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Identification of the *WRKY* gene family and functional analysis of two genes in *Caragana intermedia*

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Abstract

Background: WRKY transcription factors, one of the largest families of transcriptional regulators in plants, play important roles in plant development and various stress responses. The WRKYs of *Caragana intermedia* are still not well characterized, although many WRKYs have been identified in various plant species.

Results: We identified 53 *CiWRKY* genes from *C. intermedia* transcriptome data, 28 of which exhibited complete open reading frames (ORFs). These *CiWRKY*s were divided into three groups via phylogenetic analysis according to their WRKY domains and zinc finger motifs. Conserved domain analysis showed that the *CiWRKY* proteins contain a highly conserved WRKYGQK motif and two variant motifs (WRKYGKK and WKYEEK). The subcellular localization of *CiWRKY26* and *CiWRKY28-1* indicated that these two proteins localized exclusively to nuclei, supporting their role as transcription factors. The expression patterns of the 28 *CiWRKY*s with complete ORFs were examined through quantitative real-time PCR (qRT-PCR) in various tissues and under different abiotic stresses (drought, cold, salt, high-pH and abscisic acid (ABA)). The results showed that each *CiWRKY* responded to at least one stress treatment. Furthermore, overexpression of *CiWRKY75-1* and *CiWRKY40-4* in *Arabidopsis thaliana* suppressed the drought stress tolerance of the plants and delayed leaf senescence, respectively.

Conclusions: Fifty-three *CiWRKY* genes from the *C. intermedia* transcriptome were identified and divided into three groups via phylogenetic analysis. The expression patterns of the 28 *CiWRKY*s under different abiotic stresses suggested that each *CiWRKY* responded to at least one stress treatment. Overexpression of *CiWRKY75-1* and *CiWRKY40-4* suppressed the drought stress tolerance of *Arabidopsis* and delayed leaf senescence, respectively. These results provide a basis for the molecular mechanism through which *CiWRKY*s mediate stress tolerance.

Keywords: WRKY gene family, *Caragana Intermedia*, Abiotic stress, Gene expression, Subcellular localization, Function analysis

Background

Transcription factors are a class of proteins that significantly regulate plant growth and development and play an important role in plant defense and stress responses [1, 2]. WRKY transcription factors are one of the largest families of transcriptional regulators in plants [2]. These transcription factors contain the WRKY domain, which

is a region of 60 amino acids that is highly conserved [3]. The WRKY domain comprises a featured WRKYGQK sequence motif and a zinc finger-like motif [4]. The WRKY sequence is occasionally replaced by WRRY, WSKY, WKRY, WVKY or WKKY in a subset of WRKY proteins [5]. The zinc finger structure is either CX₄₋₅CX₂₂₋₂₃HXXH (C₂H₂-type) or CX₇CX₂₃HXC (C₂HC-type) [5]. At least one WRKY domain is present in all known WRKY proteins [3].

WRKY proteins can be classified into three main groups (group I, group II and group III) according to the number of WRKY domains along with features of the

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zinc finger-like motif [3]. Group I proteins have two WRKY domains: an N-terminal WRKY domain and a C-terminal WRKY domain, with C₂H₂ zinc finger type CX₄CX₂₂₋₂₃HXH. Group II proteins harbor a single WRKY domain and a C₂H₂-type CX₄₋₅CX₂₃HXH zinc finger. Group III proteins contain a single WRKY domain and a C₂HC-type CX₇CX₂₃HXC zinc finger [3]. Group II WRKY proteins were further divided into five subgroups on the basis of their primary amino acid sequences: IIa, IIb, IIc, IId and IIe [5]. Furthermore, WRKY proteins specifically bind to the W box (C/T)TGAC(T/C) DNA sequence [3, 4], and this binding requires both WRKY and zinc finger domains [4].

The first 4 WRKY cDNAs (*SPF1*, *ABF* (1, 2), *PcWRKY* (1, 2, 3) and *ZAPI*) were cloned from sweet potato (*Ipomoea batatas*), wild oat (*Avena fatua*), parsley (*Petroselinum crispum*) and *Arabidopsis thaliana*, respectively [3]. Numerous WRKY proteins have been studied in various plant species. For example, there are 72 identified members in *A. thaliana* [6], 182 in soybean (*Glycine max*) [7], 127 in apple (*Malus domestica* Borkh.) [8], 80 in grape (*Vitis vinifera*) [9], 86 in *Brachypodium distachyon* [10], 85 in cassava (*Manihot esculenta*) [11], 95 in carrot (*Daucus carota*) [12], 61 in *Salvia miltiorrhiza* [1], 103 in rice (*Oryza sativa*) [13], and 32 in Broomcorn millet (*Panicum miliaceum* L.) [14]. In maize (*Zea mays*), 136 WRKY proteins encoded by 119 WRKY genes have been identified [15]. In cotton, 116 and 102 WRKY genes have been identified from *Gossypium raimondii* and *Gossypium hirsutum* [16], respectively.

WRKY transcription factors are involved in multiple plant processes, such as seed development [17, 18], dormancy [19] and germination [20], trichome development [21], root development [22], leaf senescence [23], plant growth and flowering [24, 25]. Notably, WRKYs have been broadly reported to be involved in plant defense responses. For instance, 49 out of 72 WRKYs from *A. thaliana* are differentially expressed after pathogen infection or SA treatment [6], and *A. thaliana* WRKYs including AtWRKY7, 11, 17, 18, 23, 25, 27, 38, 40, 41, 48, 53, 58, 60, and 62 serve as negative regulators of defense signaling [26]. OsWRKY62 and OsWRKY76 of rice negatively regulate defense upon pathogen infection [27]. Overexpression of *OsWRKY13* [28] and *OsWRKY45-2* [29] in rice increases resistance to bacterial blight and fungal blast caused by *Xanthomonas oryzae* pv. *Oryzae* and *Magnaporthe grisea*, respectively, while overexpression of *OsWRKY42* decreases rice resistance to *M. oryzae* [30]. The transcriptional regulatory cascade of WRKY13, WRKY42, and WRKY45-2 is necessary for defending rice against fungal pathogens [30]. In cotton (*G. hirsutum* L.), GhWRKY15 is involved in disease resistance [31]. Overexpression of grape *VvWRKY1* in tobacco results in enhanced resistance to

various fungi [32]. The roles of WRKYs in plant responses to abiotic stresses such as drought [33–36], cold [37], salinity [37], heat [38, 39], low Pi [40, 41], ultraviolet B (UV-B) [42], osmotic stress [43], and ABA [33, 36, 44] have been widely reported. These studies suggested that WRKYs are involved in multiple stress responses.

Caragana intermedia, a native desert perennial shrub belonging to the Leguminosae family, is distributed in sandy grasslands and desert regions of west and north-west China and Mongolia, and is widely valued due to its high forage value and sand-fixing capacity, along with its strong resistance to drought and salinity [45–48]. *C. intermedia* is considered to be an ideal plant for elucidating the mechanisms of abiotic stress tolerance [49], and research on its molecular mechanisms has been increasing in recent years. For instance, to normalize quantitative real-time PCR (qRT-PCR) data from *C. intermedia* under different abiotic stress conditions, suitable reference genes were screened, which was useful for further gene expression analysis [50]. The transcription of two NAC transcription factors, *CiNAC3* and *CiNAC4*, was found to be induced under treatment with ABA and various abiotic stresses, and ectopic expression of *CiNAC3* and *CiNAC4* in *Arabidopsis* reduces the inhibition of seed germination by ABA and enhances the salt tolerance of the transgenic plants [51]. The expression patterns of miR2118 and its target genes (*CiDRI* and *CiDR2*) from *C. intermedia* are altered under drought stress, and constitutive overexpression of *cin-miR2118* in tobacco enhances the plant's tolerance to drought stress [46]. Abiotic stresses (NaCl, ZnSO₄, CdCl₂, high/low temperature, and dehydration) induce the expression of the glutamate decarboxylase-encoding genes *CiGAD1* and *CiGAD2*, except for *CiGAD2* under Cd stress, and ABA has been shown to be involved in regulating the expression of *CiGADs* in response to salt stress [52]. However, there is limited knowledge of the function of WRKYs in *C. intermedia*. In this study, we identified 53 *CiWRKY* genes based on transcriptomic data from *C. intermedia*, performed phylogenetic analysis and WRKY domain alignment, and examined the subcellular localizations of two *CiWRKYs* and the expression patterns of 28 of *CiWRKYs* in different tissues and under various abiotic stresses. Furthermore, we overexpressed *CiWRKY75-1* and *CiWRKY40-4* in *A. thaliana* and characterized their function. This work provides a basis for exploring the molecular roles of WRKYs and facilitates further investigation of the molecular mechanisms of abiotic stress tolerance in *C. intermedia*.

