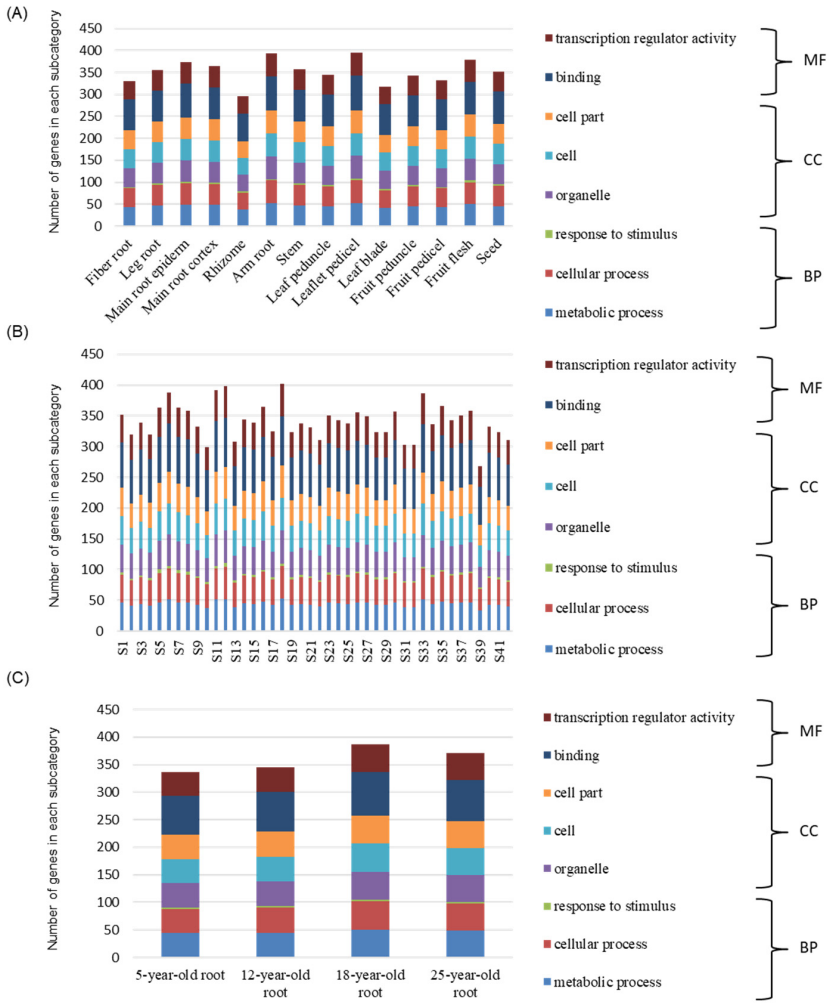

Figure 5. Variation of the functional categories of the *PgWRKY* genes by Gene Ontology (GO). (**A**) Variation of the functional categories of the *PgWRKY* genes among 14 tissues of four-year-old ginseng; (**B**) Variation of the functional categories of the *PgWRKY* genes among the 42 farmers' cultivars roots of ginseng; (**C**) Variation of the functional categories of the *PgWRKY* genes among different aged roots.

3.6. Expression Pattern and Characteristics Analysis of PgWRKYs

The expression profiles of 14 different tissues, 42 different farmers' cultivars, and 4 different-aged roots (Table S3) were used to perform the expression analysis. The genes expressed in one tissue were much more than those expressed in another two tissues (Figure 6A); the expression frequency of the total of 118 *PgWRKYs* reached 42% in 14 tissues, while in 1 tissue it reached 3%. Additionally, 8% of genes were expressed specifically at one of the four different-aged roots. The developmental stage-specific transcripts varied among the 5-, 25-, 18-, and 12-year-old roots. Of the total of 118 *PgWRKYs*, 57% expressed in four developmental stages, while another 15% did not express (Figure 6B); genes expressed in different tissues were much greater than those expressed at different-aged roots in ginseng.

