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Genome-wide identification of *WRKY* gene family and expression analysis under abiotic stresses in *Andrographis paniculata*

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Abstract: *Andrographis paniculata* (*A. paniculata*) is a Chinese herbal medicine that clears away heat, reduces inflammation, protects the liver, and promotes choleretics. The WRKYs of *A. paniculata* are still not well characterized, although many WRKYs have been identified in various plant species. In the present study, 59 *A. paniculata WRKY* (*ApWRKY*) genes were identified and renamed on the basis of their respective chromosome distribution. These *ApWRKYs* were divided into three groups via phylogenetic analysis according to their WRKY domains and combined with WRKY of *Arabidopsis*. The 59 identified *ApWRKY* transcription factors were non-uniformity distributed on 23 chromosomes of *A. paniculata*. From the structural analysis of the conserved motifs, different *ApWRKYs* structures showed different biological functions, and the *ApWRKY* transcription factor had certain species-specificity in the evolutionary process. The expression patterns of the 41 *ApWRKYs* were examined through quantitative real-time PCR (qRT-PCR) in various tissues and under abiotic stresses (salt). The results showed that most of the *ApWRKY* had different reactions to salt treatment. In addition, the content of the four main secondary metabolites in *A. paniculata* leaves was determined under salt stress. The results show that under a low concentration of salt treatment, the synthesis of andrographolide can be improved.

Introduction

Andrographis paniculata (Burm. f.) Nees is an herbaceous plant, which is commonly known as the 'King of Bitters' in the Acanthaceae family. *A. paniculata* is wildly distributed in Southern Asia (Dai *et al.*, 2019) and widely distributed in Southern China. In China, *A. paniculata*, also known as Chuanxinlian in Chinese, is used for medicine for the dry ground part. Andrographolide, a natural diterpene lactone, is the main active compound distributed in *A. paniculata* (Zhang *et al.*, 2019). It contains the active phytochemicals from the aerial parts (leaves and stems), such as diterpenoids and 2'-oxygenated flavonoids, including andrographolide (AP), neoandrographolide (NAP), 14-deoxy-11,12didehydroandrographolide (DHAP), 14-deoxyandrographolide (DOAP), isoandrographolide, homoandrographolide, rographolidegraphan, andrographosterin, and stigmasterol (Chao and Lin, 2010).

In *Arabidopsis*, WRKY is almost as complicated as the well-known transcription factor families such as myeloblastosis (MYB) and NAC. Transcription factors can be involved in regulating various developmental and physiological processes of plants by directly or indirectly binding cis-acting elements of genes involved in signal transduction process of stress.

Abiotic stress responses and gene regulation have been studied in a number of plant species, including *Arabidopsis*, rice, maize, and tomato. Several families of genes are particularly associated with significant improvements in abiotic stress tolerance, including the WRKY, NAC, and ethylene response factor (ERF) gene families (Shen *et al.*, 2017, Huang *et al.*, 2015, Fan *et al.*, 2016). Previous studies have demonstrated that WRKY genes are expressed strongly and rapidly in response to particular abiotic stresses, including wounding, waterlogging, drought, and salt stress (Madhunita and Ralf, 2014). As the seventh largest transcription factor family in higher plants, *WRKY* is generally involved in various biological processes, including plant growth and development as well as biological and

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abiotic stress responses (Zhang and Wang, 2005). Therefore, the analysis of the function of the WRKY transcription factor has always been one of the hotspots in the study of plant functional genes. SPF1 protein isolated from sweet potato was the first WRKY protein discovered (Ishiguro and Nakamura, 1994). Subsequently, researchers detected numerous WRKY family members in crops, including *Arabidopsis*, rapeseed, and rice, among which 74 and 126 WRKY family members were detected in *Arabidopsis* and rice, respectively (Berri *et al*., 2009).

The genome size of *A. paniculata* is 269 Mb, gene annotation predicted 25,428 protein-coding genes. Full genome sequencing of *A. paniculata* was completed in 2019 (Sun *et al*., 2019). However, to date, no genome-wide characterization of the WRKY family has been conducted in *A. paniculata*. So, it is of the utmost interest to carry out a genome-wide survey of this gene family in *A. paniculata*. This study focused on the whole genome-wide identification and expression analyses of WRKY in *A. paniculata* under abiotic stresses. The phylogenetic relationship of the WRKY proteins was investigated according to the WRKY information from *Arabidopsis* and rice. The genomic structure, chromosome localization, conserved domain, gene structure, and other structural features were also explored. However, 41 *WRKY* genes were selected for analyzing their expression patterns in response to abiotic stresses using quantitative real-time PCR. The objective of this study was to establish a solid foundation for understanding the regulatory mechanism of *WRKY* genes and to explore new strategies for the improvement in *A. paniculata*.

Materials and Methods

Identification and sequence analysis of WKRY genes in A. paniculata

The *A. paniculata* genome sequences were downloaded from a database (Sun *et al*., 2019). The *WRKY* sequence data of *Arabidopsis* were obtained from TAIR release 10 http://plants.ensembl.org/Arabidopsis_thaliana/Info/Index. A Hidden Markov Model (HMM) search was performed against the *A. paniculata* protein database using the WRKY-domain PF03106 http://hmmer.org/ An e-value ≤ $1.1e^{-25}$ was used as the criterion, The primary candidate sequences were submitted in the Pfam database http://pfam.xfam.org and NCBI Conserved Domains Database https://www.ncbi.nlm.nih.gov/cdd for verification. Finally, 59 WRKY proteins were screened out in the *A. paniculata* genome (Tab. 1). The isoelectric point and protein molecular weight of WRKY proteins in *A. paniculata* were predicted using the pI/Mw tool in ExPASy proteomics server http://web.expasy.org/protparam.

Multiple sequence alignment and phylogenetic analysis

A ClustalW analysis was used with the default option to implement the multiple sequence alignment on the WRKY domain sequences of the *WRKY* genes from two species, namely, *A. paniculata* and *Arabidopsis*. A Maximum Likelihood (ML) phylogenetic tree in MEGA X with the following parameters: Use all sites and bootstrap analysis with 1000 replicates for the reliability of interior branches (Kumar *et al*., 2018). The sequences for phylogenetic trees are shown in Suppl. Tab. S1.

Chromosomal locations of the ApWRKY genes

The location of *ApWRKY* was obtained by using Perl script to extract mRNA locations from *A. paniculata* GFF files, and Mapchart2.32 software was used to map to 24 chromosomes in the *A. paniculata* genome (Voorrips, 2002).

Protein properties and sequence analysis

Fifty-nine ApWRKYs were analyzed by MEME online software for conversed motif prediction with the following criteria (http://meme-suite.org/tools/meme): Select the site distribution: Zoops, 20 motifs with an optimum motif width between 10 and 50 residues, and any number of repetitions. The location information of CDS and UTR in the mRNA of *ApWRKYs* was screened from the GFF file of the whole genome of *A. paniculata*, and the gene structure was identified.

Plant materials and abiotic stress treatments

The medicinal plant *A. paniculata* was obtained from Zhejiang Province, China (30.26N,120.21E.) The plant material used in this study was formally identified by Dr. Ling Xu (Zhejiang Sci-Tech University, Hangzhou, China) and Prof. Zongsuo Liang (Zhejiang Sci-Tech University, Hangzhou, China). *A. paniculata* seeds were sown in plastic pots (2:1:1 ratio) of nutritive soil, perlite, and vermiculite, and are grown in normal sunlight at an average temperature of 30°C. Three-month-old plants were employed for the abiotic stress treatments, as previously described (Talei *et al*., 2013). A total of 9 pots were cultivated, one plant for each pot, including 3 pots in the control group, 3 pots treated with 100 mL 50 mmol/L NaCl, and 3 pots treated with 100 mL 100 mmol/L NaCl. After 9 days of treatment, the roots, stems, and leaves were sampled. Samples were snap-frozen in liquid nitrogen and stored at −80°C until they were employed for total RNA extraction. All experiments were repeated three times.

