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Genome-Wide Identification and Analysis of the WRKY Gene Family in the Xerophytic Evergreen *Ammopiptanthus nanus*

Xin Hao ^{1,†} , Shuyao Wang ^{1,†}, Yingying Chen ¹, Yue Qu ¹, Hongjun Yao ^{2,*} and Yingbai Shen ^{1,*}

¹ College of Biological Sciences and Technology, Beijing Forestry University, Beijing 100083, China; haoxin0331@bjfu.edu.cn (X.H.); shuyao0331@bjfu.edu.cn (S.W.); B20173040294@cau.edu.cn (Y.C.); yuequ1011@bjfu.edu.cn (Y.Q.)

² National Engineering Laboratory for Tree Breeding, Beijing Forestry University, Beijing 100083, China

* Correspondence: hongjuny@bjfu.edu.cn (H.Y.); ybshen@bjfu.edu.cn (Y.S.)

† These authors contributed equally to this work.

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Abstract: The WRKY family of transcription factors plays important roles in plant growth and responses to biotic and abiotic stresses. *Ammopiptanthus nanus*, the only evergreen broadleaf shrub endemic to the desert and semi-desert regions of northwestern China, is highly tolerant to various stresses. However, a systematic study of WRKY proteins in *A. nanus* has not been reported. In the present study, we identified 63 WRKY genes in the *A. nanus* genome. Based on the conserved WRKY domains, zinc finger structures, and phylogenetic relationships in their encoded proteins, we classified these genes into four groups (group I–IV) and several subgroups (subgroup IIa–IIe). Conserved motif analysis showed that all motifs except those within the WRKY domains had a subfamily-specific distribution. Expression analysis revealed that the *AnWRKY* genes had distinct expression patterns, with some being more responsive to herbivory and drought stresses than others. Based on the results of our current study, we speculate that *AnWRKY40* and *AnWRKY48* are positive regulators of the plant's response to drought and herbivory stresses, respectively. Our results indicate that *AnWRKY* genes contribute to the ability of *A. nanus* plants to withstand harsh, dry conditions.

Keywords: *Ammopiptanthus nanus*; biotic and abiotic stresses; expression analysis; gene structure; WRKY

1. Introduction

Ammopiptanthus nanus (Popov) Cheng F, a relic plant of the Tertiary period, belongs to the tribe Thermopsidae and the family Fabaceae, and it is a xerophytic leguminous shrub endemic to Central Asian deserts [1]. It is the only broadleaved evergreen that is endemic to the desert areas in northwestern China. It could be a good sand fixing plant with salt alkali, drought, and sand resistances. Moreover, with beautiful tree shape, many butterfly-shaped tubular flowers, golden yellow corolla, and white fluff on the twigs, it could be an ideal greening tree species [2]. In addition, its oil can be used, and some genes in the branches and leaves may produce a medicinal effect [3]. It has the effect of dispelling wind, warming, and dampness, promoting blood circulation and dispersing blood stasis from the perspective of traditional Chinese medical science [3]. In conclusion, it has ecological, agronomic, and medicinal value. *A. nanus* occurs only in the southwestern part of the Xinjiang Uygur Autonomous Region and is classified as a II-class nationally protected species in China [3,4]. The genome of *A. nanus* was sequenced in 2018 and the final size of the assembled this genome was close to the 889 Mb, in which the gene annotation integrality was estimated by BUSCO [1]. Due to the harsh growing environment, *A. nanus* is subjected to a variety of environmental stresses [4,5], including an arid climate (annual

precipitation <200 mm; annual evaporation >2000 mm), extreme temperatures (from -30°C to 47.6°C), and serious pests [6]. Drought and pests pose increasingly serious environmental threats to plant growth and revegetation in northwestern China. The main pest of *A. nanus* is *Orgyia ericae* Germar, a lepidopteran distributed across northwestern China and parts of Europe [7]. This pest gnaws on leaf buds, leaves, and flower buds, affecting plant growth and fruitage and even causing death.

The WRKY genes are transcriptional regulators that play important roles in plant defense and stress responses [8]. However, the identification of WRKY transcription factors in *A. nanus* and its roles in stress response are still unclear. The first reported WRKY gene was identified in sweet potato (*Ipomoea batatas*) in 1994, which could regulate the expressions of sporamin and β -amylase-related genes [9]. WRKY transcription factors were named for the highly conserved 60-amino-acid domain they all contain, which includes a conserved WRKYGQK heptapeptide, followed by a distinctive zinc finger-like motif, C2H2 (C-X₄₋₅-C-X₂₂₋₂₃-H-X-H) or C2HC (C-X₇-C-X₂₃-H-X-C) [8,10]. Based on the number of WRKY domains and the type of zinc finger motifs, WRKY proteins can be divided into four groups (I–IV) [11,12]. Group I proteins usually contain two WRKY domains and C2H2 type zinc finger motifs, whereas groups II–IV proteins contain a single WRKY domain. Group II is characterized by having C2H2 type zinc finger motifs and is further subdivided into five subgroups (IIa, IIb, IIc, IId, and IIe) based on phylogeny [8]. Group III proteins have C2HC zinc fingers and group IV proteins lack a complete zinc finger structure [13].