Results

Identification and cloning WRKY genes from *C. intermedia*

To identify WRKYs in the drought-treated transcriptome of *C. intermedia*, the sequences of the *A. thaliana*

WRKY family were used to query homologous *C. intermedia* sequences. After Blast searches against the sequences in NCBI using blastX to predict conserved domains and remove redundant sequences, 53 sequences with apparent WRKY domains were annotated as *C. intermedia* WRKY genes. Among these genes, 21 WRKYs with complete open reading frames (ORFs) were identified. Using the rapid-amplification of cDNA ends (RACE) technique, an additional 7 WRKYs were cloned, and their full-length ORFs were obtained. The ORF lengths of these 28 genes ranged from 489 bp to 2229 bp, and their amino acid lengths ranged from 163 to 743 aa (details are provided in Table 1). The 28 WRKYs with complete ORFs were used for further expression analysis.

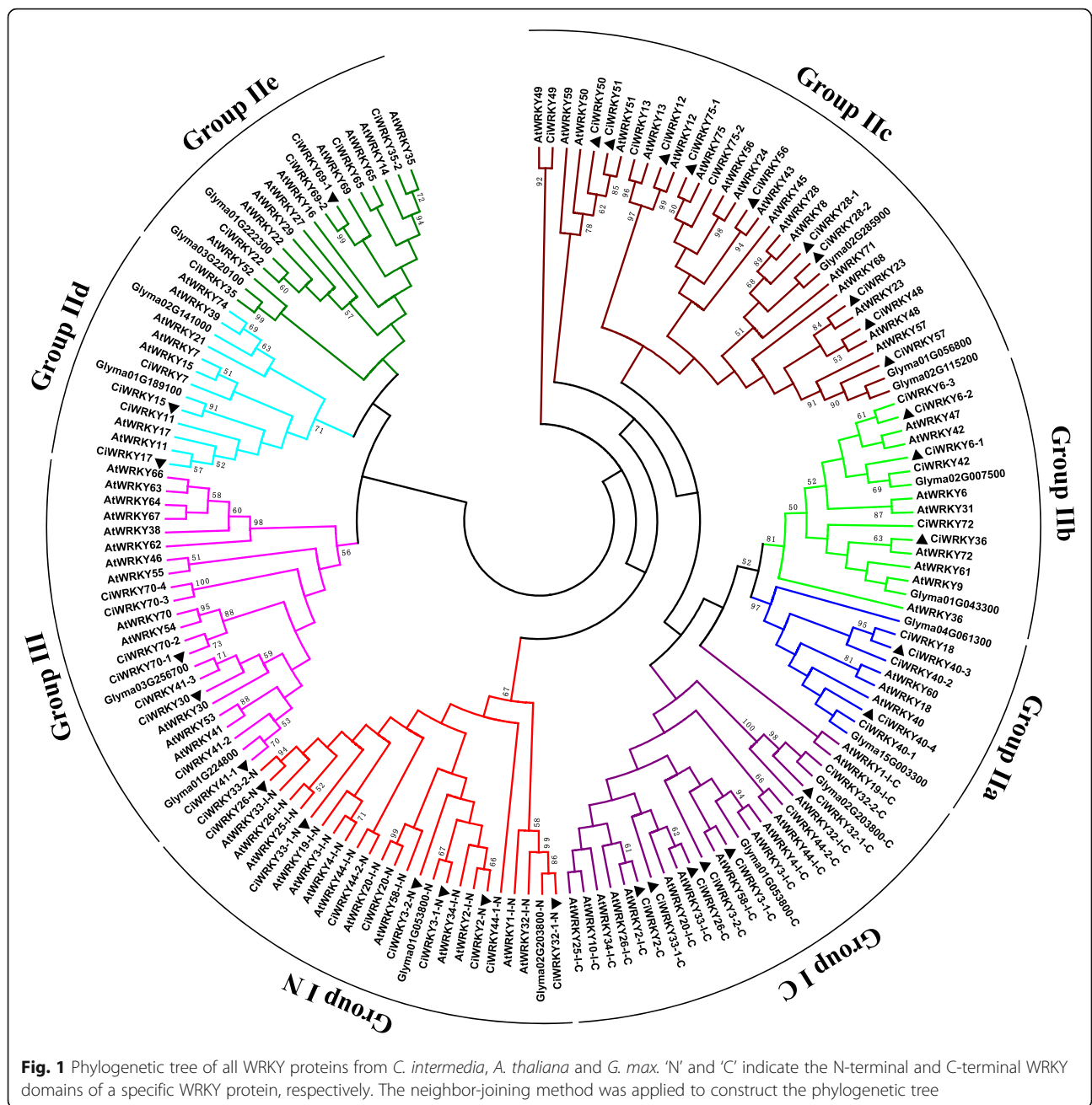
Phylogenetic analysis and multiple sequence alignment of WRKY domains

To further analyze the evolution of these CiWRKYs, we constructed a phylogenetic tree based on a total of 140 WRKYs (72 from *A. thaliana*, 53 from *C. intermedia* and 15 from *G. max* distributed in different groups) (Fig. 1). According to the number of WRKY domains and the pattern of zinc finger structures, the 53 CiWRKY proteins were distributed in three main groups. Eleven CiWRKYs were assigned to group I. Thirty-four CiWRKYs were distributed in group II, which was further classified into five sub-groups: IIa, IIb, IIc, IId, and IIe, which contained 5, 6, 13, 4 and 6 CiWRKYs, respectively. The remaining 8 CiWRKYs belonged to group III.

Table 1 Characteristics of WRKY genes in *C. intermedia*

Gene name	cDNA length	Aa	Mw (KDa)	Group	WRKY domain	Zinc finger	<i>Arabidopsis</i> ortholog
<i>CiWRKY2</i>	2229	743	80.46	Group I	WRKYGQKx2	CX ₄ CX ₂₂₋₂₃ HXH	<i>AtWRKY2</i>
<i>CiWRKY3-1</i>	1383	461	50.31	Group I	WRKYGQKx2	CX ₄ CX ₂₂₋₂₃ HXH	<i>AtWRKY3</i>
<i>CiWRKY3-2</i>	1536	512	55.86	Group I	WRKYGQKx2	CX ₄ CX ₂₂₋₂₃ HXH	<i>AtWRKY3</i>
<i>CiWRKY6-1</i>	1656	552	59.97	Group IIb	WRKYGQK	CX ₅ CX ₂₃ HXH	<i>AtWRKY6</i>
<i>CiWRKY6-2</i>	1830	610	65.56	Group IIb	WRKYGQK	CX ₅ CX ₂₃ HXH	<i>AtWRKY6</i>
<i>CiWRKY12</i>	696	232	26.32	Group IIc	WRKYGQK	CX ₄ CX ₂₃ HXH	<i>AtWRKY12</i>
<i>CiWRKY15</i>	1005	335	36.43	Group IId	WRKYGQK	CX ₅ CX ₂₃ HXH	<i>AtWRKY15</i>
<i>CiWRKY17</i>	1017	339	36.59	Group IId	WRKYGQK	CX ₅ CX ₂₃ HXH	<i>AtWRKY17</i>
<i>CiWRKY23</i>	957	319	35.71	Group IIc	WRKYGQK	CX ₄ CX ₂₃ HXH	<i>AtWRKY23</i>
<i>CiWRKY26</i>	1722	574	63.28	Group I	WRKYGQKx2	CX ₄ CX ₂₂₋₂₃ HXH	<i>AtWRKY26</i>
<i>CiWRKY28-1</i>	999	333	36.98	Group IIc	WRKYGKK	CX ₄ CX ₂₃ HXH	<i>AtWRKY28</i>
<i>CiWRKY28-2</i>	975	325	37.19	Group IIc	WRKYGQK	CX ₄ CX ₂₃ HXH	<i>AtWRKY28</i>
<i>CiWRKY30</i>	1083	361	40.79	Group III	WRKYGQK	CX ₇ CX ₂₃ HXC	<i>AtWRKY30</i>
<i>CiWRKY32-1</i>	1521	507	54.68	Group I	WRKYGQKx2	CX ₄ CX ₂₂₋₂₃ HXH	<i>AtWRKY32</i>
<i>CiWRKY33-1</i>	1614	538	59.57	Group I	WRKYGQKx2	CX ₄ CX ₂₃ HXH	<i>AtWRKY33</i>
<i>CiWRKY36</i>	1785	595	64.36	Group IIb	WRKYGQK	CX ₅ CX ₂₃ HXH	<i>AtWRKY36</i>
<i>CiWRKY40-1</i>	990	330	36.78	Group IIa	WRKYGQK	CX ₅ CX ₂₃ HXH	<i>AtWRKY40</i>
<i>CiWRKY40-3</i>	825	275	30.97	Group IIa	WRKYGQK	CX ₅ CX ₂₃ HXH	<i>AtWRKY40</i>
<i>CiWRKY40-4</i>	897	299	33.66	Group IIa	WRKYGQK	CX ₅ CX ₂₃ HXH	<i>AtWRKY40</i>
<i>CiWRKY41-1</i>	1083	361	41.12	Group III	WRKYGQK	CX ₇ CX ₂₃ HXC	<i>AtWRKY41</i>
<i>CiWRKY48</i>	1155	385	42.15	Group IIc	WRKYGQK	CX ₄ CX ₂₃ HXH	<i>AtWRKY48</i>
<i>CiWRKY50</i>	489	163	19.22	Group IIc	WRKYGKK	CX ₄ CX ₂₃ HXH	<i>AtWRKY50</i>
<i>CiWRKY51</i>	558	186	21.55	Group IIc	WRKYGKK	CX ₄ CX ₂₃ HXH	<i>AtWRKY51</i>
<i>CiWRKY56</i>	678	226	25.61	Group IIc	WRKYGQK	CX ₄ CX ₂₃ HXH	<i>AtWRKY56</i>
<i>CiWRKY57</i>	834	278	30.68	Group IIc	WRKYGQK	CX ₄ CX ₂₃ HXH	<i>AtWRKY57</i>
<i>CiWRKY69-1</i>	798	266	28.89	Group IIe	WRKYGQK	CX ₅ CX ₂₃ HXH	<i>AtWRKY69</i>
<i>CiWRKY70-1</i>	939	313	35.24	Group III	WRKYGQK	CX ₇ CX ₂₃ HXC	<i>AtWRKY70</i>
<i>CiWRKY75-1</i>	567	189	21.08	Group IIc	WRKYGQK	CX ₄ CX ₂₃ HXH	<i>AtWRKY75</i>