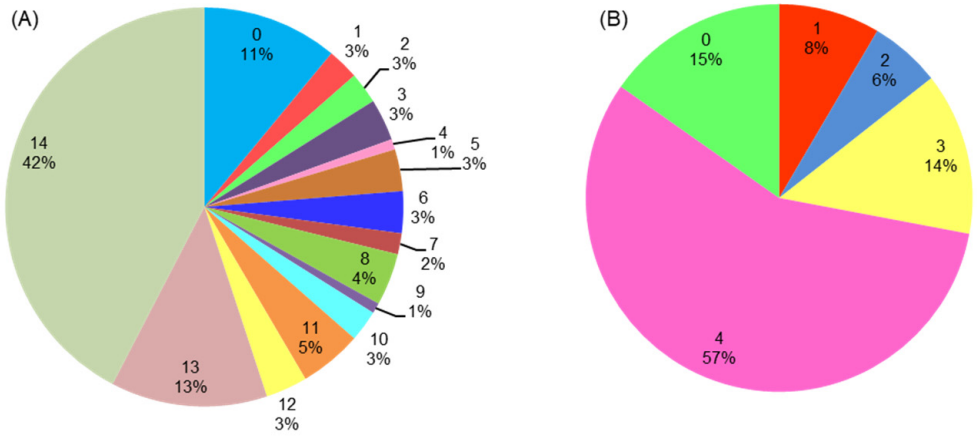

Figure 6. Expression of the *PgWRKY* in ginseng. (**A**) Expression of the *PgWRKY* in different tissues of ginseng. (**B**) Expression of the *PgWRKYs* in different 4 different-aged roots. The percentages of each part of the pie indicates the percentage of the 118 *PgWRKYs*; the number behind the percentage in each part of the pie indicates the number of (**A**) the 14 tissues (**B**) or the number of 4 year-olds roots.

We found that 118 *PgWRKY* genes had identical expression patterns across the 14 tissues, 4 different developmental stages, or 42 different farmers' cultivars, suggesting that they were co-regulated. With different tissues, 118 *PgWRKY* genes shared different expression patterns (Figure 7A): 35 members in groups I, IIb, IIc, and IIe were up-expressed in roots (arm root, fiber root, and leg root); 20 *PgWRKYs* in groups I, IIb, IId, and III did not express at any other tissue exclusively up-expressed in fruit dedicel; and 7 *PgWRKYs* belonging to group IId possessed the highest expression in stem.

The expression of the same gene shared a different expression pattern in different farmers' cultivars (Figure 7B); moreover, different genes may have shared similar expression profiles. A total of 118 *PgWRKY* genes was expressed in the four different-aged roots (Figure 7C). A total of 23 *PgWRKYs* involved in every group of 5-year-old roots shared higher expression. A further 11 *PgWRKYs* involved in every group except group IIb in 18-year-old roots possessed higher expression. Additionally, 28 *PgWRKYs* present in every group except for group IIa shared higher expression in 25-year-old roots.

WRKY TFs exhibited divergent expression patterns in different tissues; analyzing the spatial and temporal expression profiles of these genes will help us target specific tissues, organs, and developmental stages to perform genetic manipulation. Those genes with high expression in all tissues would be considered as the integral TFs in growth, developmental, and metabolic procedures.