Total RNA extraction and cDNA synthesis

RNA was extracted using the TIANGEN kit, for tissue-specific expression, RNA was extracted from different tissues, including the roots, stems, and leaves. RNA integrity was examined through agarose gel electrophoresis, and RNA purity was determined based on the OD_{260}/OD_{280} and OD_{260}/OD_{230} nm ratios. The RNA extracted was reversely transcribed into cDNA using the TaKaRa (Dalian, China) reverse transcription kit. The cDNA was then diluted 10-fold to be employed as a template for qRT-PCR analysis.

Transcriptional analysis by real-time quantitative PCR (RT-qPCR)

RT-qPCRs were performed using the ChamQ Universal SYBR qPCR Master Mix (Vazyme, China) and carried out in triplicate for each tissue sample. Gene-specific primers (Suppl. Tab. S2) were designed using Real-time PCR (TaqMan) Primer and Probes Design Tool https://www.genscript.com/tools/real-time-pcr-taqman-primer-design-tool. The length of amplicons is between 150 bp and 200 bp. ApUBC was selected as a reference gene as described

TABLE 1

Sequence features of *ApWRKYs* in *A. paniculata*

Name	Gene ID	AA Len	Linkage	pl	MW (Da)	WRKY domain	Group	Arabidopsis
ApWRKY1	CXN00000500-RA	256	chr12	9.71	28885.4	WRKYGQK	IIa	AtWRKY18
ApWRKY2	CXN00000896-RA	205	chr17	4.81	23163.7	WRKYGKK	IIc	AtWRKY50
ApWRKY3	CXN00001046-RA	278	chr17	10.52	29800.8	WRKYGQK	IId	AtWRKY21
ApWRKY4	CXN00001084-RA	275	chr17	6.6	30737.8	WRKYGQK	IIa	AtWRKY60
ApWRKY5	CXN00001534-RA	301	chr22	5.48	34401	WRKYGQK	III	AtWRKY70
ApWRKY6	CXN00001535-RA	233	chr22	5.59	25803.2	WRKYGQK	III	AtWRKY70
ApWRKY7	CXN00002456-RA	236	chr8	9.07	25502.5	WRKYGQK	IIc	AtWRKY71
ApWRKY8	CXN00002475-RA	283	chr8	4.26	31445.2	WRKYGQK	IIe	AtWRKY69
ApWRKY9	CXN00002611-RA	495	chr8	4.98	53047.7	WRKYGQK	I	AtWRKY33
ApWRKY10	CXN00002815-RA	290	chr10	4.62	31756.4	WRKYGQK	IIe	AtWRKY69
ApWRKY11	CXN00003233-RA	308	chr4	8.35	34533.3	WRKYGQK	IIc	AtWRKY13
ApWRKY12	CXN00003789-RA	217	chr7	8.2	24221.2	WRKYGQK	IIc	AtWRKY12
ApWRKY13	CXN00004229-RA	326	chr19	5.12	35518.7	WRKYGQK	IIc	AtWRKY57
ApWRKY14	CXN00004661-RA	424	chr16	9.77	46590.1	WRKYGQK	III	AtWRKY30
ApWRKY15	CXN00004894-RA	285	chr11	4.41	32742.5	WRKYGQK	IIe	AtWRKY65
ApWRKY16	CXN00004902-RA	402	chr11	6.36	44091	WRKYGQK	IIc	AtWRKY23
ApWRKY17	CXN00005372-RA	478	chr7	7.1	52295.6	WRKYGQKx2	I	AtWRKY44
ApWRKY18	CXN00005788-RA	447	chr10	6.86	48108.4	WRKYGQK	IIb	AtWRKY36
ApWRKY19	CXN00005815-RA	345	chr13	6.05	38499.4	WRKYGQK	III	AtWRKY55
ApWRKY20	CXN00006953-RA	329	chr15	9.29	35608.6	WRKYGQK	IId	AtWRKY15
ApWRKY21	CXN00007657-RA	321	chr18	4.99	35232.2	WRKYGQK	IIc	AtWRKY12
ApWRKY22	CXN00008448-RA	308	chr15	9.32	33255	WRKYGQK	I	AtWRKY44
ApWRKY23	CXN00009022-RA	314	chr9	10.02	35522.9	WRKYGQK	IIa	AtWRKY18
ApWRKY24	CXN00009478-RA	489	chr1	7.72	54161.7	WRKYGQKx2	I	AtWRKY20
ApWRKY25	CXN00009767-RA	314	chr2	6.7	34614.6	WRKYGQK	IIc	AtWRKY8
ApWRKY26	CXN00010773-RA	210	chr1	11.47	24397.2	WRKYGQK	IIc	AtWRKY71
ApWRKY27	CXN00010935-RA	451	chr10	5	48820.8	WRKYGQK	IIe	AtWRKY27
ApWRKY28	CXN00011208-RA	478	chr20	7.68	52892	WRKYGQK	IIb	AtWRKY61
ApWRKY29	CXN00011338-RA	231	chr2	8.71	27049.5	WRKYGQK	IIc	AtWRKY45
ApWRKY30	CXN00012073-RA	403	chr14	6.8	44988.5	WRKYGQK	I	AtWRKY49
ApWRKY31	CXN00012169-RA	703	chr14	7.97	77465.7	WRKYGQKx2	I	AtWRKY32
ApWRKY32	CXN00013114-RA	403	chr6	7.13	43101.4	WRKYGQK	I	AtWRKY33
ApWRKY33	CXN00014018-RA	468	chr8	6.78	51339.3	WRKYGQK	IIb	AtWRKY9
ApWRKY34	CXN00014261-RA	285	chr20	6.92	32280.7	WRKYGQK	III	AtWRKY70
ApWRKY35	CXN00014703-RA	420	chr14	8.31	47395.8	WRKYGQKx2	I	AtWRKY26
ApWRKY36	CXN00015346-RA	419	chr23	10.58	47256.7	WRKYGQK	IId	AtWRKY21
ApWRKY37	CXN00015731-RA	298	chr7	4.64	33142.4	WRKYGQK	III	AtWRKY55
ApWRKY38	CXN00016370-RA	349	chr16	10.2	38688.8	WRKYGQK	IId	AtWRKY21
ApWRKY39	CXN00016482-RA	80	chr24	8.7	8969.1	WRKYGQK	IId	AtWRKY15
ApWRKY40	CXN00016532-RA	456	chr2	5.16	48509.7	WRKYGQK	IIe	AtWRKY35
ApWRKY41	CXN00017907-RA	440	chr2	9.31	47525	WRKYGQK	IIb	AtWRKY72
ApWRKY42	CXN00018131-RA	294	chr3	6.1	32865.1	WRKYGQK	IIe	AtWRKY27
ApWRKY43	CXN00018343-RA	283	chr21	10.06	31285.7	WRKYGQK	IIa	AtWRKY18
ApWRKY44	CXN00019292-RA	301	chr17	5.5	34449.9	WRKYGQK	III	AtWRKY70

(Continued)

Table 1 (continued).