WRKY transcription factors control a range of physiological and biochemical processes and responds to a variety of abiotic and biotic stresses, such as drought, salinity, herbivory, and pathogens [10,14]. Most WRKY proteins regulate gene expression by specifically binding to a W-box element (TTGAC(C/T)) in the target gene promoter [8]. Previous studies showed that the Siberian crabapple (*Malus baccata*) *MbWRKY5* gene can improve drought and salt tolerance when heterologously expressed in tobacco (*Nicotiana tabacum*) by increasing proline and ascorbic acid content and enhancing the activities of peroxidase, superoxide dismutase, and catalase [15]. In *Arabidopsis thaliana*, *AtWRKY18* and *AtWRKY60*, working cooperatively with *AtWRKY40*, regulate abscisic acid (ABA)-mediated drought and salinity stress responses. These transcription factors bind to the W-box in the promoters of *ABI4* and *ABI5*, ABA signaling pathway genes that affect plant stress resistance [16]. In rice (*Oryza sativa*), overexpression of *OsWRKY11*, *OsWRKY30*, or *OsWRKY47* significantly improved drought tolerance by activating the expression of drought-responsive genes [17–19]. Many studies show that WRKY transcription factors are also involved in biotic stress responses. For example, overexpression of *OsWRKY13* in rice enhanced resistance to blast disease and bacterial blight, increased the accumulation of salicylic acid, and inhibited the expression of jasmonic acid (JA) response genes [20]. Similarly, constitutive overexpression of *OsWRKY45* significantly enhanced blast resistance [21]. Wang showed that *OsWRKY89* overexpression plants had a reinforced epidermis that enhanced resistance against both rice blast fungus and rice white-back planthopper [22]. In tobacco plants that were attacked by the moth *Manduca sexta*, *NtWRKY3* and *NtWRKY6* were strongly upregulated, thereby potentiating and/or maintaining active JA levels during continuous insect attacks [23]. In addition, recent studies showed that *VviWRKY40* expression pattern was negatively correlated with that of *VviGT14* in grape berries (*Vitis vinifera* L.) and reported that ABA could downregulate the transcript level of *VviWRKY40* in order to control monoterpenoid glycosylation [24].

WRKY genes have been identified in many species, resulting in large gene families that include up to 72 members in *Arabidopsis* [8], 96 in rice [25], 55 in cucumber (*Cucumis sativus*) [26], 59 in grapevine [13], and 127 in apple (*Malus domestica* Borkh.) [27], and the mechanisms by which WRKY proteins regulate some biological process have been explored. However, no studies have examined the WRKY gene family in *A. nanus*. It is regarded as a plant that can withstand harsh conditions, it is of both scientific and practical interest to comprehensively identify and analyze the WRKY gene family in this plant and to explore the molecular mechanisms underlying its stress tolerance.

2. Materials and Methods

2.1. Database Search and Identification of WRKY Family Members in *A. nanus*

The sequences of *Arabidopsis* WRKY proteins were downloaded from the *Arabidopsis* database (TAIR, <http://www.arabidopsis.org/>). The *A. nanus* genome was downloaded from the GigaScience database (GigaDB, <http://gigadb.org/>; accession number 100466). First, a local protein database was established containing the proteome of *A. nanus* using BioEdit 7.2.0 software (Ibis Therapeutics, Carlsbad, USA) [28]. To identify WRKY proteins in *A. nanus*, a local BlastP search was performed using a hidden Markov model (HMM) profile of the WRKY domain (PF03106) as query sequences. Default parameters were used and the expectation (E) value was less than 0.001. The search results were used to identify candidate genes, and then conserved domain analysis was conducted with the SMART program [29]. After removing low-reliability and redundant sequences, the complete set of *A. nanus* WRKY family proteins was obtained. To further analyze the zinc finger structure of *A. nanus* WRKY proteins, candidate protein sequences were aligned in the DNAMAN program (Lynnon BioSoft, San Ramon, CA, USA). In addition, the theoretical isoelectric point (pI) and molecular weight (MW) were analyzed using the ProtParam program [30].

2.2. Phylogenetic Analysis

Multiple amino acid sequence alignment was performed using Clustal X 1.81 (<http://www.clustal.org/>) with default parameters [31]. A phylogenetic tree was constructed with the whole WRKY proteins of *Arabidopsis* and *A. nanus* using the neighbor-joining method in MEGA 7.0 software [32], with the following parameters: Poisson correction, partial deletion, and bootstrap (1000 replicates). All of the *A. nanus* WRKY genes were classified into subgroups based on the zinc finger structure. The gene IDs of AtWRKYs used are as follows: AtWRKY7: AT4G24240, AtWRKY8: AT5G46350, AtWRKY9: AT1G68150, AtWRKY14: AT1G30650, AtWRKY18: AT4G31800, AtWRKY21: AT2G30590, AtWRKY22: AT4G01250, AtWRKY25: AT2G30250, AtWRKY31: AT4G22070, AtWRKY33: AT2G38470, AtWRKY41: AT4G11070, AtWRKY44: AT2G37260, AtWRKY45: AT3G01970, AtWRKY47: AT4G01720, AtWRKY50: AT5G36170, AtWRKY54: AT2G40750, AtWRKY55: AT2G40740, AtWRKY60: AT2G25000, AtWRKY65: AT1G29280, and AtWRKY72: AT5G15130.

2.3. Exon–Intron Structure and Conserved Motif Analysis

The DNA sequence and coding sequence (CDS) of *A. nanus* WRKY genes were downloaded from the GigaScience database (accession number 100466), and the exon–intron distribution patterns were analyzed using online GSDS software [33]. The conserved motifs were analyzed using MEME [34], a software tool for predicting conserved sequences of genes, with the following parameters: amino acid width >6 and <200, maximum number of motifs: 15.