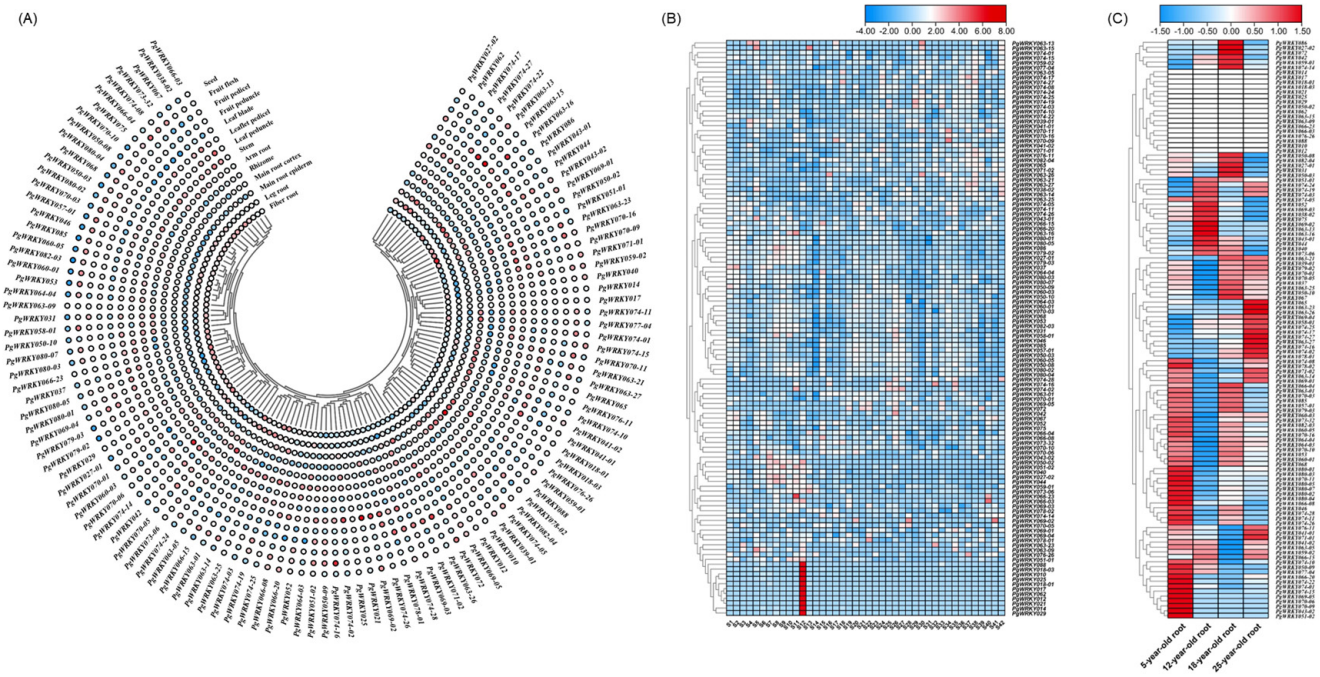

Figure 7. Expression heatmaps of the *PgWRKY* gene family transcripts in *Panax ginseng*. (**A**) The 118 *PgWRKY* genes expressed in the 14 tissue of 4 years old ginseng. (**B**) The 118 *PgWRKY* genes expressed in the 42 farmers' cultivars of ginseng. (**C**) The 118 *PgWRKY* genes expressed in the 4 different aged roots.

3.7. Co-Expression Network Analysis of PgWRKYs

The co-expression network of *PgWRKY* genes in the 4-year-old roots of 42 different farmers' cultivars was at a $p \leq 0.05$ (Figure 8). Consequently, 78 of the 118 *PgWRKY* genes formed a co-expression network that was composed of 78 nodes, 1276 edges (Figure 8A), and 7 clusters (Figure 8B) to form a network. The co-expression network of the *PgWRKY* genes, nodes, and edges was much more than that constructed from the 78 randomly selected unknown ginseng genes at all significance levels from $p \leq 0.05$ through $p \leq 1.0 \times 10^{-8}$ (Figure 8C,D). A statistical analysis confirmed that *PgWRKY* genes had a greater tendency and were more likely to form a co-expression network than randomly selected unknown ginseng transcripts (Figure 8E,F). *PgWRKY* genes were located in the network, rather than those randomly selected unknown genes, suggesting that the *WRKY* family forms a co-expression network and participates in regulating the growth, development, and stress response of *Panax ginseng*.