Name	Gene ID	AA Len	Linkage	pI	MW (Da)	WRKY domain	Group	Arabidopsis
ApWRKY45	CXN00019385-RA	299	chr1	8.74	33346.5	WRKYGQK	IIb	AtWRKY36
ApWRKY46	CXN00019605-RA	309	chr1	7.33	33265.1	WRKYGQK	I	AtWRKY26
ApWRKY47	CXN00019899-RA	173	chr13	11.64	20064.1	WRKYGKK	IIc	AtWRKY51
ApWRKY48	CXN00020137-RA	654	chr2	7.46	72013	WRKYGQK	IIc	AtWRKY71
ApWRKY49	CXN00020327-RA	531	chr10	8.02	57317.2	WRKYGQK	IIb	AtWRKY36
ApWRKY50	CXN00020882-RA	258	chr3	7.12	28918.8	WRKYGQK	IIa	AtWRKY60
ApWRKY51	CXN00021127-RA	375	chr17	6.73	42143.3	WRKYGQK	IIe	AtWRKY27
ApWRKY52	CXN00021336-RA	471	chr1	7.41	49786.1	WRKYGQK	IIb	AtWRKY47
ApWRKY53	CXN00021801-RA	324	chr3	10.09	35029.2	WRKYGQK	IId	AtWRKY11 AtWRKY17
ApWRKY54	CXN00022088-RA	189	chr19	8.62	20928	WRKYGQK	IIc	AtWRKY75
ApWRKY55	CXN00022391-RA	387	tig00000239	6.83	42696.9	WRKYGQK	IIc	AtWRKY48
ApWRKY56	CXN00022686-RA	316	tig00005375	5.14	35816.5	WRKYGQK	III	AtWRKY46
ApWRKY57	CXN00023451-RA	477	chr17	8.32	51101.1	WRKYGQKx2	I	AtWRKY1
ApWRKY58	CXN00023860-RA	397	chr2	7.7	43240.7	WRKYGQK	IIb	AtWRKY61
ApWRKY59	CXN00024805-RA	465	chr11	6	51220.9	WRKYGQK	I	AtWRKY32

Note: pI proteins' isoelectric point, MW molecular weight.

previously (Li *et al*., 2013). We selected 59 genes for RT-qPCR experiments, of which 18 genes were expressed very low. So, we used the remaining 41 genes for mapping. Three independent biological replicates were performed. The obtained cDNA was used as a template for the RT-qPCR analysis using the QuantStudio TM Flex6 System (ABI, Alexandria, America). Briefly, standardization of gene expression data was performed from three biological replicates as described. The $2^{-\Delta\Delta Ct}$ method was used to achieve results for relative quantification. For statistical analysis, analysis of variance (ANOVA) was done calculated using SPSS (Version 20.0, IBM, USA).

Metabolite extraction and HPLC analysis

The whole plant was treated with NaCl, and the contents of andrographolide (AP, NAP, DHAP and DOAP) under different salt stress concentrations were determined by HPLC (Fig. 7). The extraction and analysis of secondary metabolites were carried out according to the following methods (Bindu *et al*., 2020), and improvements were made on this basis. Four diterpenoid lactones (AP, NAP, DHAP and DOAP) were investigated. Preparation of test solution: 0.2 g of *A. paniculata* powder (passed through a 60-mesh sieve) was accurately weighed, placed in a stoppered Erlenmeyer flask, filled with 10 mL of methanol, tightly stoppered, weighed, immersed for 30 min, and then ultrasonically extracted for 30 min, when the solution temperature reaches room temperature, make up the weight with methanol, centrifuge, take the supernatant, pass through a 0.22 μm filter membrane, and continue the filtrate as the test solution. Waters HPLC system (Milford, MA, USA) contained a 1525 binary pump, an automatic sample injector, and a Waters 2998 photodiode array detector (PDA). HPLC separation was performed with a SunFire C18 column (4.6 mm × 250 mm, 5 μm particle size) at 30°C. Empower 3 software (Milford, MA, USA) was used for data acquisition and analysis. The sample injection volume was 20 μL; the PDA detection wavelength for the diterpenoids was 255 nm. Separation was achieved by elution using a linear gradient with solvent-A (acetonitrile) and solvent-B (0.4% phosphoric acid solution) gradient elution: 0–35 min, 5–45% A(v/v), 35–60 min, 45–52% A (v/v), 60–62 min, 52–5% A (v/v) flow rate: 1 mL/min. Standards of secondary metabolite compounds were purchased from Shanghai Bio-Technology Co., Ltd., Shanghai, China.

Results

Identification and classification of WRKY gene family of A. paniculata

In total, 67 *WRKY* genes were predicted protein sequences without a WRKY domain were excluded. After comparison to a Hidden Markov Model (HMM) search using the WRKY-domain PF03106, a total of 59 WRKY proteins were identified in the *A. paniculata* genome. The 59 *A. paniculata* WRKY proteins ranged from 80 (ApWRKY39) to 703 (ApWRKY31) amino acid (aa) in length, with an average length of approximately 352 aa. The molecular weights (MWs) ranged from 8969.9 Da (ApWRKY39) to 77465.7 Da (ApWRKY31). The isoelectric points (pIs) of the WKRY proteins ranged from 4.26 (ApWRKY8) to 11.64 (ApWRKY47), with 26 pIs <7 and the remaining pIs >7 (Tab. 1). Similar observations were made in sesame (Li *et al*., 2017), which showed 71 SiWRKYs with pIs ranging from 4.81 to 9.74 and MWs ranging from 14.44 kDa to 125.94 kDa. Total 59 ApWRKY proteins contained one or two identical WRKYGQK domains (Tab. 1). Although the WRKYGQK domain is highly conserved in WRKY protein, ApWRKY2 and ApWRKY47 differ from other ApWRKYs in that glutamine residues are replaced by lysine residues

(Meng *et al*., 2016, Eulgem *et al*., 2000, He *et al*., 2012). This change was also observed in *Arabidopsis* and another plant WRKY family.

Phylogenetic analysis of the ApWRKY genes

To further analyze the evolution of these ApWRKYs, we constructed a phylogenetic tree based on a total of 131 WRKYs (72 from *A. thaliana* and 59 from *A. paniculata* distributed in different groups) (Fig. 1).

Combined with the classification of *Arabidopsis* WRKY transcription factors, the conserved domains of candidate ApWRKY were identified and analyzed. The results showed that WRKY family protein sequences of *A. paniculata* can also be divided into three Groups I, II, and III, of which there are 11 ApWRKYs domains in Group I, and five *ApWRKYs* (*ApWRKY17, ApWRKY24, ApWRKY31, ApWRKY35, ApWRKY57*) have two WRKYGQK conserved domain structures (Figs. 1 and 2; Tab. 1). Forty ApWRKYs were distributed in Group II, which was further classified into five subgroups: IIa, IIb, IIc, IId and IIe, which contained 5, 8, 14, 6 and 7 ApWRKYs, respectively. The remaining 8 ApWRKYs belonged to Group III. Overall results indicated that different WRKY subgroups contained different evolutionary rates.

In this study, ApWRKYs transcription factors could be divided into three groups, among which the number of Group II could be further subdivided into five subgroups at most. By analyzing the structural characteristics of the WRKY domain, it was found that most ApWRKY proteins in Group I had two WRKYGQK conserved domains without variation. However, it was found in Group IIc that the WRKYGQK conserved domain of ApWRKY2 and ApWRKY47 were mutated from 'Q' to 'K' (WRKYGKK) (Fig. 2) (Chanwala *et al*., 2020, Qin *et al*., 2020). There is one WRKYGQK conservative domain for each ApWRKY in Group III.