2.4. Plant and Insect Materials, Growth Conditions, and Stress Treatments

A. nanus seeds were collected from Wuqia County in Xinjiang Province in northwestern China. The seeds were surface sterilized for 7 min in 2% (*w/v*) KMnO₄, soaked in water for 12 h, and then sown in sterilized soil (sand: vermiculite: perlite = 4:1:1). Seedlings were grown in a growth chamber under a 14 h light (29 °C)/10 h dark (25 °C) photoperiod and relative humidity of 60–70% [35]. Wild type *Arabidopsis* (wt, Columbia ecotype) and *atwrky48* lines (SALK_066438, kindly provided by Prof. Diqui Yu; Chinese Academy of Sciences, China) were incubated in the chamber under a 16 h light (22 °C)/8 h dark (20 °C) photoperiod and relative humidity of 70%. The plants used were about five weeks old.

Orygia ericae cocoons were collected from Alxa Left Banner, China. *Plutella xylostella* L. eggs were purchased from Jiyuan Baiyun Industry Co., Ltd. (Jiyuan, China). Cocoons or eggs were hatched at 28 °C or conserved at 4 °C. For feeding assays, the insect-inoculated plants were placed in a clear plastic cups covered with plastic net chamber to facilitate the exchange of gases. The *O. ericae* in the second- or third-instar stage was placed on *A. nanus* leaves of four-week-old plants without blossoming, and one

or two larvae were reared on each of 10 plants per biological replicates ($n = 3$). One day later, RNA was extracted from the attacked leaves for qRT-PCR analysis. The newly hatched *Plutella xylostella* L. were placed on rosette leaves of five-week-old *Arabidopsis*, and three larvae were reared on each of 60 plants per genotype. The larval weights and lengths were measured, and the specimens were photographed after six days.

For drought treatment, RNA was extracted for qRT-PCR analysis from the leaves of five-week-old *A. nanus* plants that had not been watered for six days.

2.5. Expression Analysis

Total RNA was isolated using an RNAPrep Pure Plant Kit (TIANGEN, Beijing, China) from *A. nanus* tender roots, non-lignified stems, young leaves, and cotyledons. RNA was quantified using NanoDrop 2000, and electrophoresis analysis revealed clear bands, indicating intact RNA products. Then 500 ng RNA was reverse transcribed using a PrimeScriptTM RT Reagent Kit (Perfect Real Time) (Takara, Dalian, China) for semi-quantitative PCR (semi-qRT-PCR) and quantitative real-time PCR (qRT-PCR) analysis. Specific primers that avoid the conserved regions of WRKY genes were designed using Primer 5.0 software (Premier Biosoft, Palo Alto, CA, USA). For semi-qRT-PCR, a 20 μ L reaction system was used containing 10 μ L 2 $\times$ Premix TaqTM (TaKaRa, Dalian, China), forward and reverse primers diluted to 0.4 mM, and 0.25 μ L template mentioned above. The reaction conditions were 29 cycles of 94 $^{\circ}$ C for 30 s, 60 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 30 s. *AnACTIN2* was used as a sufficiently stable internal control gene for *A. nanus*. PCR products were run on 2% agarose gels. For qRT-PCR analysis, the reaction system contained 10 μ L of 2 $\times$ SYBR Premix Ex Taq II (TaKaRa, Dalian, China) as the enzyme and the other reagents were the same as those used in the semi-qRT-PCR. The reaction conditions were 95 $^{\circ}$ C for 30 s, followed by 40 cycles of 95 $^{\circ}$ C for 5 s, and 60 $^{\circ}$ C for 30 s, and fluorescence was collected after each cycle. The relative expression of genes was calculated using the $2^{-\Delta\Delta C_t}$ method. Three technical and three biological replicates were performed for each gene.

3. Results

3.1. Identification and Annotation of WRKY Genes in *A. nanus*

To identify *A. nanus* WRKY family genes, the HMM (PF03106) profile was applied as a query to search against the local protein database. After further analysis using the SMART program, missing and redundant sequences were removed and 63 WRKY genes were confirmed in the *A. nanus* genome. In order to better reflect the orthologous relationship between AnWRKY and AtWRKY proteins, we named each AnWRKY gene based on their phylogenies corresponding to individual AtWRKY (Figure S1).

The characteristics of the AnWRKY genes and their predicted protein products, including subgroup, location, length, PI, MW, and intron number are annotated in Table 2. The PIs ranged from 4.79 (*AnWRKY15*) to 9.91 (*AnWRKY11*), and the MWs ranged from 13.3 kDa (*AnWRKY45*) to 83.5 kDa (*AnWRKY25*).

Table 1. *A. nanus* WRKY genes and their characteristics.

Gene Name	Subgroup	Gene ID	Contig	Location	Length	PI	MW	Introns
<i>AnWRKY1</i>	I	EVM0036550.1	Contig00249	881267-885956	531	9.04	58.9	4
<i>AnWRKY2</i>	I	EVM0025925.1	Contig00134	185096-190006	749	6.06	80.8	5
<i>AnWRKY3</i>	I	EVM0026202.1	Contig00060	673753-678833	495	6.29	54.4	3
<i>AnWRKY4</i>	I	EVM0024746.1	Contig00347	1152356-1159915	480	6.54	53.1	3
<i>AnWRKY5</i>	IIa	EVM0007876.1	Contig00285	1145300-1147375	306	9.24	34.2	4
<i>AnWRKY6</i>	IIb	EVM0028523.1	Contig00068	4100063-4103327	626	6.03	68.1	5
<i>AnWRKY7</i>	IIc	EVM0029026.1	Contig00309	3026901-3029798	351	9.74	38.4	2
<i>AnWRKY8</i>	IIc	EVM0031818.1	Contig00172	2727817-2730284	288	8.20	32.5	2
<i>AnWRKY9</i>	IIb	EVM0013215.1	Contig00144	291495-294545	500	5.62	55.6	4
<i>AnWRKY10</i>	IIc	EVM0030112.1	Contig00147	1095121-1098490	467	5.68	51.3	3

Table 2. *A. nanus* WRKY genes and their characteristics.