3.8. Response of PgWRKY Genes to Salt Stress in Ginseng Adventitious Roots

The evolutionary tree is similar in affinity and function. We plotted the evolutionary tree by downloading the nucleic acid sequences of WRKY transcription factors that have been validated to function in salt stress—*TaWRKY10* [38], *DgWRKY3* [39], *NbWRKY79* [40], *GmWRKY12* [41], and *GmWRKY45* [42] genes—and plotting these sequences against *PgWRKY* gene transcripts (Figure 9A). Finally, we obtained *PgWRKY39-01*, *PgWRKY062*, *PgWRKY065*, and three *PgWRKY* genes from ginseng. The expression of *PgWRKY062* and *PgWRKY065* genes was significantly increased at 90 mM NaCl compared with the control, and the expression of the *PgWRKY39-01* gene was significantly increased at 80 mM NaCl compared with the control (Figure 9B). The expression of the *PgWRKY39-01* gene was significantly higher in 80 mM, 90 mM, and 100 mM NaCl than the control group (Figure 9B). These results suggest that the three *PgWRKY* genes (*PgWRKY39-01*, *PgWRKY062*, and *PgWRKY065*) play important roles in the resistance to salt stress in ginseng.

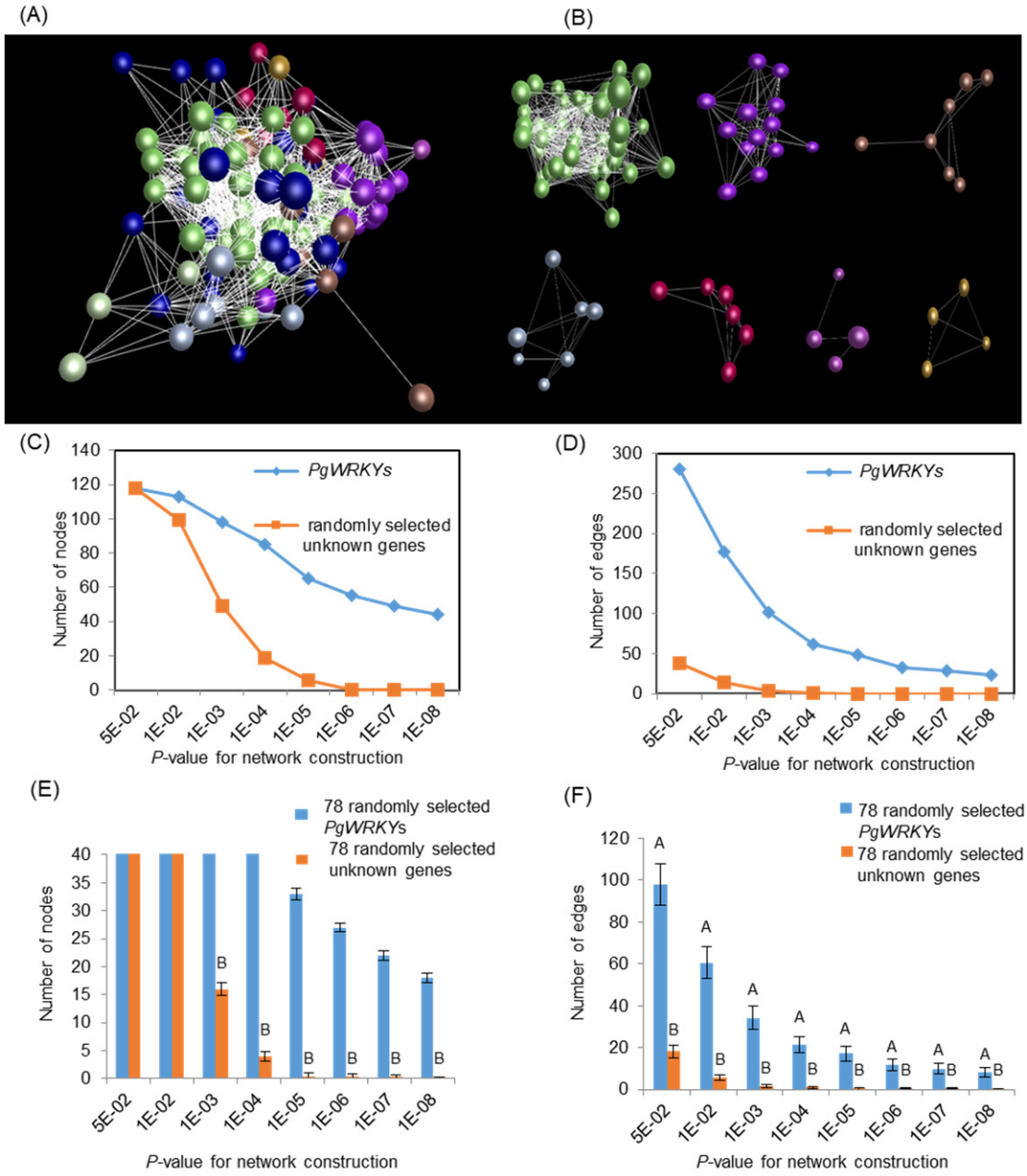

Figure 8. Network analysis of the *PgWRKY genes* expressed in the 4-year-old roots of 42 farmers' cultivars. (**A**) The co-expression network constructed from the 118 *PgWRKYs*. The network was constructed at $p \leq 0.05$. (**B**) The three clusters constituting the network. Different clusters are indicated by different colors. (**C**) Tendency that these *PgWRKYs* form a network, with the randomly-selected ginseng unknown genes as controls: variation in number of nodes. (**D**) Tendency that these *PgWRKYs* form a network, with the randomly-selected ginseng unknown genes as controls: variation in number of edges. (**E**) Statistical analysis of variation in number of nodes in the network. (**F**) Statistical analysis of variation in number of edges in the network. Different capital letters, significant at $p \leq 0.01$. Error bar, standard deviation for 20 replications.

