BMC Genomics

RESEARCH

Open Access

Genome-wide identification and analysis of the *WRKY* gene family in Sainfoin (*Onobrychis viciifolia*) and their response to drought, salt, and alkali stress

Wenxiang Li[1,2†], Yinuo Yan[1†], Yanxia Liang[1,3†], Shiyao Yue[1,5], Yaling Liu[6], Feng Yuan[6], Mengrong Xu[1,4], Dengxia Yi[1], Jun Li[3], Yan Zhao[4], Lili Nan[2*] and Wenbo Jiang[1,7*]

Abstract

Background The *WRKY* gene family plays important roles in plant growth, development, and response to various stresses. Despite the significance of Sainfoin (*Onobrychis viciifolia*) as an important forage legume species, *WRKY* genes in this species have not been studied thoroughly. The aim of this study was to identify *WRKY* genes family in Sainfoin genome, particularly those linked to response to drought, salt, and alkali stresses.

Results A total of 253 *WRKY* genes (*OvWRKY*) were identified in Sainfoin. Phylogenetic analysis and conserved domain analysis divided these genes into seven subfamilies with similar conserved motifs but different gene structures. Collinearity analysis identified 232 duplicated gene pairs, including four tandem repeat pairs, indicating role of segmental duplication in the expansion of the *OvWRKY* gene family. Transcriptome analysis revealed that highly expressed genes under drought, salt, and alkali stress mostly belonged to groups I, III, IId, IIa, and IIb. *OvWRKY89* was down-regulated, and *OvWRKY147* was up-regulated by drought, salt, and alkali treatments. *OvWRKY240* and *OvWRKY164* were down-regulated by both drought and salt stress, and *OvWRKY36*, *OvWRKY107*, *OvWRKY65*, and *OvWRKY200* were down-regulated by both salt and alkali stress. Protein association network analysis using STRING suggested that OvWRKY89 is functionally associated with five other WRKY proteins and four stress-related proteins.

Conclusions In this study, we identified and analyzed the *WRKY* gene family members in Sainfoin for their physicochemical properties, gene structures, conserved motifs, phylogenetic relationships, and expression under drought, salt, and alkali stresses. Some key genes were identified based on enrichment analysis, potential protein network, and the expression under drought, salt, and alkali stress. This study provides insights into the diversity of the *WRKY* gene family and their roles in drought, salt, and alkali stress responses in Sainfoin.

Keywords Sainfoin, WRKY family, Drought, Salt, Alkali, Genome-wide analysis

†Wenxiang Li, Yinuo Yan and Yanxia Liang contributed equally to this work.

*Correspondence:
Lili Nan
958032689@qq.com
Wenbo Jiang
jiangwenbo@caas.cn
Full list of author information is available at the end of the article

Background

Sainfoin (*Onobrychis viciifolia*) is a perennial forage belonging to the family Leguminosae [1, 2]. It is known for non-bloating, anthelmintic, and anti-methanogenic properties, as well as high resistance to alfalfa weevil. Its protein content is comparable to that of alfalfa (*Medicago sativa*) [3]. Furthermore, Sainfoin possesses a thick, deep taproot that harbors a significant population of rhizobia, facilitating biological nitrogen fixation, which enhances soil nutrient content and minimizes the leaching of inorganic nitrogen into the environment [4]. These qualities render Sainfoin a promising candidate for high-quality forage production. Research on Sainfoin is primarily focused on aspects such as forage yield, genetic diversity, and morphological traits [5–9]. However, in recent years, significant advancements have been achieved in genome assembly, genetic marker development, and omics analysis [10–13]. These advancements provide valuable tools for identifying resistance genes and elucidating the molecular mechanisms underlying stress responses at the genomic level in Sainfoin.