Mw Molecular weight, Aa amino acid length



Multiple sequence alignments of the core domains of the CiWRKYs showed that, in group I, with the exception of CiWRKY44–1, which exhibited a WKYEEK variant sequence, all others harbored the conserved WRKYGQK sequence, and all displayed the apparent C₂H₂-type zinc finger (CX₄CX₂₂₋₂₃HXH). Among the 34 group II proteins containing one WRKY domain, 32 CiWRKYs carried the conserved WRKYGQK sequence, while CiWRKY50 and CiWRKY51 exhibited the WRKYGKK sequence instead. The C₂H₂-type zinc finger (CX₄₋₅CX₂₃HXH) was observed in 33 CiWRKYs. Seven of the eight group III WRKYs harbored the WRKYGQK

sequence, while the variant sequence WRKYGKK was present in CiWRKY41–2. The other group III CiWRKYs exhibited C₂HC-type zinc finger (CX₇CX₂₃HXC) with the exception of CiWRKY41–3 (Fig. 2).

Subcellular localization of CiWRKYs

To examine the subcellular localization of the CiWRKY proteins, one of the proteins containing two WRKY domains (CiWRKY26) and one of the proteins containing one-WRKY-domain (CiWRKY28–1) were fused with green fluorescent protein (GFP). The roots of the T2 generation of 35S::CiWRKYs-GFP transgenic *Arabidopsis*

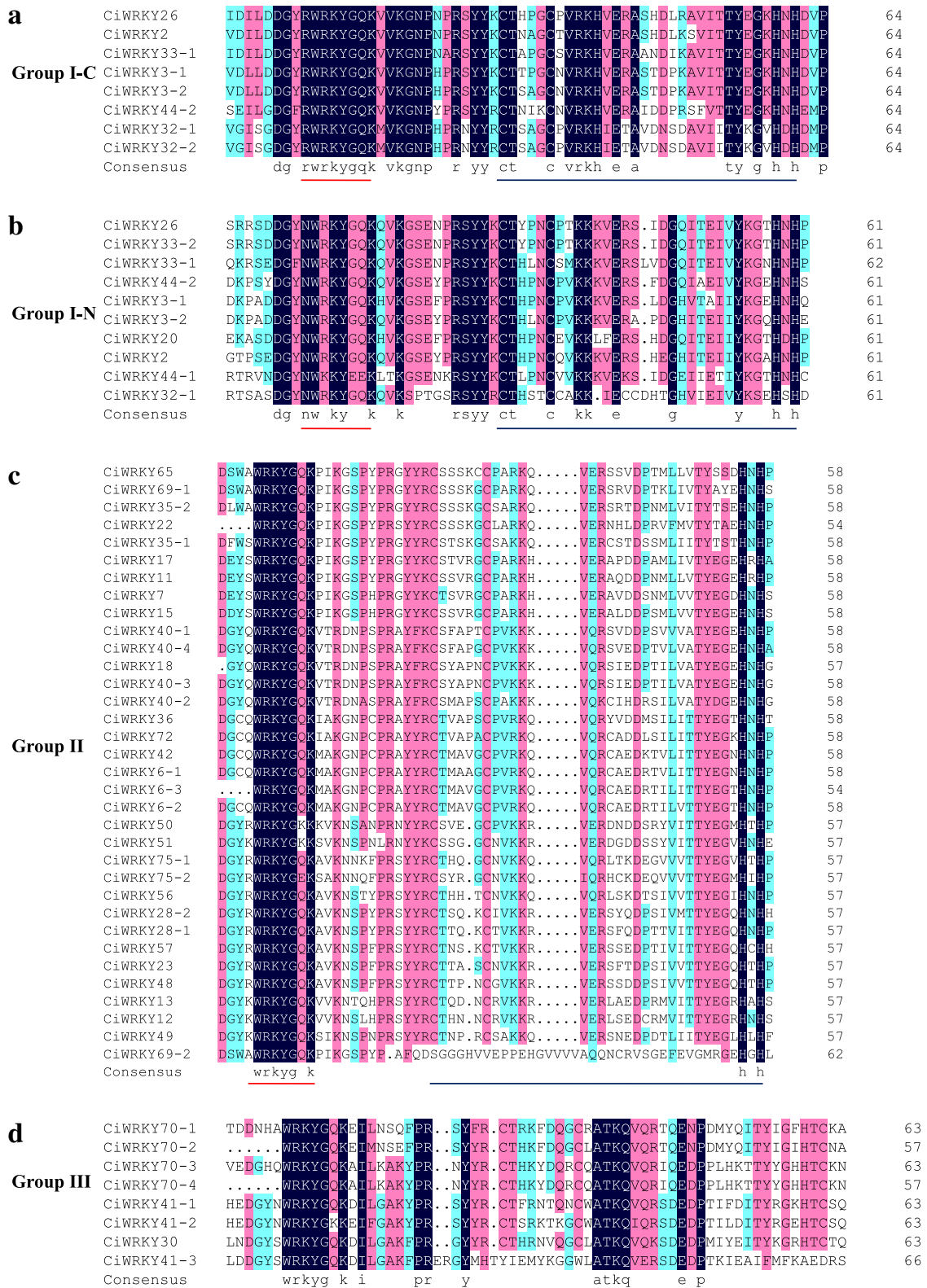


Fig. 2 Multiple sequence alignments of the WRKY domains of from CiWRKYs. **a** C-terminal WRKY domains of group I proteins. **b** N-terminal WRKY domains of group I proteins. **c** WRKY domains of group II proteins. **d** WRKY domains of group III proteins. The conserved WRKY amino acid signature is indicated by red bars, and the zinc finger motif is indicated by black bars

seedlings were used to examine GFP activity. GFP fluorescence was exclusively observed in the nuclei of the transgenic *Arabidopsis* seedlings, while GFP was ubiquitously present throughout the cells of the *35S::GFP* transgenic lines (Fig. 3). The nuclear localization of these two *CiWRKYs* is in agreement with their putative roles as transcription factors.

Expression patterns of *CiWRKYs* in *C. intermedia*

The expression patterns of the 28 *CiWRKYs* with complete ORFs in wild *C. intermedia* tissues, including the roots, stems and leaves, were examined via qRT-PCR. As shown in Fig. 4 and Additional file 1: Table S1, four genes (*CiWRKY3-2*, *CiWRKY6-2*, *CiWRKY23* and *CiWRKY26*) exhibited relatively high expression levels in all examined tissues, especially in the leaves. *CiWRKY32-1*, *CiWRKY17* and *CiWRKY15* were also ubiquitously expressed, but at relatively low levels compared with the above four genes. In contrast, the transcripts of eight genes, including *CiWRKY36*, *CiWRKY75-1*, *CiWRKY12*, *CiWRKY50*, *CiWRKY28-1*, *CiWRKY48*, *CiWRKY40-4* and *CiWRKY6-1*, were detectable, but showed extremely low abundance in all tissues. *CiWRKY28-2* and *CiWRKY56* were mainly expressed in stems, while *CiWRKY40-1* and *CiWRKY30* were predominantly found in leaves. *CiWRKY69-1* showed higher transcript abundance in the roots than in other tissues. The other *CiWRKY* genes showed lower expression globally, and most of them exhibited lower transcript abundance in the roots than in the leaves or stems.

Expression patterns of *CiWRKYs* under abiotic stresses

Malondialdehyde (MDA), proline (PRO) and soluble total sugar (SS) are indicators of plant physiological status. As shown in Additional file 2: Figure S1, drought treatment induced the accumulation of MDA, PRO and SS, whose levels peaked at 48 h. These findings indicated that the applied drought treatment was effective.