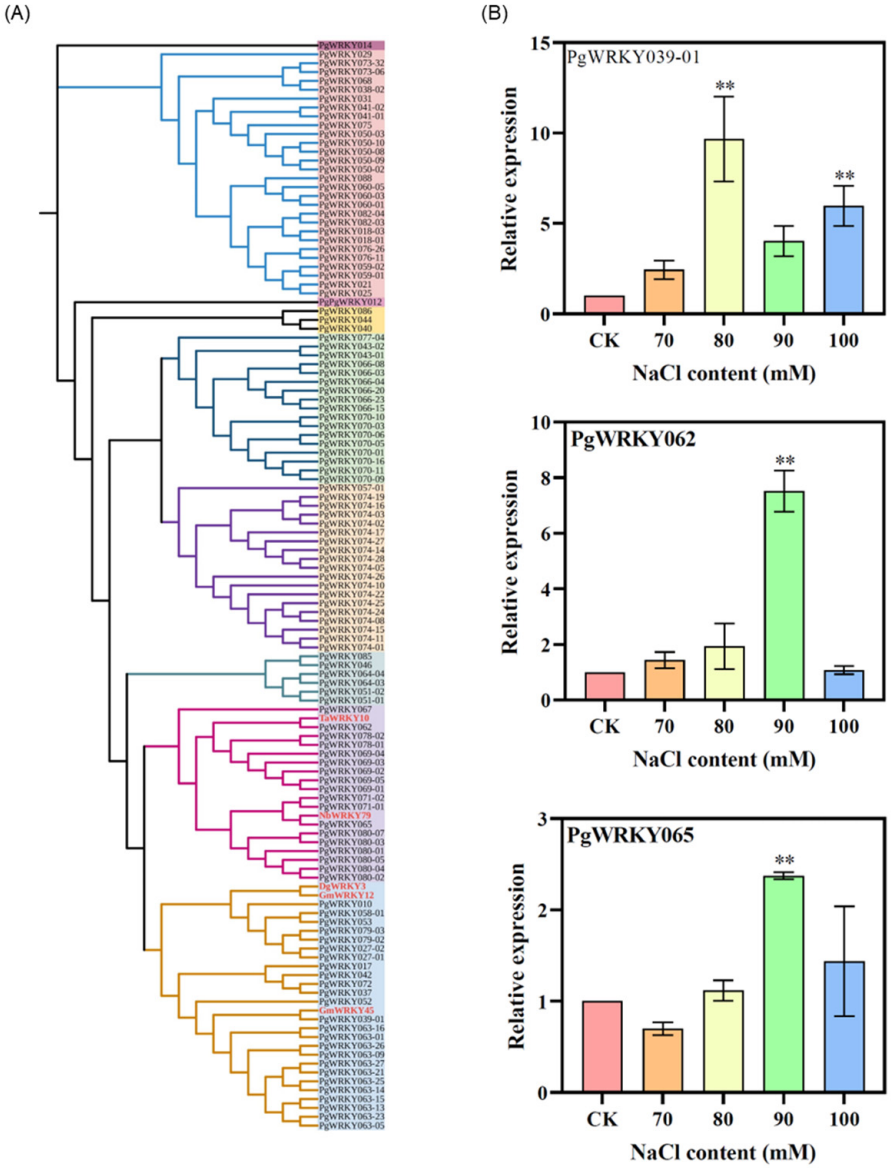

Figure 9. The expressions analysis of *PgWRKY39-01, PgWRKY062, and PgWRKY065* genes in the ginseng adventitious roots treated with salt stresses using the RT-qPCR. (**A**) The evolutionary tree of *PgWRKY* genes with five *WRKY* genes known to have salt stress function, included *TaWRKY10*, *DgWRKY3*, *NbWRKY79*, *GmWRKY12*, and *GmWRKY45* genes. (**B**) The expression of *PgWRKY39-01*, *PgWRKY062*, and *PgWRKY065* genes in the ginseng adventitious roots treated with and without salt stresses. The $2^{-\Delta\Delta Ct}$ method was used to evaluate the relative expression, and the expression levels of genes in the control were defined as "1". Each value is the average of three replicates, and error bars represent ± SD. "**" Significant at $p \leq 0.01$; "*" significant at $p \leq 0.05$; the remaining is not significant at $p \geq 0.05$.

4. Discussion

Panax ginseng is one of the most important species in traditional Chinese medicine (TCM). The recently published genome sequence of ginseng [43] provides a great opportunity for the study of medicinal chemistry, genetics, and genomics, although there is still much that needs to be done. There are few scaffolds included in the genome map draft. Transcriptome sequencing provides more useful information [28].

Transcription factors (TFs) have been considered as the best candidate genes for the manipulation of complex traits in crop plants [44]. More and more research has raised interest in the *WRKYs* in ginseng. In 2015, 48 *WRKY* transcripts from methyl jasmonate

(MeJA)-treated adventitious root were identified and cloned to one of the transcripts known as *PgWRKY1.