Distribution of ApWRKY genes from A. paniculata chromosomes

Except that two WRKY genes *ApWRKY55* and *ApWRKY56* could not be located on any of the chromosomes, namely *ApWRKY55* and *ApWRKY56*, the others distributed on chromosomes 1 to 24 (Fig. 3, Tab. 1). Chr2 and Ch17 contained the greatest number of *A. paniculata* 6 *ApWRKY* genes, whereas Chr4, Chr6, Chr9, Chr12, Chr18, Chr21, and Chr24 contained only one gene each. The other chromosomes varied from 2 to 5 genes. The analysis showed that both *ApWRKY15* and *ApWRKY16* were on Chr11, with a distance difference of 30403 bases. Similarly, both *ApWRKY5* and *ApWRKY6* were on Chr22, with a distance difference of 6,166 bases. The preliminary judgment was the tandem repeat between genes, which needs further analysis (Fig. 3).

Motif composition and gene structure of A. paniculata WRKY gene families

The structure diagram of ApWRKY protein was constructed based on the results of MEME motif analysis. Among them,

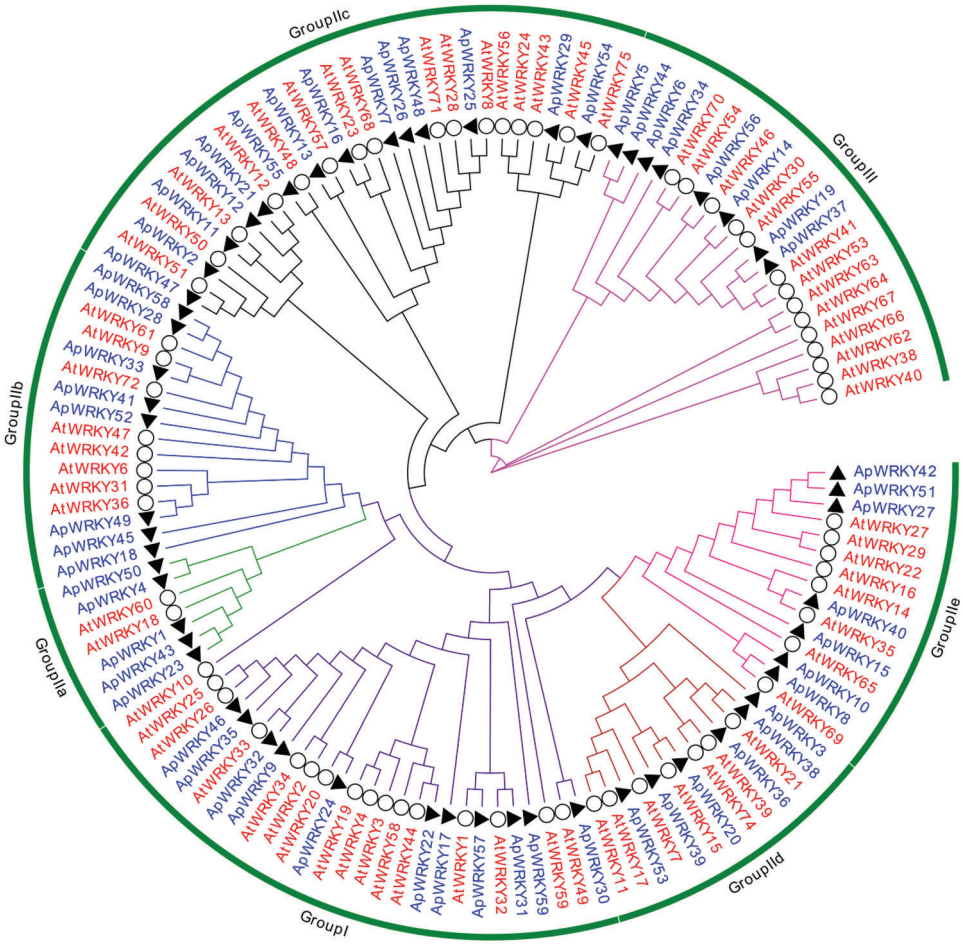

FIGURE 1. Phylogenetic analysis of WRKYs proteins from *A. paniculata* and *A. thaliana*.
The tree was constructed from amino sequences using MEGA X via the Maximum Likelihood (ML) method with 1000 bootstrap replicated. The empty circles, black triangles represent WRKYs from *A. paniculata* and *A. thaliana*, respectively. Clades with different colors represent diverse groups.

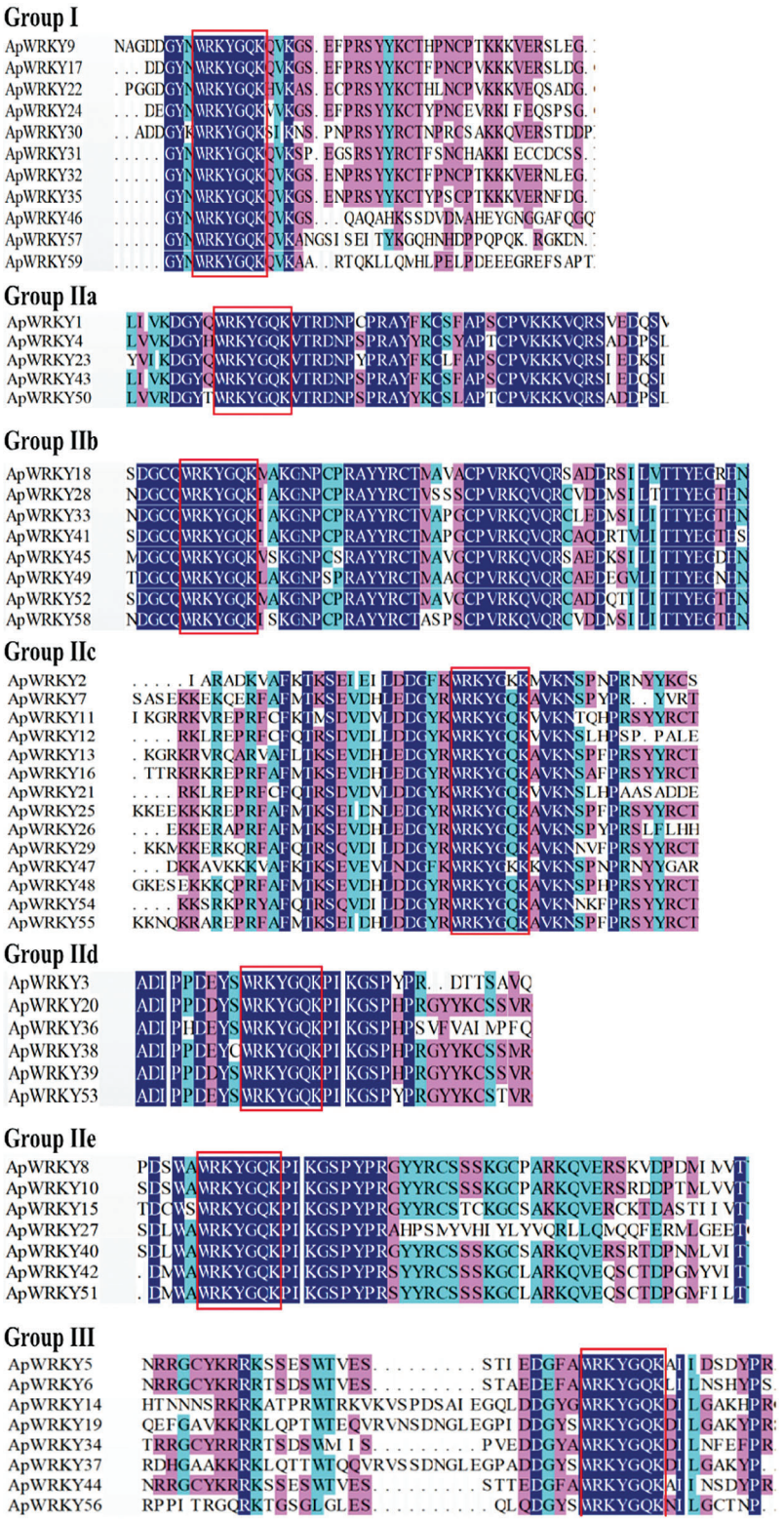

FIGURE 2. Multiple sequence alignment of the WRKY domain from *ApWRKYs*.
Red box indicates WRKY Domain.