The expression patterns of the 28 of *CiWRKYs* were further assessed using qRT-PCR, under drought treatment as well as other abiotic stresses, including salt, abscisic acid (ABA), cold, and high-pH treatments (Table 2). The gene expression of twenty-four *CiWRKYs* genes was found to be induced by drought treatment, with changes of 2- to 149-fold. In contrast, the expression of *CiWRKY33-1*, *CiWRKY15* and *CiWRKY69-1* was inhibited by drought treatment, and no obvious change in the expression of *CiWRKY32-1* was observed (Fig. 5). Salt treatment down-regulated the expression of 18 *CiWRKYs* and up-regulated the expression of 5 *CiWRKYs* (Additional file 3: Figure S2). Under ABA treatment, the expression of 22 *CiWRKYs* was increased, while that of *CiWRKY3-1* and *CiWRKY33-1* was decreased, and *CiWRKY2*, *3-2*, *32-1* and *69-1* were not obviously affected (Additional file 4: Figure S3). Under cold treatment, the expression of 14 *CiWRKYs* was up-regulated, and the transcripts of 11 *CiWRKYs* were down-regulated. The expression of three other *CiWRKYs* presented no change compared with the expression seen in the control (Additional file 5: Figure S4). High-pH treatment enhanced the expression of 12 *CiWRKYs*.

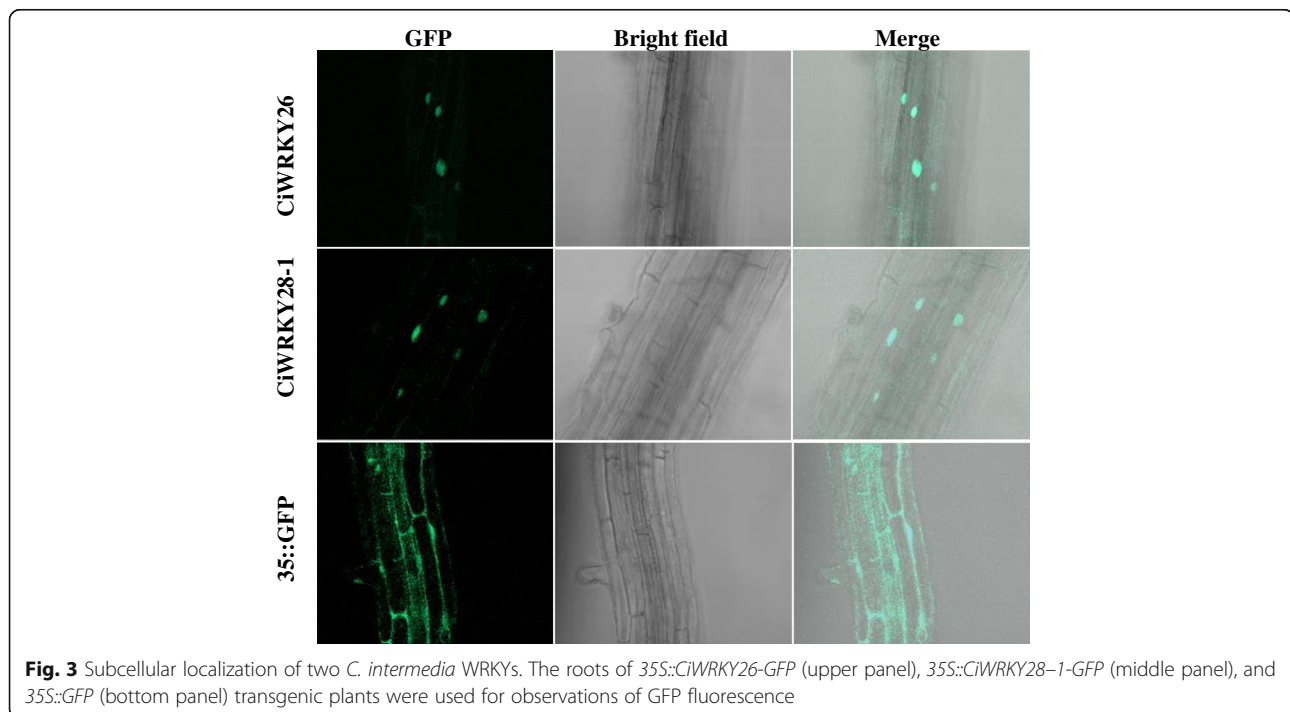
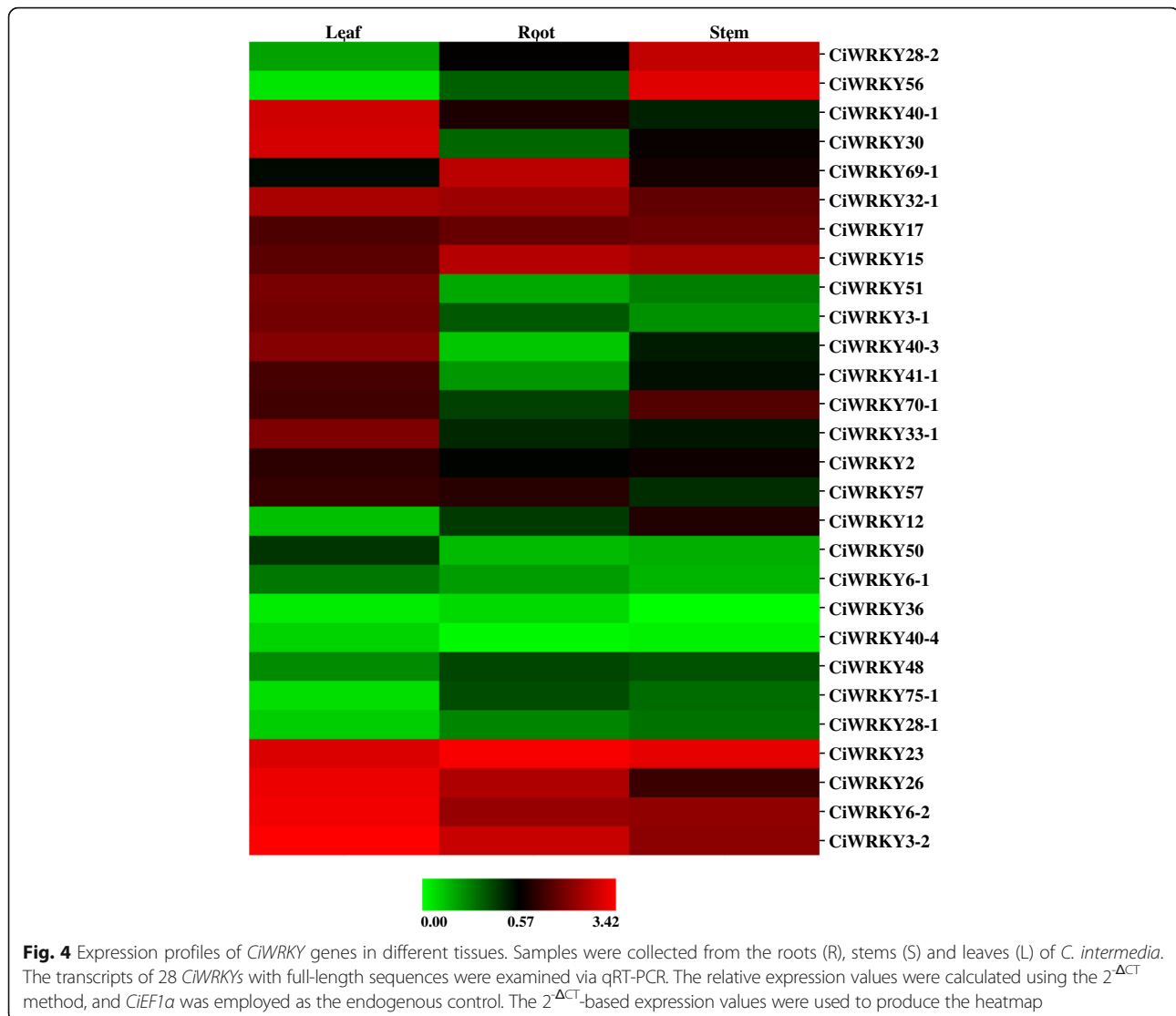


Fig. 3 Subcellular localization of two *C. intermedia* WRKYs. The roots of *35S::CiWRKY26-GFP* (upper panel), *35S::CiWRKY28-1-GFP* (middle panel), and *35S::GFP* (bottom panel) transgenic plants were used for observations of GFP fluorescence



Notably, the maximal expression observed for *CiWRKY33-1*, *41-1*, *50*, and *40-4* corresponded to increases of 111-, 365-, 388-, and 109-fold, respectively, whereas the expression of the other *CiWRKY*s was not affected or even down-regulated after high-pH treatment (Additional file 6: Figure S5). The results indicated that each of these *CiWRKY* genes responded to at least one stress treatment.