Gene Name	Subgroup	Gene ID	Contig	Location	Length	PI	MW	Introns
AnWRKY11	IV	EVM0013235.1	Contig00133	619190-622048	253	9.91	27.6	1
AnWRKY12	IIc	EVM0034422.1	Contig00030	346313-358646	234	6.32	26.6	3
AnWRKY13	IIc	EVM0021276.1	Contig00096	4921639-4926137	237	9.21	26.4	2
AnWRKY14	IIe	EVM0008136.1	Contig00144	223409-227393	438	5.58	48.2	2
AnWRKY15	IIe	EVM0019948.2	Contig00172	2368271-2371101	261	4.79	29.4	2
AnWRKY16	IIb	EVM0023616.1	Contig00021	2183821-2186821	532	5.97	57.6	5
AnWRKY17	IIId	EVM0019864.1	Contig00567	2286577-2289261	304	9.36	33.5	2
AnWRKY18	IIa	EVM0003639.1	Contig00190	6269580-6272469	255	7.69	29.0	3
AnWRKY19	IIb	EVM0018318.3	Contig00210	855716-860907	556	7.91	60.6	3
AnWRKY20	I	EVM0020336.1	Contig00178	1212361-1218212	590	6.16	64.1	5
AnWRKY21	IIe	EVM0010357.1	Contig00260	7444503-7446533	263	5.26	29.2	2
AnWRKY22	IIe	EVM0015516.1	Contig00044	3972223-3973753	246	5.70	27.9	2
AnWRKY23	IIc	EVM0022911.1	Contig00044	3902508-3905588	328	5.78	37.2	2
AnWRKY24	IIc	EVM0021945.1	Contig00260	4579044-4581468	219	9.08	25.0	2
AnWRKY25	I	EVM0014036.1	Contig00408	529863-534523	763	6.61	83.5	4
AnWRKY26	I	EVM0013267.1	Contig00181	6605357-6608548	554	7.26	60.8	4
AnWRKY27	IIe	EVM0005883.1	Contig00252	246512-248278	387	8.67	43.0	2
AnWRKY28	IIc	EVM0008616.1	Contig00342	469047-471651	348	6.83	38.4	2
AnWRKY29	IIe	EVM0034521.1	Contig00655	985219-987118	227	7.06	25.7	1
AnWRKY30	III	EVM0025502.1	Contig00271	172850-180802	371	5.82	41.2	2
AnWRKY31	IIb	EVM0010766.2	Contig00237	2363259-2369053	661	5.95	71.4	4
AnWRKY32	I	EVM0030310.1	Contig00606	1628533-1634405	502	8.59	54.8	4
AnWRKY33	I	EVM0015108.1	Contig00321	1171470-1175612	573	6.81	63.2	4
AnWRKY34	I	EVM0015833.1	Contig00264	1234107-1237865	521	6.58	58.1	4
AnWRKY35	IIe	EVM0033995.1	Contig00556	2322207-2326210	522	5.89	56.4	2
AnWRKY36	IIb	EVM0004781.1	Contig00573	2918455-2921696	501	5.89	55.5	4
AnWRKY37	IIa	EVM0001679.1	Contig00145	1300181-1302883	290	7.69	32.5	3
AnWRKY38	III	EVM0026515.1	Contig00096	6112779-6115591	332	5.85	37.5	2
AnWRKY39	IIId	EVM0020784.1	Contig00044	6968734-6971484	348	9.64	38.9	2
AnWRKY40	IIa	EVM0026094.1	Contig00190	6349068-6351253	291	8.69	32.6	3
AnWRKY41	IV	EVM0006002.1	Contig00026	2668094-2669212	288	4.88	33.3	4
AnWRKY42	IIb	EVM0015185.1	Contig00172	3548090-3553038	499	6.92	54.0	6
AnWRKY43	IIe	EVM0026729.1	Contig00237	5846780-5852712	265	5.29	29.2	2
AnWRKY44	I	EVM0032970.3	Contig00115	5503144-5508551	463	8.86	51.2	5
AnWRKY45	IIc	EVM0036516.1	Contig00260	2357799-2363839	110	9.86	13.3	1
AnWRKY46	III	EVM0032312.1	Contig00096	6105731-6109309	359	6.11	39.7	2
AnWRKY47	IIb	EVM0037234.1	Contig00276	750183-761903	461	7.63	51.0	5
AnWRKY48	IIc	EVM0006677.1	Contig00607	1571628-1573738	362	5.51	40.2	2
AnWRKY49	IIc	EVM0027357.1	Contig00027	6165824-6168441	318	5.45	35.4	2
AnWRKY50	IIc	EVM0003197.1	Contig00190	4832963-4834879	160	6.21	18.3	2
AnWRKY51	IIc	EVM0028564.1	Contig00096	5038138-5041729	192	6.15	21.9	2
AnWRKY52	IIb	EVM0005061.1	Contig00417	2832100-2835526	602	6.48	65.3	5
AnWRKY53	III	EVM0014815.1	Contig00655	1517724-1519031	360	5.41	40.8	2
AnWRKY54	III	EVM0034840.1	Contig00001	122763-125652	361	5.17	40.5	2
AnWRKY55	III	EVM0010299.1	Contig00181	8058953-8062299	368	6.21	40.2	2
AnWRKY56	IIc	EVM0012573.1	Contig00260	4718556-4723016	179	6.15	20.7	2
AnWRKY57	IIc	EVM0018636.1	Contig00556	1464035-1465952	389	5.19	42.5	2
AnWRKY58	I	EVM0000741.1	Contig00164	630769-636130	527	6.66	57.2	3
AnWRKY59	IIc	EVM0024952.1	Contig00145	2079202-2080594	154	5.27	17.6	2
AnWRKY60	IIa	EVM0008017.1	Contig00629	2362396-2365475	304	7.53	33.8	4
AnWRKY61	IIb	EVM0006376.1	Contig00260	14884-20418	614	6.31	66.6	4
AnWRKY62	III	EVM0029994.1	Contig00181	8049570-8056182	331	6.76	37.3	2
AnWRKY63	III	EVM0034027.1	Contig00181	6684863-6688460	430	6.26	48.4	2