motif 1, motif 2, and motif 3 were widely distributed on 46, 49, and 39 ApWRKYs proteins, respectively. It may be related to the important function of the WRKY transcription factor. It was found that the ApWRKY members of each group usually had similar motif composition. For example, motif 18 was unique to Group IIb, and motif 6 was specific to Groups IIa and IIb. Further, each ApWRKY contained its specificity; for example, Motif16 only existed in ApWRKY; in ApWRKY17, ApWRKY24, and ApWRKY35, there were two motifs, 1 and 2 (Fig. 4b). ApWRKY protein showed a similar motif arrangement in the subgroup, indicating that the protein structure is conserved in a specific subfamily. The function of most conservative motifs remained to be clarified. In general, the conservative motif composition and similar gene structure of the same WRKY members, as well as phylogenetic tree analysis results, strongly supported the credibility of this group of taxa.

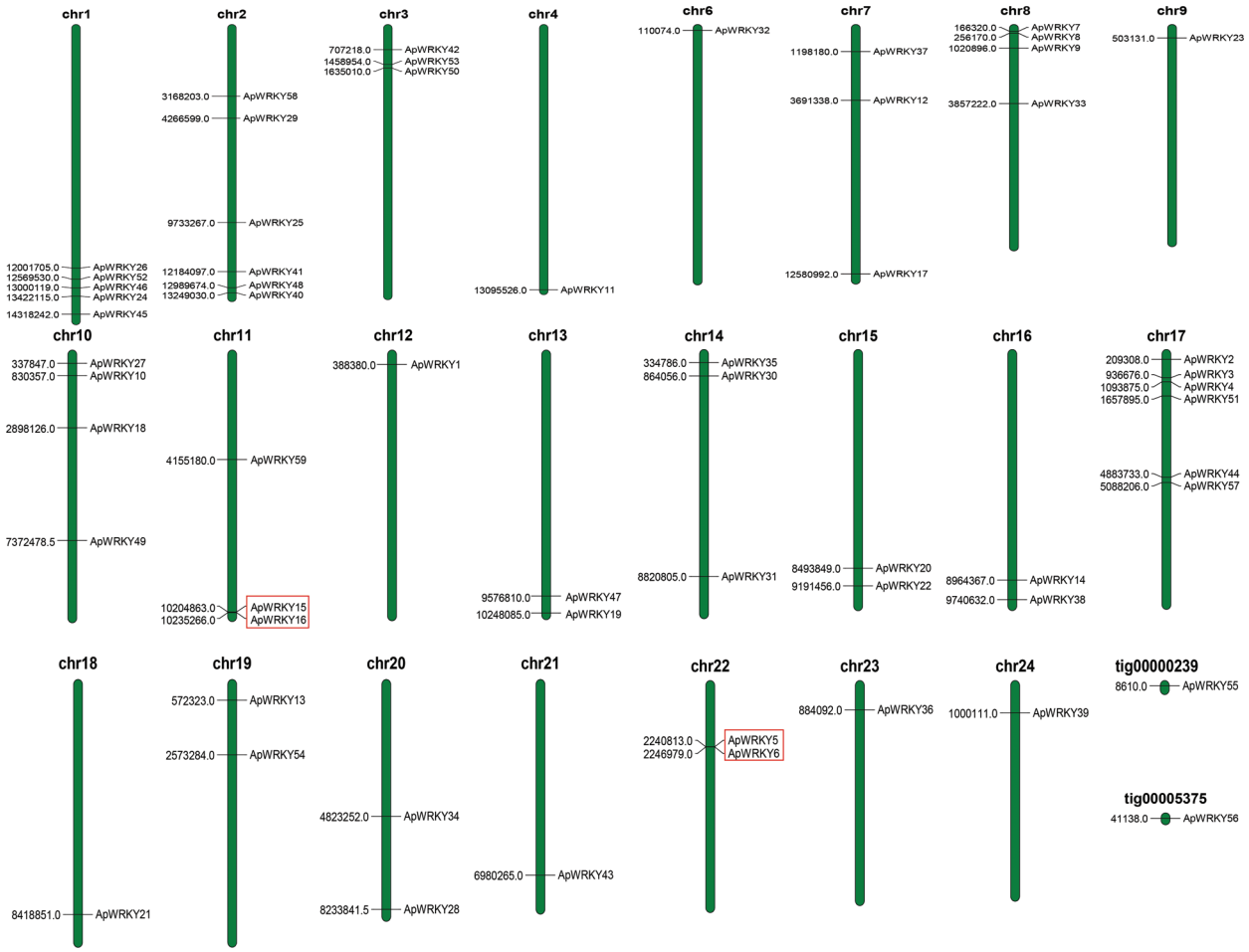

FIGURE 3. Distribution of *ApWRKY* genes within the *A. paniculata* chromosomes (Chrs).
Vertical bars represent the Chrs within the *A. paniculata* genome. The Chr number is indicated at the top of each Chrs. The units of numbers in the figure are 1 base. The red boxes represent the possibility of tandem repeats.

The exons of all the identified *ApWRKY* genes were examined to gain more insight into the evolution of the *WRKY* family in *A. paniculata* (Fig. 4c). The mRNA structure of all the identified *ApWRKY* were also examined. All *ApWRKY* genes possessed two to seven CDS (nine with two CDS regions, 20 with three CDS regions, 13 with four CDS regions, 11 with five CDS regions, five with six CDS regions, and one with seven CDS regions). Further observation revealed that *ApWRKY* mRNA in the three CDS regions accounted for 1/6 of the universal. mRNA within the same group usually had similar structures; for example, all Group III members contained almost three CDS regions.

Differential expression of ApWRKY gene under salt stress

In order to verify whether the expression of *ApWRKY* gene was affected by abiotic stress, we selected 41 *ApWRKY* genes (Fig. 5) from 59 *A. paniculata* WRKY genes, and the transcription level was relatively higher in different tissues. To illustrate the analysis results, we divided 41 *ApWRKY* genes into four clusters. In Cluster I and Cluster IIa, 21 *ApWRKY* transcription factors were sensitive to NaCl in the stems of *A. paniculata*. In Cluster IIb and Cluster IIc, 20 *ApWRKY* transcription factors were sensitive to NaCl in the roots of *A. paniculata* (Fig. 5). Most of the *WRKY* genes were upregulated in the roots and stems of *A. paniculata* after treatment, while WRKY transcript levels were depressed in the leaves. These findings indicated that WRKY gene transcripts were mainly induced in the roots and stems of *A. paniculata* after salt treatment. In the roots and stems, the expression level of most WRKY genes increased with the increase of Na^+ level, with some exceptions including *ApWRKY9*, *ApWRKY20*, *ApWRKY53* and *ApWRKY59*. Suggesting that *WRKY* genes play a crucial role in the salt stress tolerance of *A. paniculata*. In addition, the transcript levels of some *WRKY* genes, such as *ApWRKY9*, *ApWRKY10* and *ApWRKY16*, were significantly induced in *A. paniculata* leaves under low salinity conditions.