***CiWRKY75-1* transgenic *Arabidopsis* is hypersensitive to drought stress**

CiWRKY75-1, which exhibited relatively high expression under all five treatments (Table 2), was over-expressed in *Arabidopsis*. Three representative overexpression lines (*CiWRKY75-1-OE5*, *CiWRKY75-1-OE6* and *CiWRKY75-1-OE8*) showing relatively high expression levels were used to perform abiotic stress tolerance tests. Four-week-old transgenic *Arabidopsis* were exposed to drought stress by withholding watering for 15 days and were then re-

watered for 3 days. The *CiWRKY75-1*-overexpressing lines were more sensitive to drought, displaying decreased survival rates and significantly increased MDA levels compared with the wild type (Fig. 6). No morphological differences were observed between the wild-type and *CiWRKY75-1*-overexpressing plants under normal growth conditions (Fig. 6). These results indicated that overexpression of *CiWRKY75-1* reduced the tolerance of *Arabidopsis* to drought stress, and *CiWRKY75-1* acted as a negative regulator in the plant response to drought.

***CiWRKY40-4*-overexpressing lines show delayed leaf senescence**

According to the observed stress-responsive expression patterns, *CiWRKY40-4* was another gene that was induced under all five treatments (Table 2). However, ectopic expression of *CiWRKY40-4* in *Arabidopsis* resulted in no obvious morphological differences from the

Table 2 Expression patterns of *CiWRKYs* under different abiotic stresses

Gene name	Drought	Salt	ABA	Cold	High-pH
<i>CiWRKY2</i>	up	N	N	down	up
<i>CiWRKY3-1</i>	up	down	down	N	N
<i>CiWRKY3-2</i>	up	down	N	up	N
<i>CiWRKY6-1</i>	up	down	up	down	N
<i>CiWRKY6-2</i>	up	up	up	up	up
<i>CiWRKY12</i>	up	down	up	down	down
<i>CiWRKY15</i>	down	down	up	up	up
<i>CiWRKY17</i>	up	down	up	down	down
<i>CiWRKY23</i>	up	N	up	N	N
<i>CiWRKY26</i>	up	up	up	up	up
<i>CiWRKY28-1</i>	up	down	up	down	down
<i>CiWRKY28-2</i>	up	N	up	down	N
<i>CiWRKY30</i>	up	down	up	up	up
<i>CiWRKY32-1</i>	N	down	N	N	down
<i>CiWRKY33-1</i>	down	down	down	up	up
<i>CiWRKY36</i>	up	down	up	down	N
<i>CiWRKY40-1</i>	up	up	up	up	up
<i>CiWRKY40-3</i>	up	down	up	up	N
<i>CiWRKY40-4</i>	up	up	up	up	up
<i>CiWRKY41-1</i>	up	N	up	up	up
<i>CiWRKY48</i>	up	down	up	down	down
<i>CiWRKY50</i>	up	down	up	down	up
<i>CiWRKY51</i>	up	down	up	down	up
<i>CiWRKY56</i>	up	down	up	down	down
<i>CiWRKY57</i>	up	down	up	up	N
<i>CiWRKY69-1</i>	down	down	N	up	N
<i>CiWRKY70-1</i>	up	N	up	up	down
<i>CiWRKY75-1</i>	up	up	up	up	up

One-month-old *C. intermedia* seedlings were subjected to drought, salt, ABA, cold and high-pH treatments, and shoot samples were collected at 0.5, 1, 3, 6, 12, 24 or 48 h. Untreated plants were employed as controls. "Up" indicates up-regulated expression with a minimum 2-fold change compared with the control. "Down" indicates down-regulated expression with a minimum 2-fold change compared with the control. "N" indicates no obvious change detected

wild type under drought stress. Unexpectedly, under normal growth conditions, *CiWRKY40-4*-overexpressing *Arabidopsis* showed delayed leaf senescence compared with the wild type (Fig. 7). To further explore the mechanism of *CiWRKY40-4* in leaf senescence, senescence related-genes, including the senescence-associated genes *SAG12*, *SAG13* and *SAG29* and the chlorophyll degradation-related genes *pheophytinase* (*PPH*), *pheophorbide a oxygenase* (*PAO*), *non-yellowing1/stay-green1* (*NYE1/SGR1*), *non-yellow coloring1* (*NYC1*) and *NYC1-like* (*NOL*) were analyzed. All of these genes were down-regulated in the *CiWRKY40-4*-overexpressing lines compared with the wild type (Fig. 8), indicating that

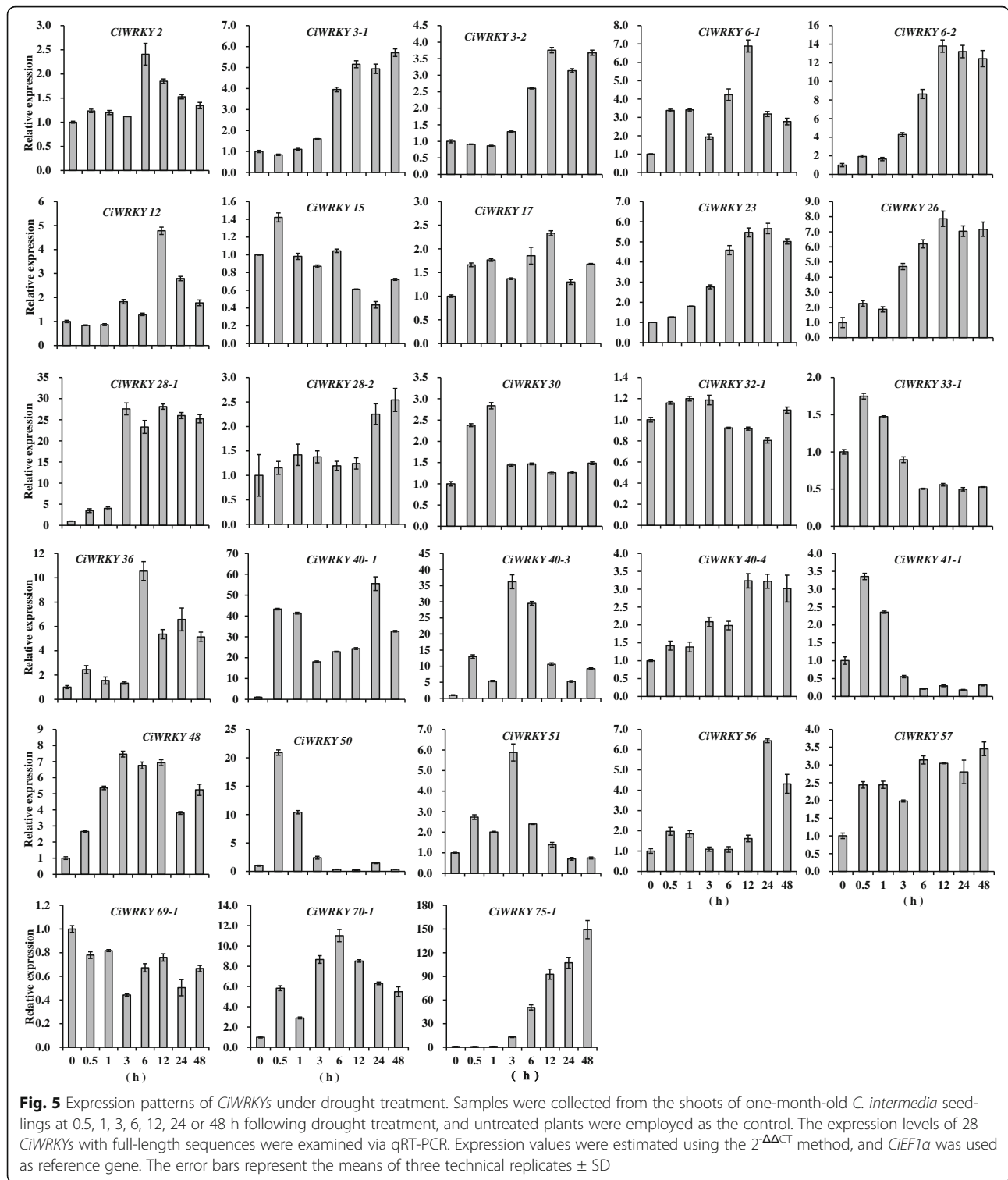
CiWRKY40-4 delayed plant senescence by negatively regulating senescence-associated gene expression in *Arabidopsis*.

Discussion

In this study, we identified 53 *CiWRKY*-encoding genes from the drought-treated transcriptome database (NCBI Sequence Read Achieve (SRA) accession number SRP121096) based on the presence of WRKY domains [3]. According to the number of their WRKY domains and the features of their zinc finger-like motifs, the *CiWRKYs* were divided into three groups, as generally described, among which the group II *CiWRKYs* accounted for the largest proportion of 64%. These findings are consistent with reports regarding the WRKYs found in *A. thaliana* [3], cassava [11], and soybean [7], which harbor the greatest numbers of group II WRKYs in the family.

Generally, WRKY proteins are defined by the conserved amino acid sequence WRKYGQK [3]; however, this sequence has been replaced by WRRY, WSKY, WKRY, WVKY or WKKY in some WRKY proteins [5]. In the present study, the WKKYEEK variant was observed in *CiWRKY44-1* proteins, and the WRKYGKK variant motif replaced the conserved WRKYGQK sequence in *CiWRKY41-2*, 50 and 51. These findings are consistent with reports describing the WRKY proteins identified in other species, such as *A. thaliana* (*AtWRKY51*, 52 and 59) [3], *V. vinifera* L. (*VvWRKY8*, 13, 14 and 24) [53], and *P. miliaceum* L. (*PmWRKY2*, 15, 23, 24, and 28) [14].