Length: amino acid length; pI: isoelectric point; MW: molecular weight.

We compared the number of WRKY genes in each subfamily in *A. nanus* to that in *Arabidopsis*, rice, grapevine, and cucumber (Table 3). The WRKY gene family in *A. nanus* has roughly the same characteristics as in other species, but also shows obvious differences. In *A. nanus*, there are only three genes in subgroup IId, which consequently accounts for a significantly smaller proportion (4.76%) of the total genes than it does in other species.

Table 3. The number and proportion of WRKY genes in *A. nanus* (An), *Arabidopsis* (At), rice (Os), grapevine (Vv), and cucumber (Cs).

Group Name	AnWRKY	AtWRKY [8]	OsWRKY [25]	VvWRKY [13]	CsWRKY [26]
Group I	12 (19.05%)	13 (18.06%)	15 (15.63%)	12 (20.34%)	10 (18.18%)
Subgroup IIa	5 (7.94%)	4 (5.56%)	4 (4.17%)	3 (5.08%)	4 (7.27%)
Subgroup IIb	10 (15.87%)	7 (9.72%)	8 (8.33%)	8 (13.56%)	4 (7.27%)
Subgroup IIc	15 (23.81%)	18 (25.00%)	15 (15.63%)	16 (27.12%)	16 (29.09%)
Subgroup IId	3 (4.76%)	7 (9.72%)	7 (7.29%)	6 (10.17%)	8 (14.55%)
Subgroup IIE	8 (12.70%)	9 (12.50%)	11 (11.46%)	6 (10.17%)	7 (12.73%)
Group III	8 (12.70%)	14 (19.44%)	36 (37.50%)	6 (10.17%)	6 (10.91%)
Group IV	2 (3.17%)	-	-	2 (3.39%)	-

“-”: not available.

3.2. Phylogenetic Analysis

To examine the phylogenetic relationships among AnWRKY proteins, we constructed a phylogenetic tree based on multiple sequence alignment of 63 AnWRKY and 20 AtWRKY proteins (as references) using MEGA 7.0 software. Consistent with previously published reports [9], the 63 AnWRKY proteins were subdivided into four subgroups, designated groups I to IV (Figure 1; group IV is not shown). Group I was divided into two independent subgroups, group I-N and I-C, based on differences between the N- and C-terminals of the WRKY domains.

To gain insight into the WRKY domain and zinc finger structure, the multiple amino acid sequence of each subgroup member was aligned using DNAMAN software. As shown in Figure S2, twelve genes with two highly conserved WRKY domains were assigned to group I, which also had C-X₄-C-X₂₂-H-X-H and C-X₄-C-X₂₃-H-X-H zinc fingers at the N- and C-terminus, respectively. Group II was subdivided into IIa (5 members), IIb (10 members), IIc (15 members), IId (3 members), and IIE (8 members), which all had a C-X₅-C-X₂₃-H-X-H zinc finger motif, except for group IIc, which had a C-X₄-C-X₂₃-H-X-H zinc finger. Group III had eight genes, which had C2HC (C-X₇-C-X₂₃-H-X-C) zinc fingers instead of the C2H2 type. Two genes, *AnWRKY11* and *AnWRKY41*, had a WRKY domain but only incomplete zinc finger structures and therefore were assigned to group IV. Although *AnWRKY10* clustered with group I-C in the phylogenetic tree, it had only one WRKY motif and should be classified as subgroup IIc based on the presence of this conserved motif. *AnWRKY49* clustered with group III genes in the phylogenetic tree, but it was assigned to subgroup IIc because of its C2H2 zinc finger motif. Although most AnWRKYs contain the highly conserved sequence WRKYGQK, this motif was replaced by WRKYGEK in *AnWRKY40* and by WRKYGKK in four other genes (*AnWRKY50*, *51*, *56*, *59*). Notably, the N-terminal WRKY domain of *AnWRKY34* was replaced by WKYGEK, which was a new consensus WRKY motif (Figure S2). Overall, AnWRKY proteins fell into four groups and had highly conserved C2H2 and C2HC zinc finger motifs, similar to what has been reported for other plants.