* The expression of *PgWRKY1* was significantly induced by salicylic acid, abscisic acid, and NaCl, but down-regulated by the MeJA treatment [29]. Soon after, in 2016, eight WRKY genes known as *PgWRKY2-9* from NGS-based transcriptome sequencing were cloned and characterized by Xiu et al. (2016) [30]. All of these works show us that *PgWRKYs* should be multiple stress-inducible genes responding to salt stress and other biotic and abiotic stresses, as well as growth and development.

A total of 118 *PgWRKYs* with a complete ORF was identified; all members in ginseng possess the WRKY domain with the WRKYGQK core sequence. There are currently many members of the *WRKY* TF gene family in plants that have been reported, including *Arabidopsis thaliana* [45], *Oryza sativa* [46], *Cucumis sativus* [47], *Brachypodium distachyon* [48], *Populus trichocarpa* [49], *Panax quinquefolius* [50], *Gossypium aridum* [51], *Lotus japonicus* [52], mulberry [53], *Brassica napus* [23], and *Daucus carota* [54] (see Table 2 for the role they play in plants). More and more members of this large family will be demonstrated and characterized in the future. In this study, we found that compared with the number of WRKY gene family members in other plants, the number of WRKY sequences in ginseng is significantly higher. Only the number of WRKY members in rapeseed [23] is higher than in ginseng. This indicates that the WRKY gene family of ginseng is relatively large and has more special regulatory functions.

A phylogenetic analysis using *Panax ginseng*, *Arabidopsis thaliana*, *Oryza sativa*, and *Panax quinquefolius* (Table S5) revealed that most sub-group members were contained in all the studied species. Group of most *PgWRKYs* followed a distribution pattern similar to other plant species except for *PgWRKY25;* they belong to group I, and while they were not allocated to the group I cluster, they were instead clustered to groups III, IIb, and IIe. This may be the reason behind those that originated the earliest and were the most divergent in group I (Figure 1; Table S3).