According to recent reports [54], several of the WRKYs have more than 2 WRKY domains or contain other domains, in addition to the WRKY domains. These other domains include, for example, the ZF_SBP TF domain, kinase domain, PAH domain, ULP_protease domain, TIR domain, LRR domains, NAC domain, ATP_GRASP domain, and B3 domain. Based on these findings, additional novel groups, beyond the three known WRKY groups, were identified [54]. In *A. thaliana*, *Fragaria vesca*, *Brassica rapa*, *G. max*, *G. raimondii*, and *Sorghum bicolor*, 1, 5, 3, 1, 2, and 3 novel WRKY proteins have been found, respectively [54]. We report here that all of the 28 identified *CiWRKYs* with ORFs contained one or two WRKY domains. In addition to the WRKY domains, NCBI conserved domain database (CDD) annotation [55] showed that *CiWRKY36* (Group Iib) contains a DUF972 (domain of unidentified function 972) domain, followed by a WRKY domain, and both *CiWRKY15* and *CiWRKY17* (Group Iid) harbor a plant_Zn_cluster domain, which is located at the N-terminus and is followed by a WRKY domain. *CiWRKY6-1* (Group Iib) exhibits a bZIP (basic region/leucine zipper) domain, followed by a WRKY domain.



DUF972 belongs to a protein family of unknown function. The plant_Zn_cluster domain is present in two *CiWRKYs* (*CiWRKY15* and *CiWRKY17*) and is located at the N-terminus of the WRKY domain. Its sequence (EHSDDVSGSGKCHCVKRRKRNVRKRTVRVPAISSKIAD)

is different from the zinc finger structure, and this domain may have additional functions. bZIP transcription factors are also one of the largest gene families in plants and are involved in multiple plant biological processes, including various stress responses [56, 57].

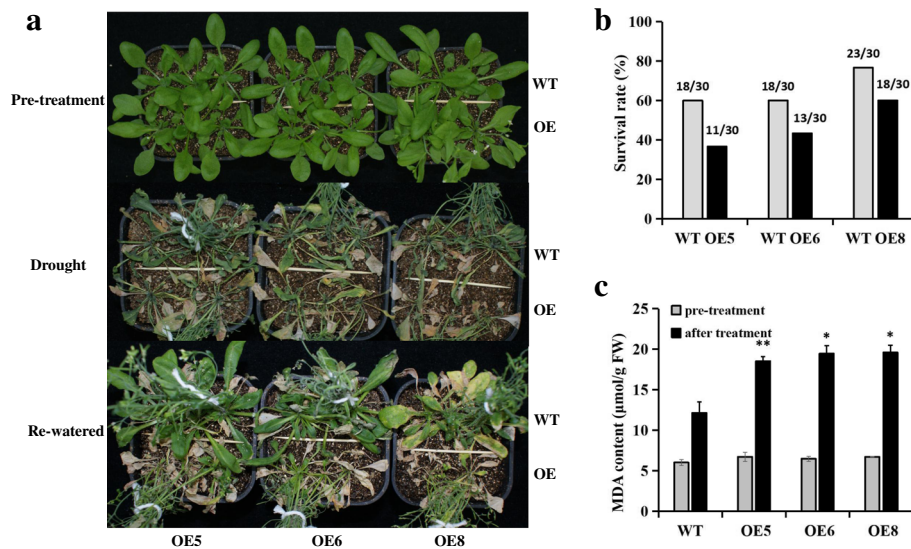


Fig. 6 Transgenic *Arabidopsis* overexpressing *CiWRKY75-1* is hypersensitive to drought stress. Four-week-old *CiWRKY75-1* transgenic and wild-type *Arabidopsis* plants were subjected to drought stress by withholding water for 15 days, followed by re-watering for 2 days. **a** Photograph of representative plants. **b** Calculation of survival rates. Thirty plants from each genotype were used for statistical analysis. **c** Measurement of MDA contents. * indicates significant differences ($P < 0.05$); ** indicates extremely significant differences ($P < 0.01$). OE represents overexpression lines, WT represents wild-type *Arabidopsis*. The experiments were repeated three times with similar results

Evidence from genome- or transcriptome-wide analyses shows that the response of *WRKYs* to abiotic stresses varies according to the species. For instance, 30 putative *WRKYs* were identified through transcriptome analysis in Chinese wild hazel (*Corylus heterophylla Fisch*), and 23 of these *WRKYs* were found to be simultaneously up-regulated by cold, drought and high-salinity stresses [58]. Twenty-eight salt-responsive *GarWRKYs* were identified among 109 *WRKY* genes in a salt-tolerant wild cotton species (*Gossypium aridum*) based on transcriptome sequencing data [59]. In *P. miliaceum L.*, 32 *WRKYs* were identified based on transcriptome analysis,

and the expression levels of 22 *PmWRKYs* were observed to be significantly altered under at least one abiotic stress treatment [14]. Among the 85 *WRKY* genes identified from the *M. esculenta* genome, 78 were found to be differentially expressed in response to drought stress [11]. In addition, 34 out of 127 *WRKYs* from the apple genome were identified as differentially expressed under water-logging stress [8].

Drought is one of the most common environmental stress factors that significantly inhibits plant growth and crop production [60]. The roles of the *WRKY* transcription factors in the drought response have been widely

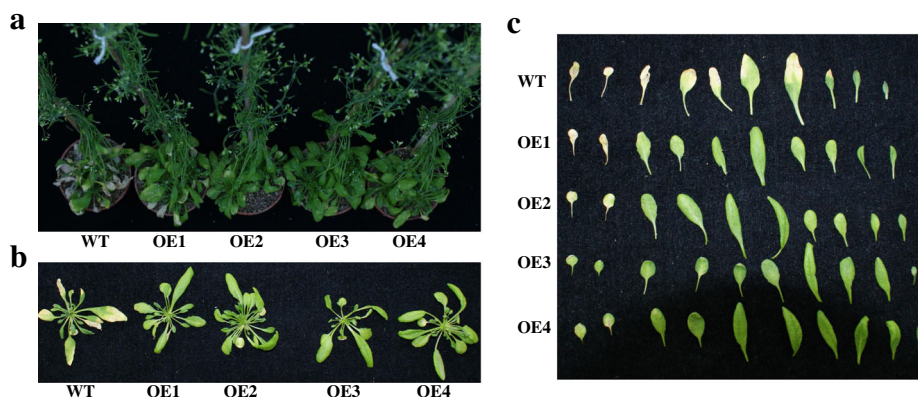


Fig. 7 Overexpression of *CiWRKY40-4* delayed *Arabidopsis* leaf senescence. Six-week-old *CiWRKY40-4*-overexpressing lines growing in normal conditions exhibited a stay-green phenotype compared with wild-type *Arabidopsis*. OE represents overexpression lines, WT represents wild-type *Arabidopsis*. **a** The adult plants of different genotypes growing under normal conditions. **b** The rosette leaves of the plants from **a**. **c** The detached rosette leaves from **b**

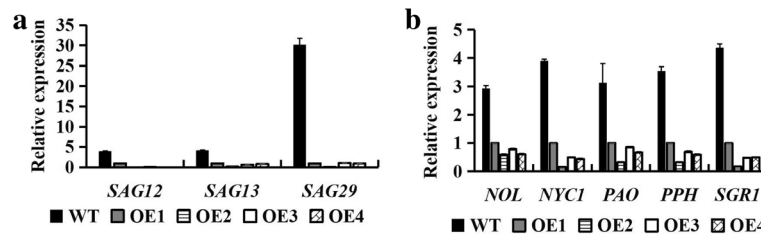


Fig. 8 Expression patterns of senescence-related genes in *CiWRKY40-4*-overexpressing and wild-type *Arabidopsis*. Leaf samples were collected from five-week-old *CiWRKY40-4*-overexpressing lines and wild-type *Arabidopsis* and were employed for expression analyses through quantitative real-time PCR. **a** Expression patterns of senescence-associated genes (*SAG12*, *SAG13* and *SAG29*). **b** Expression patterns of chlorophyll degradation-related genes (*PPH*, *PAO*, *SGR1/NYE1*, *NOL* and *NYE1*). Expression values were estimated using the $2^{-\Delta\Delta CT}$ method, and *AtEF1a* was used as the reference gene. The error bars represent the mean of three technical replicates \pm SD. The experiments were repeated three times with similar results

reported. Activated or constitutive expression of *WRKY57* confers drought tolerance in *A. thaliana* [34], and the expression of *AtWRKY57* is increased by abiotic stresses such as ABA, dehydration, and mannitol treatment [34]. Overexpression of *AtWRKY53* results in hypersensitivity to drought stress by inhibiting stomatal closure [61]. Overexpression of *TaWRKY1* and *TaWRKY33* in *Arabidopsis* leads to enhanced plant drought tolerance [62]. *WRKY20* transcripts accumulate in wild soybean (*G. soja*) under ABA, salt, cold, and drought stresses, and overexpression of *GsWRKY20* increases the drought tolerance in *A. thaliana* [33].