In this study, the expression of 20 *ApWRKY* genes was significantly altered under the salinity stress (Fig. 6a). The greatest increase in expression (nearly 45-fold) was detected for under 100 mmol/L NaCl treatment as compared with the control in roots. *ApWRKY10*, *ApWRKY16* and *ApWRKY25* transcript levels were downregulated in the leaves of *A. paniculata* under salinity stress (Fig. 6c). *ApWRKY17*, *ApWRKY29*, *ApWRKY33*, *ApWRKY37*, *ApWRKY48*, *ApWRKY50*, *ApWRKY51*, and *ApWRKY54* were significantly upregulated in the roots in response to different levels of salinity stress. *ApWRKY29*, *ApWRKY48*, *ApWRKY50*, and *ApWRKY54* genes clearly reduced

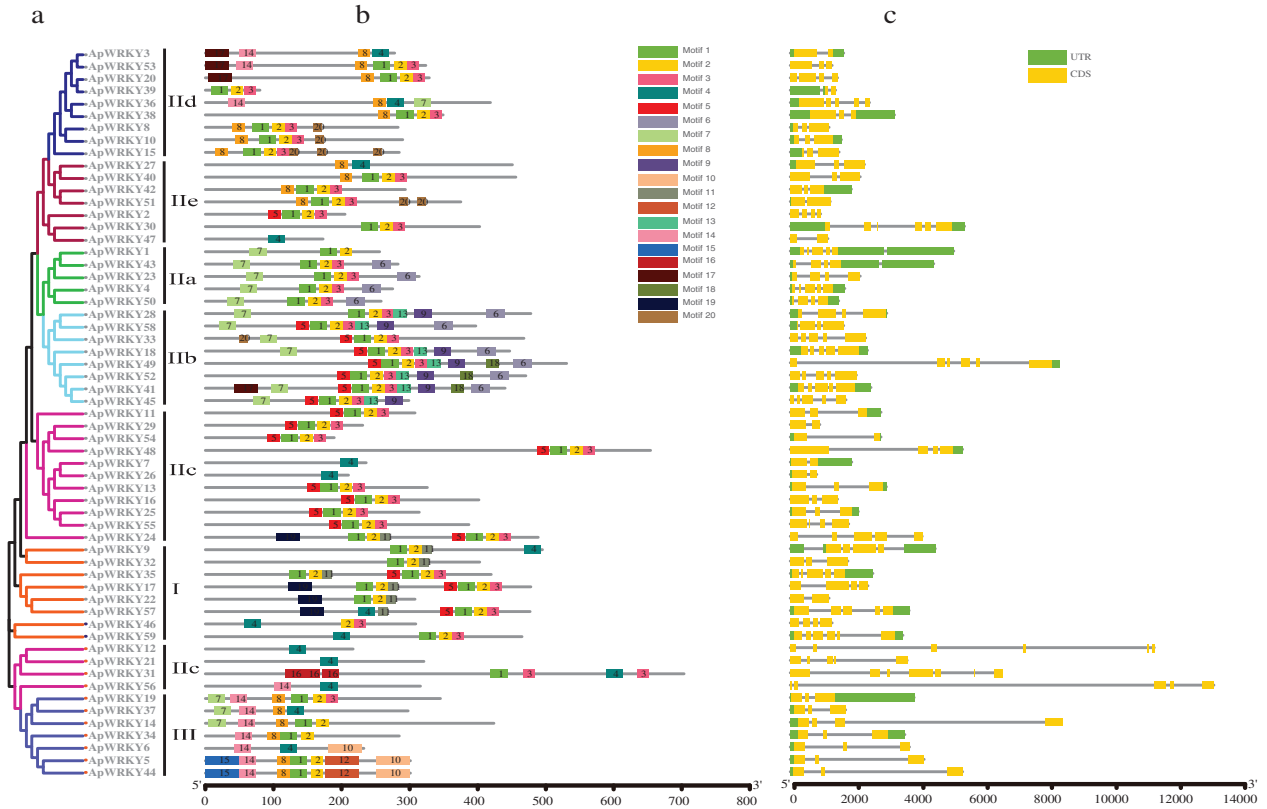

FIGURE 4. Phylogenetic relationships, the architecture of conserved protein motifs and gene structure in *WRKY* genes from *A. paniculata*. (a) The phylogenetic tree was constructed based on the full-length sequences of *A. paniculata* WRKY proteins using MEGA 7.0 software. Details of branch are shown in different colors. (b) The motif composition of *A. paniculata* WRKY proteins. (c) The motifs, numbers 1 to 20, are displayed in different colored boxes. The sequence information and length of the motif are provided at the bottom of the image.

transcript levels in the leaves under the 50 mmol/L salinity concentration and even decreased to undetectable levels in roots. In contrast, *ApWRKY29*, *ApWRKY48*, *ApWRKY50*, and *ApWRKY54* genes were significantly induced, and their high levels were maintained in the roots under high salinity stress. The transcripts of *ApWRKY4* and *ApWRKY34* exhibited an obvious increase in the stems with the enhancement of salinity concentration (Fig. 6b). In addition, under 50 mmol/L NaCl stress, the transcription level in the stems of *A. paniculata* was significantly increased compared with the control group (Fig. 6c). The salt tolerance mechanism of different *ApWRKY* genes was obviously different.

The active ingredient changes of A. paniculata under salt stress

Stems and leaves of *A. paniculata*, which are regarded as a promising system with which to produce AP, NAP, DHAP, and DOAP. Expression levels of biosynthesis genes were different after treatment with different concentrations of salt.

The accumulation of secondary metabolism in these environments was quantified. Interestingly, we found that the content of AP, NAP, DHAP and DOAP were obviously elevated by 50 mmol/L NaCl treatment after, and the contents of all four lactones are the highest. However, after 100 mmol/L NaCl stress, the contents of four andrographolides were lower than those of the control group. The results showed that a certain concentration of salt could promote or inhibit the biosynthesis of diterpenoid lactones in *Andrographis paniculata* leaves. According to Figs. 6 and 7, there can be seen that in the stem, under the salt stress of 50 mmol/L, *ApWRKY1, 26, 32* and *52* are significantly up-regulated in the stem, while *ApWRKY9* is significantly up-regulated in the leaf, which binds to under 50 mmol/L salt stress, *A. paniculata* has the highest content of active ingredients. We speculate that *ApWRKY1, 9, 26, 32* and *52* may be involved in the synthesis of four kinds of andrographolides.

Discussion

WRKY transcription factors participate in many physiological and biochemical processes in higher plants, not only in the growth and development processes of plant stem elongation, seed development, leaf senescence, root development, and trichome formation but also in the biological and abiotic stress processes of plants (Eulgem *et al.*, 2000). Since *WRKY* genes play an important regulatory role in plant defense and disease resistance, understanding these genes is of great significance to plant research, plant breeding, and crop improvement. From the perspective of evolutionary relationship, the clustering results of the WRKY protein phylogenetic tree are highly consistent with the classification. The structure of the ApWRKY transcription factor phylogenetic tree is basically the same as that of the *Salvia miltiorrhiza* WRKY transcription factor phylogenetic tree of *Salvia miltiorrhiza* and sunflower (Yu *et al.*, 2018, Li *et al.*, 2020), Group IId, Group IIe, and Group III members clustered on the same branch, indicating that *A. paniculata*