Figure 1. Phylogenetic tree of AnWRKY proteins. The tree was constructed using the neighbor-joining method, implemented in MEGA 7.0 software. The bootstrap value was 1000. Selected AtWRKY proteins serve as representatives for the different subgroups. Triangles indicate *Arabidopsis* WRKY proteins; squares indicate *A. nanus* proteins. The black intermittent arcs indicate different groups and subgroups. For group I proteins, “NT” and “CT” indicate the N-terminal and C-terminal WRKY domains, respectively.

3.3. Analysis of AnWRKY Gene Structure

We explored the structure of the *A. nanus* WRKY genes by analyzing the positions of conserved motifs, exons, and introns (Figure 2). Motif 1 indicates a WRKY motif and motif 2 represents a zinc finger motif, which together comprise an integrated WRKY/zinc finger domain. These motifs were present in all of the AnWRKY genes except AnWRKY11 and AnWRKY41, which had WRKYGQK motifs (motif 12) but incomplete zinc finger structures. Motif 3 and motif 7, which were present only in group I genes, are a WRKY motif and a zinc finger motif, respectively, that together form a second, N-terminal WRKY domain in these genes. The distribution of other conserved motifs among the AnWRKY genes exhibited familial specificity. Motif 4 and motif 9 were present only in subgroups IIa and IIb. The highly conserved motif 8 was present only in subgroup IIb, immediately downstream of motif 2. Motif 10 was present only in group I, whereas motif 13 was present in all group III genes except AnWRKY41. Motif 12 was present in most genes in groups IId, IIe, III, and IV.

Figure 2. Conserved motifs and exon–intron distribution of *AnWRKY* genes. Conserved motifs (left panel) and exon–intron structure (right panel) were analyzed using MEME and GSDS software, respectively. The different groups and subgroups are indicated at the far left. Motifs 1 to 15 are indicated by different colored boxes in the left panel. In the right panel, introns and exons are represented by thin black lines and thick yellow lines, respectively. Thick blue lines represent UTRs. Note that the two panels are drawn to different scales: protein length on the left and DNA length on the right.

Based on our analysis of exon–intron structure, *AnWRKY* genes that clustered together in the evolutionary tree often had similar exon–intron structures. Genes of subgroup IIb have complex exon–intron structures, whereas those of subgroups IId and IIe are simple. Among the 63 *AnWRKY* genes we identified, three genes had one intron, eight genes had five or six introns, and the remainder had two to four introns.

3.4. Expression of *AnWRKY* Genes in Different Tissues

To investigate the expression pattern of *AnWRKY* genes, we used a semi-qRT-PCR method to determine the transcript levels of 63 *AnWRKY* genes in roots, stems, leaves, and cotyledons. *AnACTIN2* was used as an internal control. The specific primer sequences, which did not overlap with any of the conserved WRKY domains, are shown in Table S1.

Most of the *AnWRKY* genes were more highly expressed in roots than in other tissues. Ten genes, *AnWRKY9*, 16, 22, 27, 29, 35, 36, 42, 48, and 61 were expressed specifically in the roots. Among these genes, five belonged to subgroup IIb and 4 to subgroup IIe. Five genes, *AnWRKY37*, 38, 40, 56, and 63, showed specific and low expression in leaves. None of the genes were specifically expressed in stems and cotyledons (Figure S3).

The patterns of gene expression showed subfamily specificity. All of the genes in group I and subgroup IId, except *AnWRKY33*, were expressed at high or moderate levels in all four tissues. Genes

in subgroup IIa and group III were generally expressed at low levels in the four tissues, except for *AnWRKY5* and *AnWRKY53*, which were highly expressed in roots and leaves, respectively. Most genes in subgroups IIb and IIe showed high expression in roots. Thirteen genes, *AnWRKY10*, 14, 18, 28, 33, 41, 45, 46, 47, 49, 50, 55, and 62, showed very low expression in all four tissues (Figure S3).

Based on their subgroup designation and expression level in the different tissues, 15 *AnWRKY* genes were selected for further study of their expression patterns with qRT-PCR. These results showed the same general trends as the semi-qRT-PCR results. Transcript levels for *AnWRKY61* were 180 times higher in roots than in leaves, and transcript levels for *AnWRKY9*, 29, and 35 were about 30–40 times higher in roots than in leaves. The expression of *AnWRKY5*, 7, 21, 23, 26, 27, and 52 was also highest in roots. Expression of *AnWRKY13* was highest in the stems, about 14 times higher than in leaves and cotyledons, whereas that of *AnWRKY53* was highest in leaves, about 10 times higher than in the other tissues (Figure 3).

Figure 3. Expression analysis of 15 *AnWRKY* genes in roots, stems, leaves, and cotyledons. *AnACTIN2* was used as an internal control. The mean and standard error (SE) were derived from three biological and three technical replicates. The different groups and subgroups are indicated above the graphs. Different letters indicate significant differences at $p < 0.05$, Student's *t*-test.

3.5. The Effect of Herbivory and Drought on Expression of *AnWRKY* Genes

To see how the *AnWRKY* genes respond to stress, we performed a preliminary semi-qRT-PCR analysis of *AnWRKY* expression in the roots and leaves of plants subjected to herbivory or drought (data not shown). Based on the results, we selected the top nine genes with the most significant

expression change for further analysis: *AnWRKY9*, 18, 27, 29, 43, 45, 48, 62, and 63 for herbivory and *AnWRKY8*, 26, 27, 29, 34, 36, 40, 59, and 61 for drought.