We performed a transcriptome analysis to profile gene expression patterns in *C. intermedia* in response to drought stress and identified 53 *CiWRKY* genes with varying expression patterns in response to drought stress (Additional file 7: Table S2). Further investigation showed that the transcripts of 28 *CiWRKYs* (with complete ORFs) responded to at least one abiotic stress when plants were subjected to drought, cold, salt, high-pH, and ABA treatments. The expression levels of fifteen genes (*CiWRKY12*, 17, 15, 28-1, 30, 33-1, 40-1, 50, 51, 48, 56, 26, 75-1, 40-4, and 6-2) were altered under all five treatments. Notably, *CiWRKY40-1* and *CiWRKY75-1* responded to all treatments intensively. In *A. thaliana*, *AtWRKY75*, which is a homologue of *CiWRKY75-1* sharing 85% amino acid sequence similarity, has been reported to regulate Pi starvation and root development [63] and to play a role in plant resistance to pathogens [64, 65]. In the present study, the overexpression of *CiWRKY75-1* in *Arabidopsis* compromised the drought stress tolerance of plants. This finding has not been reported by other researchers, and suggests that *CiWRKY75-1* serves as a negative regulator under drought stress. Both the mRNA and protein of *AtWRKY53* are reported to accumulate in *Arabidopsis* following drought stress, and *AtWRKY53* overexpression enhances hypersensitivity to drought stress compared with that in the wild type, via inhibition of stomatal

closure to increase the water loss rate [61]. The *AtWRKY46* transcript is also induced by drought stress in *Arabidopsis* [66], and the *AtWRKY46/54/70* signaling complex negatively regulates plant drought tolerance by repressing dehydration-inducible gene expression, which is likely achieved via the simultaneous function of *BES1* [67]. However, all four *Arabidopsis* *WRKYs* belong to group III, while *CiWRKY75-1* belongs to group IIc; thus, the mechanism underlying the regulation of the drought tolerance signaling pathway by *CiWRKY75-1*, either through direct or indirect interaction with downstream genes, remains unclear. Additionally, no morphological differences during root development were observed between the overexpression lines and wild-type *Arabidopsis*, and the functions of *CiWRKY75-1* in pathogen infection and Pi starvation still require further investigation.

CiWRKY40-4 is in the same group IIa clade as *WRKY40*, *WRKY18*, and *WRKY60* of *A. thaliana* and is most similar to *AtWRKY40*. In *A. thaliana*, *WRKY18*, *WRKY40* and *WRKY60* play partially redundant roles in response to *P. syringae* and *B. cinerea* infection [68]. The *wrky18wrky40* double mutant displays enhanced resistance towards the powdery mildew *Golovinomyces orontii*. This mutant is also involved in PAMP-triggered basal defense and acts as a negative regulator of positive defense regulators such as *CYP71A13*, *EDS1* and *PAD4* [69]. In addition, the expression of *WRKY40*, *WRKY18*, and *WRKY60* is elevated under ABA, NaCl and PEG treatments [44]. The *wrky40* knockout mutant of *A. thaliana* exhibits a sensitive phenotype, while *WRKY40*-overexpressing lines show a less sensitive phenotype than the wild type under ABA-induced inhibition of seed germination [44]. *WRKY40* represses the ABA-responsive genes, such as *ABI5*, and functions as negative regulator of in ABA signaling by interacting with *ABAR* [70]. *AtWRKY40* acts as a negative regulator of pathogen-induced cell death regulated by *NAC4* [71]. The transcription of *WRKY40*, *WRKY46*, *WRKY51*, *WRKY60*, *WRKY63*,

and *WRKY75* is elevated in a loss-of-function mutant of *FtSH4*, which encodes a mitochondrial protease that is involved in SA-mediated leaf senescence [72]. In the present study, overexpressing *CiWRKY40-4* in *A. thaliana* did not alter drought tolerance but delayed leaf senescence by down-regulating the expression levels of senescence-related genes. Senescence is the final step in leaf development and allows nutrients from old leaves to be salvaged [73]. *SAGs*, which are markers of senescence, are up-regulated in the senescing leaves of *A. thaliana* [74]. Overexpression of *SAG29* has been shown to result in accelerated senescence, and osmotic stresses, such as high salinity, cold, and drought, induce the accumulation of its transcripts [75].

NYC1 and *NOL* encode chlorophyll *b* reductase, which is involved in the biochemical pathway of chlorophyll degradation [76] and accelerates the reduction reaction of chlorophyll *b* in conversion to chlorophyll *a* [73, 76]. *PPH* is an essential enzyme for chlorophyll breakdown that catalyzes the transformation of pheophytin *a* (Mg-free chlorophyll *a*) into pheophorbide *a* [77], which is then converted to a red chlorophyll catabolite by *PAO* [78]. The activity of *PAO* is modulated by *NYE1*, which is also known as *SGR1* [79, 80]. In *A. thaliana*, a *PPH*-deficient mutant (*pph-1*) exhibits a stay-green phenotype due to the prevention of chlorophyll degradation during senescence [77]. The *Atnye1*, *Atpao1*, *Osnol1* and *Osnyc1* mutants all exhibit a stay-green phenotype under natural and/or induction conditions [73, 76, 79, 81]. The down-regulation of *SAGs* (12, 13 and 29), *NYC1*, *NOL*, *PPH*, *PAO* and *NYE1/SGR1*, in *CiWRKY40-4*-overexpressing *Arabidopsis* indicates that *CiWRKY40-4* delays *Arabidopsis* leaf senescence by directly or indirectly down-regulating these genes; however, further investigation will be required to elucidate the mechanism.

Taken together, these results indicate that different *CiWRKYs* play different roles in plant responses to abiotic stress and developmental processes. Hence, there is an urgent need for examination of the function of *CiWRKYs* in tolerance against abiotic stresses in *C. intermedia* in future studies.

Conclusions

In this study, 53 *WRKY* sequences were retrieved from the drought-treated transcriptome of *C. intermedia*, among which 28 *CiWRKYs* exhibited full-length sequences. This is the first study to analyze *WRKY* family genes in *C. intermedia* based on transcriptome data. The predicted proteins were grouped via phylogenetic tree analysis with *WRKYs* from *A. thaliana* and *G. max*, and their *WRKY* domains were characterized through multiple sequence alignment of the *CiWRKY* genes. Furthermore, the subcellular localization of both *CiWRKY28-1*, with one *WRKY* domain, and *CiWRKY26*, with two

WRKY domains, is nuclear. The majority of the 28 *CiWRKYs* with full-length sequences are expressed in more than one tissue. The expression patterns of the 28 *CiWRKYs* in response to different abiotic stresses (drought, salt, cold, high-pH and ABA) were examined, and the abiotic stress-responsive genes were further evaluated. Additionally, overexpression of *CiWRKY75-1* and *CiWRKY40-4* in *A. thaliana* resulted in hypersensitivity to drought stress and delayed leaf senescence, respectively, compared with the wild type. The results of this study will be useful for understanding the involvement of *WRKY* genes in stress resistance and plant development and will provide the basis for future functional studies on *WRKYs*.

Methods

WRKY identification, phylogenetic analysis and multiple sequence alignment

CiWRKY sequences were derived from a drought-treated RNA-seq database of *C. intermedia*, in which dehydration treatment was performed by placing the whole seedlings on filter paper for 1, 3 or 12 h. The dataset was submitted to NCBI SRA under the accession number SRP121096. The *WRKY* sequences of *A. thaliana* and *G. max* were downloaded from the TAIR database (The Arabidopsis Information Resource: <http://www.arabidopsis.org/>) and the JGI database (<https://phytozome.jgi.doe.gov>), respectively.

The conserved protein domains were subjected to Blast searches against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) using blastx. After the removal of redundant sequences and *WRKY* domain prediction, a total of 53 putative *CiWRKYs* containing the *WRKY* domain were obtained, which were named according to their similarity with *Arabidopsis* *WRKYs*.

A phylogenetic tree of *CiWRKYs* was constructed using MEGA6.0 and the neighbor-joining (NJ) method, with 1000 bootstrap replications, based on the amino acid sequences of *A. thaliana* and *G. max* *WRKY* proteins.

DNAMAN7 software was used to analyze the core sequences of the *WRKY* domain via multiple sequence alignment.

Plant materials and abiotic stress treatments

Seeds from wild *C. intermedia* were collected from Wulanchabu City, Inner Mongolia Autonomous Region, China (41.44N, 111.69E). No specific field permissions were required to collect the plant samples. The plant material used in this study had been formally identified by Dr. Liwang Qi (Chinese Academy of Forestry), Prof. Ling Yan (Inner Mongolia Agricultural University) and Prof. Meng Ji (Inner Mongolia Academy of Forestry).