Genome duplication events result in gene expansion and often lead to functional divergence. To analyze the genome duplication of *PgWRKY* genes, we mapped the *PgWRKYs* onto each scaffold based on publicly available ginseng genome data. The results showed that the duplication events of the *WRKY* gene display different spatial patterns in ginseng (Figure 7). A total of 56 *PgWRKYs* was mapped onto 49 scaffolds (Table S6). Eight *PgWRKY* genes were identified based on phylogenetic relationships, gene structures, and conserve motifs (Table 2). The *Ka*/*Ks* substitutions were calculated to verify Darwinian position selection. The *Ka/Ks* ratios were all lower than 1.0 at *PgWRKY* paralogous pairs, indicating that the *PgWRKY* family had undergone purifying selection pressure. Evolutionary processes such as duplication could be extended to gene family members. On the other hand, mutations in CDS regions and upstream/downstream sites can affect the regulatory and expression system of members of a gene family and lead to a diversity of expression [55,56].

GO is widely used to standardize gene function classification at transcriptome analysis [57]. Our study shows that *PgWRKY* gene family members share a divergent function. All of these eight GO items were allocated to three main functions—cell components (CC), molecular functions (MF), and biological processes (BP) (Table S6)—following the cells, metabolic process, response to stimuli, biological regulation, binding, nucleic acid binding transcription factor activity, and other functions. Using a ginseng database of 248,993 transcripts as the control, we performed a level 2 enrichment analysis based on the GO annotation; all sub-components of these eight sub-components comprised of the 248,993 equivalent level had significant differences (Figure 4B). Among them, the cellular process, metabolic process' regulation of the biological process, biological regulation, nucleic acid binding transcription factor activity, and binding were much larger than the expected 248,993 control. We found that 84.7% of the members of the WRKY gene family of ginseng are annotated to the functions of metabolism, cellular processes, and response to stimulation mechanisms under the functions of biological processes, and 57.6% of the sequences are annotated to the functions of transcriptional activity regulation. At the same

time, they also have similar functions in *Arabidopsis* [57] and rice [58], which indicates that WRKY transcription factors can perform similar functions among different species.

The function of WRKY mainly concerns the biological process and abiotic stress, as well as occasional organ development in plants. Plants work against the stress by activating secondary metabolic pathways to synthesize the secondary metabolite, e.g., terpenoid and phenolics provide a reference for the analysis of the molecular function of plants, especially the majority of medicinal plants, on the basis of molecular biology. Therefore, we analyzed the response of *PgWRKY39-01*, *PgWRKY062*, and *PgWRKY064* genes to salt stress treatment using RT-qPCR. The expression of three genes had an upward trend after different salt concentrations treatment, indicating that some PgWRKY transcription factors members in ginseng were involved in responses to salt stresses, which supported their roles in abiotic stresses, growth, and development. However, the mechanism behind the *PgWRKY* genes' participation in abiotic stress responses needs to be further studied in the future.

5. Conclusions

In this study, transcriptome-wide identification, a phylogenetic analysis, structural variation, functional differentiation, and a co-expression analysis of *WRKY* genes in *Panax ginseng* provided us with functional gene resources and dates in ginseng. All of these factors will help us to precisely understand the role of *PgWRKY* genes, especially their role in the synthesis of pharmaceutical activity ingredients. This study further demonstrates that the *WRKY* gene family members of ginseng can respond to salt stresses, and it provides functional gene resources to ginseng gene breeding and improving ginseng quality in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8090756/s1, Table S1: Expressions of *PgWRKY* gene transcripts in 14 tissues, 42 cultivar roots, and 4 aged roots (TPM); Table S2: Nucleic acid sequences and protein sequences of *PgWRKY* gene transcripts; Table S3: Physical and chemical properties of the *PgWRKY* gene family; Table S4: Classification, annotation, and GO functional categorization of the *PgWRKY* gene transcripts; Table S5: Identified genes used as evolutionary controls for the *PgWRKY* gene phylogenetic analysis; Table S6: *PgWRKY* gene transcript mapping onto the scaffolds of ginseng genome sequences.

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