The effect of herbivory on *AnWRKY* expression was strongest in leaves of the treated plants, where the expression of all nine genes was upregulated by about 2- to 14-fold (Figure 4). In leaves, the expression of *AnWRKY18* was upregulated by about 5.2-fold (statistical significance at *** $p < 0.001$). The expression of *AnWRKY45*, 48 and 63 were respectively increased by about 14.1-, 17.6-, and 4.2-fold (** $p < 0.01$), and the significance of *AnWRKY48* not reached to *** $p < 0.001$, which may be due to more error between biological replicates. The expression of other genes was also upregulated (* $p < 0.05$) in leaves. In roots, the expression of *AnWRKY9*, 43, and 48 genes were significantly upregulated. Three genes (*AnWRKY18*, 45, and 62) were downregulated in roots by about 1.7- to 4-fold, but *AnWRKY45* has no statistical significance (Figure 4). Interestingly, the expression levels of these nine genes was low relative to other *AnWRKY* genes in untreated leaves (Figure S3).

Figure 4. qRT-PCR assays of the expression of *AnWRKY9*, 18, 27, 29, 43, 45, 48, 62, and 63 in response to herbivory in roots and leaves. Black columns represent data for the one-day herbivory treatment, and gray columns represent the control data. HW: herbivory wounding. *AnACTIN2* was used as an internal control. The mean and SE were derived from three biological and three technical replicates. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, Student's *t*-test.

The effect of drought on *AnWRKY* expression showed four upregulated genes (*AnWRKY8*, 26, 40, 59) and five markedly downregulated genes (*AnWRKY27*, 29, 34, 36, 61) in both roots and leaves of the treated plants. It should be noted that the expression level of *AnWRKY27* and *AnWRKY29* was downregulated by about six fold in roots (*** $p < 0.001$), but there were no obvious changes in leaves. In addition, both *AnWRKY34* and *AnWRKY59* showed great changes in leaves, but they

have no statistical significance, which may be caused by more error between biological replicates. The expression of *AnWRKY40* was remarkably upregulated in the roots, but it hardly changed in leaves. *AnWRKY8* was strikingly enhanced in leaves, but it did not change significantly in roots (Figure 5). In general, different from herbivory treatment, *AnWRKY* genes showed an inconsistent variation trend after drought treatment.

Figure 5. qRT-PCR assays of the expression of *AnWRKY8*, 26, 27, 29, 34, 36, 40, 59, and 61 in response to drought in roots and leaves. Black columns show data for the six-day drought treatment, and the gray columns show data for the controls. *AnACTIN2* was used as an internal control. The mean and SE were derived from three biological and three technical replicates. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, Student's t -test.

In general, the expression of *AnWRKY* genes showed regular change in the treated plants subjected to herbivory or drought, especially herbivory treatment induced upregulated expression in leaves. These results indicated that *AnWRKY* genes was involved in the expression regulation under biotic and abiotic stresses.

3.6. Insect Feeding Trials in *atwrky48*

Based on the strong upregulation of *AnWRKY48* expression after the herbivory treatment, we hypothesized that this gene might be involved in plant defense responses to insect feeding. *AnWRKY48* is homologous to *AtWRKY48* in *Arabidopsis*, but neither gene has been examined for its role in insect resistance. To verify this hypothesis, wild type (WT) and *atwrky48* *Arabidopsis* plants were performed feeding assays simultaneously. As shown in Figure 6a,b, the average weight and length of larvae raised on *atwrky48* plants (42.4 ± 0.2 mg, 7.5 ± 0.2 mm) were significantly higher than those of larvae raised on WT plants (37.9 ± 0.2 mg, 7.2 ± 0.2 mm), (* $p < 0.05$). At the same time, the *Plutella*

xylostella L. larvae reared on *atwrky48* were significantly lower than that of larvae reared on WT plants (Figure 6c), indicating that the mutant is less resistant to herbivore attack.

Figure 6. Insect feeding trials on the *atwrky48* mutant. Average weight (a) and length (b) of *Plutella xylostella* L. larvae reared on WT and *atwrky48* mutant plants for six days. The mean and SE were derived from 60 biological replicates. * $p < 0.05$, Student's *t*-test. (c) Photographs of representative *Plutella xylostella* L. larvae recovered from WT and *atwrky48* mutant plants after six days of feeding. Scale bar = 1 mm.

4. Discussion

Transcription factors in the WRKY superfamily play important roles in physiological and developmental processes of plants. In this study, we identified 63 novel *AnWRKY* genes in *A. nanus*. Only three of these genes (*AnWRKY7*, 17, and 39) belonged to subgroup IId. As a previous study showed that WRKY genes of subgroup IId could directly interact with calcium-modulated protein [36], we speculate that these three genes may play an important role in the regulation of calcium ions in *A. nanus*. Furthermore, we analyzed the conserved motifs of *AnWRKY* genes and established that the distribution of the conserved motifs showed subgroup specificity; most conserved motifs (e.g., motifs 2, 5, 7, 8, 11, and 12) were located near the WRKY region, suggesting that these motifs may be critical for the function of WRKY proteins.

WRKYGQK, a highly conserved domain, was replaced with WRKYGEK in *AnWRKY45* and with WRKYGKK in *AnWRKY50*, 51, 56, and 59. These genes all belong to subgroup IIC. Similar phenomena were reported in *Arabidopsis* and cucumber [8,26]. As a previous study demonstrated that the WRKYGKK domain failed to bind to the W-box [37], it needs further experimental verification about whether *AnWRKY45*, 50, 51, 56, and 59 can be combined with the W-box. The molecular mechanisms by which these family members function in plants remain to be elucidated. In addition, *AnWRKY34* contained motif 3 and 7, which formed the WRKY domain and C2H2 type zinc finger structure of the N-terminal. Although the WRKYGQK domain in the N-terminal was replaced by a new consensus WRKY motif, WKYGEK [38], *AnWRKY34* still belongs to group I. In chrysanthemum (*Chrysanthemum morifolium*), *CmWRKY17* contains the WKYGEK domain, but belongs to group II. This protein binds strongly to the W-box and weakly to the mutant W-box [39]. Therefore, whether *AnWRKY34* will have the same situation as *CmWRKY17* also needs further experimental verification.