The seeds were sown in pots containing peat soil and vermiculite (1:2 v/v) under long-day conditions (16-h-light/8-h-dark cycle) at 22 °C. One-month-old plants were employed for the abiotic stress treatments, as previously described [51, 82]. Briefly, prior to the drought, salt, high-pH (10) and ABA treatments, whole seedlings were removed from soil and cleaned with tap water. For drought treatment, whole seedlings were placed on filter paper for 0.5, 1, 3, 6, 12, 24 or 48 h at room temperature. For the salt, high-pH (10) and ABA treatments, the roots of the seedlings were soaked with solutions containing 300 mM NaCl, 200 mM NaHCO₃ (using NaOH to adjust the pH to 10) or 100 μM ABA, respectively, for 0.5, 1, 3, 6, 12, 24 or 48 h. For cold treatment, pots containing seedlings were transferred to 4 °C and then cultured for 0.5, 1, 3, 6, 12, 24 or 48 h. Untreated plants were used as controls. At each time point in each treatment, the shoots of three plants were harvested as one sample; each plant was only used once for tissue harvesting, at one time point, and was not subjected to any further treatment thereafter. 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.

Additionally, the roots, stems, and leaves of wild *C. intermedia* were collected during the flowering stage for tissue-specific expression analysis.

The leaves of four-week-old wild-type and transgenic *Arabidopsis* were used for relative gene expression analysis.

PRO, SS and MDA measurements

One-month-old *C. intermedia* seedlings cultured in growth chambers were subjected to drought treatment, and physiological indices were measured at different time points (0, 1, 3, 6, 12, 24 or 48 h). The contents of PRO, MDA and SS were determined in colorimetric assays, using the ninhydrin coloration, thiobarbituric acid and anthrone methods, respectively [83].

Total RNA extraction and cDNA synthesis

Total RNA was isolated from samples using the TRIzol reagent. For tissue-specific expression, RNA was extracted from different tissues, including the roots, stems, and leaves. For the examination of abiotic stress-responsive expression, RNA was extracted from shoots. For gene expression analysis in *Arabidopsis*, RNA was extracted from leaves. RNA integrity was examined through agarose gel electrophoresis, and RNA purity was determined based on the A_{260 nm}/A_{280 nm} and A_{260 nm}/A_{230 nm} ratios.

After pretreatment with RNase-free DNase I (Takara, Dalian, China), total RNA (1 μg), was used to synthesize first-strand cDNA employing an M-MLV reverse

transcriptase kit (Takara) according to the manufacturer's instructions. The cDNA was then diluted 16-fold to be employed as a template for qRT-PCR analysis.

qRT-PCR analysis of gene expression

qRT-PCR was performed using SYBR Premix Ex Taq II (Takara) on a LightCycler 480 Real Time PCR system (Roche, Basel, Switzerland), as previously described [84]. The thermal cycling program was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 15 s. *CiEF1α* (GenBank: KC679842) [51] was employed as a reference gene to normalize target gene expression levels in the *C. intermedia* samples, and the 2^{-ΔΔCT} and 2^{-ΔCT} methods [85] were used to calculate the relative expression levels of stress-responsive and tissue-specific genes, respectively. *AtEF1α* (At5G60390) [84] was employed as a reference gene for the normalization of target gene expression levels in *Arabidopsis* samples via the 2^{-ΔΔCT} method [85]. Three technical replicates for each reaction were performed. The primers employed for qRT-PCR analysis are listed in Additional file 8: Table S3.

CiWRKY transgenic *A. thaliana*

To generate the recombinant *CiWRKY* overexpression vector, the full-length CDSs of *CiWRKY75-1* and *CiWRKY40-4* were amplified using PCR product from wild *C. intermedia* cDNA and cloned into the expression vector pCanG-HA using the restriction enzymes *SalI*/*SpeI* and *SacI*/*SalI*, respectively.

To generate the recombinant *CiWRKY* and *GFP*-infused vectors, the coding sequences of *CiWRKY26* and *CiWRKY28-1* without the stop codon were amplified using PCR, and the PCR products were then cloned into the N-terminus of the *GFP*-encoding sequence in the pCambia1302 vector driven by the cauliflower mosaic virus (CaMV) 35S promoter. The primers used for the construction of all of the recombination vectors are listed in Additional file 8: Table S3.

The recombinant vectors were expressed in wild-type *A. thaliana* using the floral dipping method [86], mediated by *Agrobacterium tumefaciens* (strain GV3101). The empty vector was used as the control.

Subcellular localization of *CiWRKYs*

The roots of 10-day-old T₂-generation transgenic seedlings were employed for observations of GFP fluorescence under an LSM510 confocal laser-scanning microscope (Carl Zeiss), with 488 nm argon excitation and a 505–530 nm band filter, as previously described [84].

Drought tolerance test

To assess the potential drought tolerance of the *CiWRKY*-overexpressing lines, four-week-old transgenic plants and wild-type plants growing in the same pots were exposed to drought stress by withholding water for 15 days, and the plants were then re-watered and allowed to grow for an additional 3 days. Survival rates were calculated using 30 plants per genotype for each experiment. The leaf samples were harvested before or immediately after drought treatment to measure physiological indices. This experiment was repeated three times with similar results.

Additional files

Additional file 1: Table S1. Expression data for *CiWRKY* genes in different *C. intermedia* tissues. The expression data were generated via qRT-PCR, and calculations were performed using the $2^{-\Delta\Delta CT}$ method; *CIEF1a* was used as an endogenous control. (XLS 22 kb)

Additional file 2: Figure S1. Changes in physiological activity in *C. intermedia* under drought treatment. The abscissa indicates the time points in the treatments, and the ordinate indicates physiological activities. (a) MDA content. (b) Pro content. (c) SS content. (PDF 112 kb)

Additional file 3: Figure S2. Expression patterns of *CiWRKYs* under salt treatment. Samples were collected from the shoots of one-month-old *C. intermedia* seedlings at 0.5, 1, 3, 6, 12, 24 or 48 h following salt treatment, and untreated plants were employed as the control. The expression levels of 28 *CiWRKYs* with full-length sequences were examined via qRT-PCR. Expression values were estimated using the $2^{-\Delta\Delta CT}$ method, and *CIEF1a* was used as reference gene. The error bars represent the means of three technical replicates \pm SD. (PDF 258 kb)

Additional file 4: Figure S3. Expression patterns of *CiWRKYs* under ABA treatment. Samples were collected from the shoots of one-month-old *C. intermedia* seedlings at 0.5, 1, 3, 6, 12, 24 or 48 h following ABA treatment, and untreated plants were employed as the control. The expression levels of 28 *CiWRKYs* with full-length sequences were examined via qRT-PCR. Expression values were estimated using the $2^{-\Delta\Delta CT}$ method, and *CIEF1a* was used as reference gene. The error bars represent the means of three technical replicates \pm SD. (PDF 254 kb)

Additional file 5: Figure S4. Expression patterns of *CiWRKYs* under cold treatment. Samples were collected from the shoots of one-month-old *C. intermedia* seedlings at 0.5, 1, 3, 6, 12, 24 or 48 h following cold treatment, and untreated plants were employed as the control. The expression levels of 28 *CiWRKYs* with full-length sequences were examined via qRT-PCR. Expression values were estimated using the $2^{-\Delta\Delta CT}$ method, and *CIEF1a* was used as reference gene. The error bars represent the means of three technical replicates \pm SD. (PDF 253 kb)

Additional file 6: Figure S5. Expression patterns of *CiWRKYs* under high-pH treatment. Samples were collected from the shoots of one-month-old *C. intermedia* seedlings at 0.5, 1, 3, 6, 12, 24 or 48 h following high-pH treatment, and untreated plants were employed as the control. The expression levels of 28 *CiWRKYs* with full-length sequences were examined via qRT-PCR. Expression values were estimated using the $2^{-\Delta\Delta CT}$ method, and *CIEF1a* was used as reference gene. The error bars represent the means of three technical replicates \pm SD. (PDF 257 kb)

Additional file 7: Table S2. Expression data for 28 *CiWRKY* genes with complete ORFs based on drought-treated transcriptome data. (XLS 22 kb)

Additional file 8: Table S3. Primers employed for qRT-PCR and the analysis of transgenic plant construction. (XLS 27 kb)

Abbreviations

ABA: Abscisic acid; GFP: Green fluorescent protein; MDA: Malondialdehyde; NOL: NYC1-like; NYC1: Non-yellow coloring1; NYE1/SGR1: Non-yellowing1/stay-green1; OE: Overexpression; ORF: Open reading frame; PAO: Pheophorbide a oxygenase; PPH: Pheophytinase; PRO: Proline; qRT-PCR: Quantitative real-time PCR; RACE: Rapid-amplification of cDNA ends; SS: Soluble total sugar

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Availability of data and materials

The transcriptome clean raw reads data that support the findings of this study have been deposited in NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/Traces/sra_sub/) under the accession number SRP121096. The sequence of *CiWRKYs* analyzed during the current study is available from the corresponding author on reasonable request. Other relevant data analyzed during this study are included in this published article and its supplementary information files.

Authors' contributions

RW, GL and YW conceived and designed the study. YW and MM performed the experiments. YW and DW integrated and analyzed the data. YW, MM and DW prepared the figures; QY, FY and MD participated in the design of the experiments. DW and YW wrote the manuscript, and GL revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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