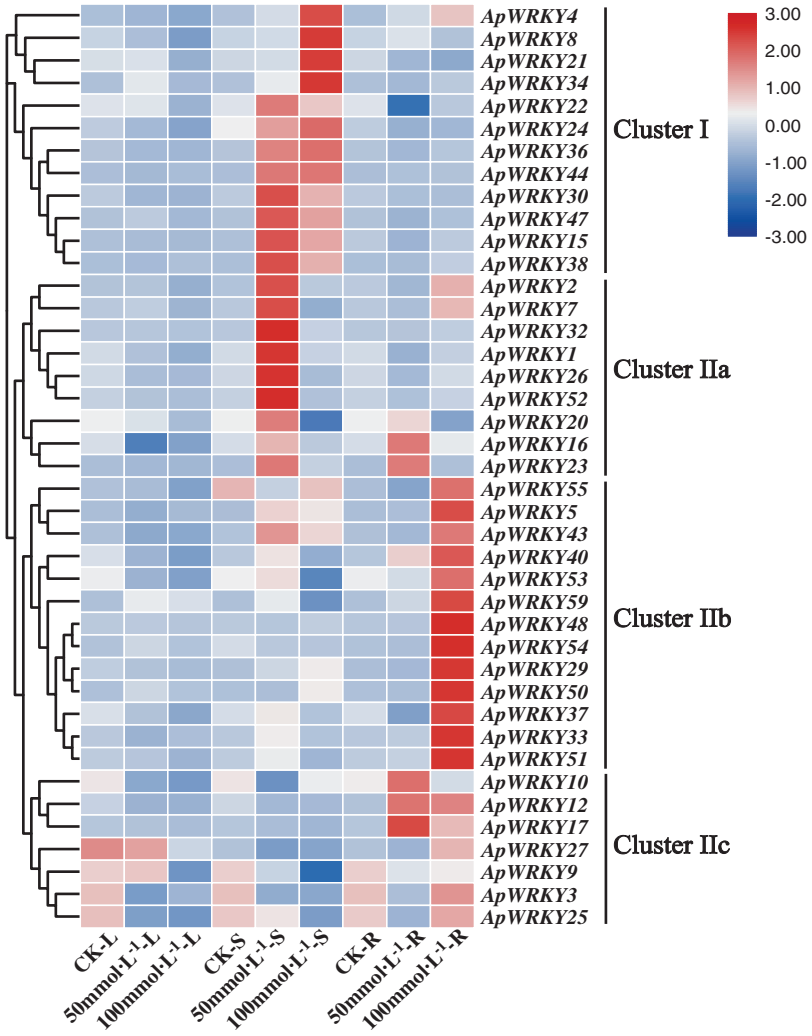

FIGURE 5. Expression patterns of 41 *ApWRKY* genes in root (R), stem (S), leaf (L) with *A. paniculata*. The gene expression level was detected via RT-qPCR. The heat map was generated using cluster software. The sources of the samples were as follows: CK-L (leaves of control), CK-S (stems of control), CK-R (roots of control), 50 mmol/L-L/S/R (leaves/stems/roots of cotton seedlings under 50 mmol/L treatment), 100 mmol/L-L/S/R (leaves/stems/roots of cotton seedlings under 100 mmol/L treatment). Blocks with colors indicate low/down expression (blue), high/up expression (red), and non-expression/no change (white).

Group IId, Group IIe, and Group III WRKY transcription factors evolved from Group I in succession. In terms of structure, the *ApWRKY* transcription factor protein is highly conserved, but there are also variations in conserved domains, indicating that the *ApWRKY* transcription factor has diversity in the course of evolution. *ApWRKY* transcription factors of Group IIc have two different conserved domain structural differentiations. Proteins with mutations in several domains cluster together, indicating that these *ApWRKY* transcription factors may have similar functions, which is consistent with the presence of SiWRKY60 in sesame seeds in Group IIc (Li *et al.*, 2017). In *Arabidopsis*, barley, and tomatoes, a similar phenomenon occurs in Group IIe members (Huang *et al.*, 2012, Mangelsen *et al.*, 2008, Eulgem *et al.*, 2000).

In terms of conserved motifs, there were two conserved motif structures in Group I, and the differentiation of different domains appears, indicating that different structures may have different biological functions. Similar structure and differentiation were also found in the *WRKY* transcription factor protein of *Caragana intermedia*, also in Group I, the same phenomenon also appeared in *Arabidopsis*, cucurbitaceous species (Jiao *et al.*, 2018, Eulgem *et al.*, 2000), indicating that the *ApWRKY* transcription factor has a certain species specificity in the evolution process.

The expression level of *WRKY* genes is usually closely related to biological stress. Comparative expression analysis of the *WRKY* genes family can understand the potential function of WRKY in the process of plant development. In the present study, high *ApWRKYs* transcript levels were observed in both roots, stems, and leaves under salinity treatment. In this study, *ApWRKY* has different expression patterns in different tissues, which is consistent with the results of other plants such as poplar (Jiang *et al.*, 2020), sunflower (Liu *et al.*, 2020), and *Artemisia annua* (De *et al.*, 2020). It can be seen from Fig. 5 that only a small part of WRKY genes is not expressed. among which while the *ApWRKY10*, *ApWRKY16*, and *ApWRKY25* genes were obviously downregulated in the leaves under salinity stress (Eulgem *et al.*, 2000). These findings indicated that these specific expression level changes might have helped protect the cell membranes system in *A. paniculata* under salinity stress (Nan *et al.*, 2020). Moreover, most of the *ApWRKY* genes were induced to express in the roots of *A. paniculata* under the salinity stress, implying a tissue-specific expression in response to the salinity condition or a possible fact that the root was the tissue directly exposed to the treatment (Goyal *et al.*, 2020, Zhao *et al.*, 2019). According to research by Chen *et al.* (2010), in *Arabidopsis*, *AtWRKY18*, *AtWRKY40*, *AtWRKY60* are expressed under abiotic stress, and *ApWRKY1, 4, 23, 43, 50* transcription