To explore the expression patterns of the *AnWRKY* genes, we analyzed the expression level of 63 *AnWRKY* genes in roots, stems, leaves, and cotyledons by semi-qRT-PCR, and selected 15 genes with representative expression patterns for qRT-PCR detection. Ten genes (*AnWRKY4*, 7, 11, 17, 23, 26, 32, 39, 52, and 58) were expressed in four different tissues (roots, stems, leaves, and cotyledons). Highly expressed WRKY genes often play key roles in plant growth and development through activating the transcription of downstream target genes [40]. Therefore, we hypothesize that these 10 WRKY genes play fundamental roles in different cell types in *A. nanus* [41]. By contrast, some *AnWRKY* genes were only expressed in individual tissues. *AnWRKY9*, 29, 35, 36, and 61 were specifically expressed in roots, and all belonged to the IIB or IIE subgroups. Similar to the situation in *Populus* [40], most *PtrWRKY* genes were highly expressed in roots, suggesting that these genes play key roles in the metabolism and development of *A. nanus* roots. Some of the *AnWRKY* genes were not expressed in the four tissues

examined and might be expressed in other tissues that were not examined or only expressed under certain conditions. For example, the expression levels of *AnWRKY40* in roots and *AnWRKY48* in leaves were relatively low, but the levels were significantly increased when *A. nanus* was subjected to drought or herbivory stress, respectively.

WRKY transcription factors not only play important roles in plant development, but also in the response to abiotic and biotic stresses. In this study, we demonstrated that the expression of *AnWRKY* genes in leaves changed greatly after herbivory treatment and tended to be upregulated. In other species [40,42,43], the studies on WRKY transcription factors were mainly focused on abiotic or pathogenic treatment, but few studies on herbivory. Only individual WRKY genes have been reported to be related to insect resistance. For instance, Yan et al. [44] revealed the inhibition roles of JAV1-JAZ8-WRKY51 (JJW) complex on expression of JA biosynthesis genes in healthy plants, and insect herbivory rapidly disintegrated JJW complex via inducing Ca^{2+} /calmodulin-dependent phosphorylation of JAV1, derepressing JA biosynthesis genes and giving rise to rapid burst of JA for plant defense. In the current study, nine genes with obvious changes were selected for further analysis by qRT-PCR, of which *AnWRKY48* was the most significantly upregulated (about 17.6 times). Insect feeding trials were performed to explore whether *AnWRKY48* affects the plant's susceptibility to insect attack. The average weight and length of larvae that fed on *atwrky48* seedlings were significantly higher than those raised on WT plants, indicating that these mutants are less resistant to herbivore attack than WT plants and that *AtWRKY48* is positively associated with resistance to herbivores (Figure 6). We speculate that *AnWRKY48*, a homolog of *AtWRKY48*, may have similar functions. In order to further verify this function, the exogenous *AnWRKY48* may be overexpressed in different model plants for testing the insect resistance ability. In the other hand, establishing a tissue culture system of *A. nanus* and transient expression technology could be used to analyze the gene functions.

Furthermore, nine genes with marked changes after drought treatment were selected for further analysis by qRT-PCR (Figure 5). These upregulated or downregulated genes might be positively or negatively correlated with drought resistance, respectively. Consistent with previous research, members in the same subgroup also can have both positive and negative roles in plant defense [45]. This suggested that transcription factor families respond differently to the same stress, even if they belong to the same subgroup. Indeed, *AnWRKY40* was the most significantly upregulated in roots, about 5.4 times compared to levels under control conditions. *AtWRKY40* (a homolog of *AnWRKY40*) promotes plant drought stress responses by interfering with the ROS-scavenging pathway and osmolyte accumulation process. The *atwrky40* mutant was less tolerant to drought than the wild type, whereas a line that overexpressed *AtWRKY40* was more drought tolerant [46]. Therefore, we speculate that *AnWRKY40* may be involved in regulating drought tolerance in *A. nanus*.

5. Conclusions

We identified 63 *AnWRKY* genes from the *A. nanus* genome, and analyzed their gene structures, conserved motifs, phylogenetic relationships, and expression patterns. The *AnWRKYs* was divided into I–IV groups, and their conserved motifs also exhibited familial specificity. Furthermore, a majority of the *AnWRKYs* are expressed in more than one tissue, while some genes are expressed only in a specific tissue. Indeed, the *AnWRKY* genes could make corresponding response according to different stresses. Combined with the results of the study, we found that *AnWRKY40* and *AnWRKY48* have significantly positive regulatory roles in the plant's tolerance to drought stress and herbivores, respectively. This work provides insight into the WRKY gene family in *A. nanus* and lays the foundation for deciphering the molecular mechanisms underlying the ability of this relic plant to withstand a range of stresses.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/11/1634/s1>, Figure S1. Phylogenetic analysis of all the *AtWRKY* and *AnWRKY* proteins, Figure S2. Multiple sequence alignment of *AnWRKY* proteins, Figure S3. *AnWRKY* genes expression in different tissues using semi-qRT-PCR, Table S1. Primer sequences.

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