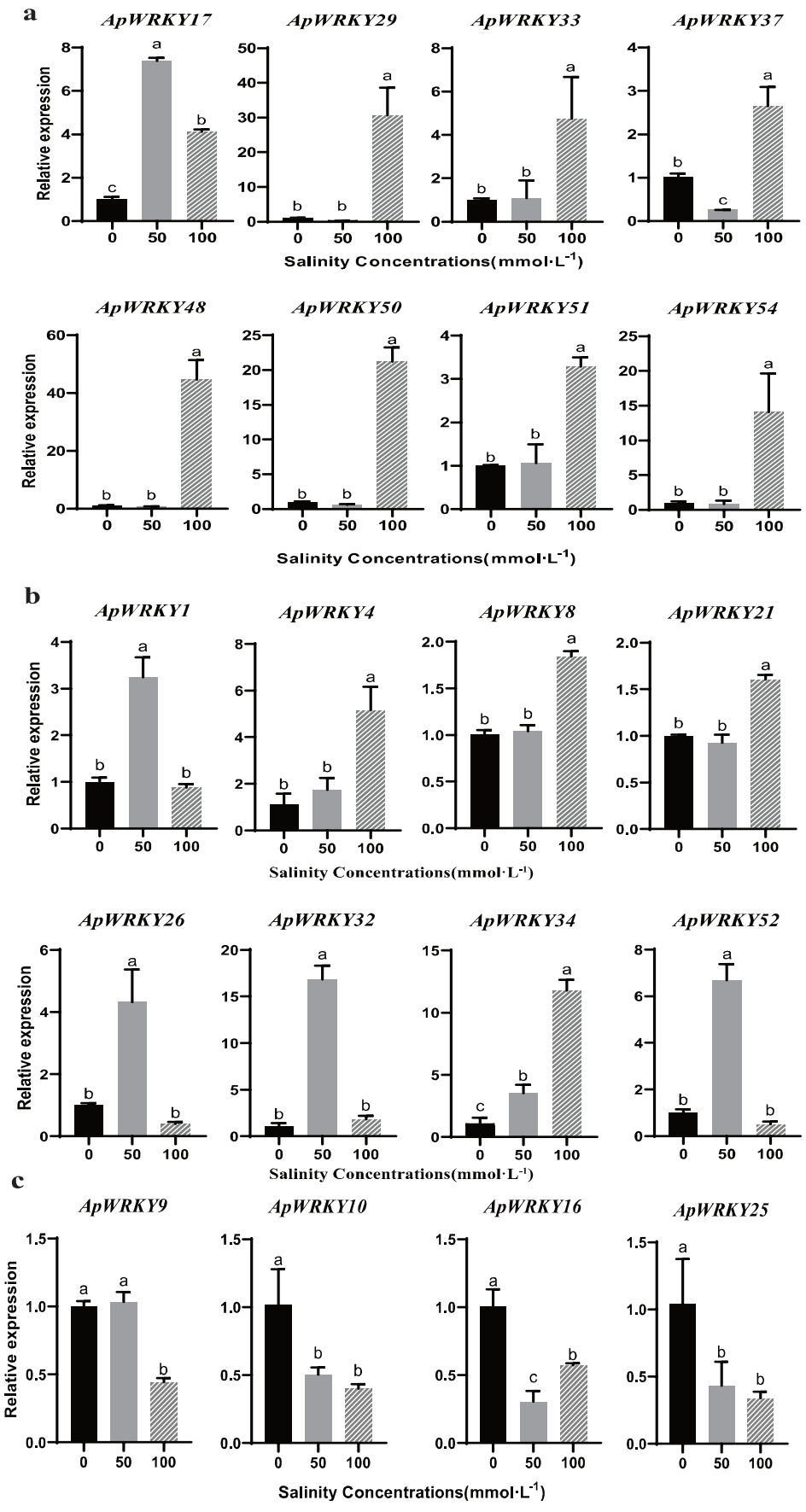

FIGURE 6. The expression patterns of selected genes in response to NaCl in different tissues of *A. paniculata*. (a) Expression patterns of some genes in roots under salt stress. (b) Expression patterns of some genes in the stem under salt stress. (c) Expression patterns of some genes in leaves under salt stress.

factors in the same branch may also form a highly interactive regulatory network (Fig. 1). By acting as a transcriptional activator or repressor to regulate gene expression in *A. paniculata* defense and stress response. Research by Li *et al.* (2011) and Jiang and Deyholos (2009) showed that *WRKY25*, *WRKY26* and *WRKY33* are part of a complex

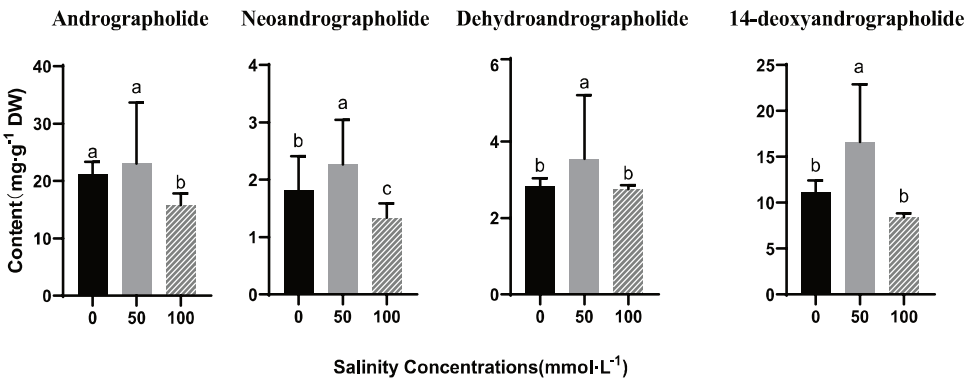

FIGURE 7. Effects of NaCl on accumulations of andrographolide, neoandrographolide, 14-deoxyandrographolide, and dehydroandrographolide in leaves of *A. paniculata*.
The error bar represents its SD value (N = 3). The lettering indicates statistically significant differences at $P < 0.05$ between the content in the NaCl stressed *A. paniculata* and that in the corresponding controls.

transcription network that regulates responses to specific abiotic stresses. The author predicts that the gene functions of *ApWRKY32* and *ApWRKY35* in the same branch are similar. Zou *et al.* (2010) found that *AtWRKY34* is specifically expressed in pollen. After low-temperature treatment, the gene expression is upregulated and reversely regulates the low-temperature sensitivity of mature *Arabidopsis* pollen. The author predicts that *ApWRKY9*, which is closely related to its relationship (Fig. 1), may also be sensitive to low-temperature stress. While negatively regulating the low-temperature sensitivity of a certain tissue. Although the functions of most WRKY genes in *A. paniculata* remain unknown, phylogenetic and expression analyses provide a solid foundation for future functional studies.

In addition, the content of the four main secondary metabolites in *A. paniculata* leaves was determined under salt stress. From the results, The accumulation of diterpenoid lactone was the highest in *A. paniculata* under 50 mmol/L NaCl stress, indicating that salt stress in this adversity environment improves the effective components of andrographolide (Talei *et al.*, 2013, Talei *et al.*, 2015, Chanwala *et al.*, 2020), which is a further reasonable and efficient breeding of *A. paniculata*. Studies have shown that in *Salvia miltiorrhiza*, *SmWRKY2* can activate the up-regulated expression of genes related to the tanshinone biosynthesis pathway, thereby increasing the content of tanshinone in the hairy roots of salvia (Deng *et al.*, 2019). *OsWRKY76* overexpressed in rice can reduce the production of rice phytoalexins (Yokotani *et al.*, 2013). The main secondary metabolites of *A. paniculata* come from the leaves. The analysis in Fig. 5 shows that the four genes *ApWRKY3, 9, 25* and *27* are upregulated in the leaves. It is speculated that these four genes may be involved in the regulation of diterpenoids in the leaves. These genes, therefore, can be used as candidate genes for the study of andrographolide biosynthetic pathways. A similar phenomenon occurs when the rice transcription factor *OsWRKY51 gene* can be induced by abscisic acid (ABA). It can inhibit the transduction of gibberellins (GA) signals in rice cells by combining with the OsWRKY transcription factor. It is an important integration factor in the two signal pathways of ABA and GA (Xie *et al.*, 2006). Among the 37 WRKY transcription factor genes expressed in Arabidopsis roots, 12 genes are specifically expressed in the tissue cells of the mature zone of the roots and may be involved in the morphogenesis of *Arabidopsis* roots (Birnbaum *et al.*, 2003).

In this study, using the latest genome data of *A. paniculata*, 59 *WRKY* family members of *A. paniculata* were systematically identified and screened, and chromosome mapping was carried out. Through the construction of the phylogenetic tree, the locations of the conserved domains and CDS and UTR regions of the genes were used to identify *ApWRKY*. The specific expression analysis of the *ApWRKY* gene family in different tissues under salt stress was carried out, and the effect of different concentrations of *A. paniculata* on the secondary metabolites under salt stress was also studied. It provides a basis for further research on the functions of *A. paniculata WRKY* family members, as well as a reference for the salt stress mechanism of *A. paniculata* and the genetic improvement of abiotic stress.

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Author Contributions: QW, LX and ZL conceived and designed this study. QW and WZ conducted experiments. BA, LX and XZ contributed the analytical tool. QW wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Availability of Data and Materials: All data generated or analyzed during this study are included in the manuscript. Data on preliminary tests are also provided in separate files as supplementary materials (Supplementary Tables and Figures). Thus, the readers can freely access all the published data with proper acknowledgment and citation. Besides, all the raw data sets generated or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Supplementary Materials

Table S1. List of sequences used for phylogenetic tree construction.

Table S2. List of RT-qPCR specific primers used for ApWRKY expression studies.