In light of rapid global climate change, drought has emerged as an important factor impacting the development of grassland livestock [2]. Also, soil salinization is increasingly exacerbated by environmental factors and human activities, affecting the growth and productivity of plants and simultaneously reducing the value of the land [14]. In arid and semi-arid regions, salinity, alkali stress, and water scarcity are the primary factors affecting plant growth and development, leading to suboptimal quality and limited resource availability [15]. Plants harbor multiple stress adaptive mechanisms at molecular, cellular, physiological and biochemical levels, including transcription factors (TF) such as the *WRKY* gene family, which regulate the expression of stress-induced genes [16, 17]. The plant WRKY TF family is characterized by the highly conserved heptapeptide sequence"WRKYGQK"located at the N-terminus, and a DNA-binding domain at the C-terminus, which consists of zinc fingers [18, 19]. In various signaling pathways, such as the MAPK signaling cascade pathway and ROS scavenging [20], WRKY TFs recognize the W-box (C/TTGACC/T) within the core promoters and play important roles in regulating the expression of key genes involved in plant responses to drought, salt, and alkali stresses [18].

The *WRKY* gene family has been studied across various forage species, including elephant grass (*Cenchrus purpureus*) [19], red clover (*Trifolium pratense*) [21], white clover (*Trifolium repens*) [22], alfalfa (*Medicago sativa*) [23, 24], and *Medicago truncatula* [25]. *MsWRKY11* exhibits high expression levels in both stem and phloem tissues and has been shown to regulate drought resistance in alfalfa by influencing stomatal density. It is also hypothesized that *MsWRKY22* may be involved in the expression of *MsWRKY11* through binding to the W-box in its promoter region[23]. Heterologous overexpression of *GsWRKY20* in alfalfa enhanced drought resistance by increasing the thickness of the cuticle layer to minimize water loss [26]. Among the 346 *WRKY* genes identified in *Tritipyrum*, *TtWRKY256* is sensitive to salt stress, and is on the same evolutionary branch as the salt tolerance genes *AtWRKY25* and *AtWRKY33* [27]. A specifically upregulated *GsWRKY23* gene was identified in salt-tolerant *Glycine soja*, and was found to be important for the regulation of ion homeostasis in plants under salt stress and improvement of plant salt-alkali tolerance by maintaining low Na^+/K^+ and Cl^-/NO_3^- ratios [28]. Several candidate genes were reported for salt-alkali tolerance in *Sorghum bicolor*, which play pleiotropic regulatory roles in growth and development, stress response, forage value, and signaling network. It provided potential resources for genetic research in salt tolerance breeding [29].

In addition, several *WRKY* genes have been associated with drought, salt, and alkali stress tolerance across different forage species, such as *LcWRKY5* in Sheepgrass (*Leymus chinensis*) [30], *HvWRKY38* in Bahiagrass (*Hordeum vulgare*) [31], and *FtWRKY6*, *7*, *31*, and *74* in Buckwheat (*Fagopyrum tataricum*) [32], *DgWRKY* in Orchardgrass (*Dactylis glomerata*) [33], *WRKY41* in Annual ryegrass (*Lolium multiflorum*) [34], and *WRKY72 A* in reed canary grass (*Phalaris arundinacea* L.) [35], *GmWRKY16* in soybean (*Glycine max*) [36], *MsWRKY33* in alfalfa [37]. The functions of *WRKY* genes in Sainfoin remain largely unexplored, particularly under drought, salt, and alkali stresses.

In this study, we identified members of the *WRKY* gene family in the Sainfoin genome and conducted a comprehensive analysis of their physicochemical properties, chromosomal distribution, gene structures, conserved motifs, and phylogenetic relationships. Gene Ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses revealed their involvement in the metabolic pathways and signaling transduction pathways. Furthermore, we examined the expression levels of the *OvWRKY* gene family members under drought, salt, and alkali stresses. We identified drought, salt, and alkali stress-responsive *WRKY* genes, providing a theoretical foundation for further investigations into the molecular mechanisms underlying WRKY-mediated stress tolerance in Sainfoin.

Results

Identification and characterization of the *OvWRKY* gene family

Hidden Markov Model (HMM) and Blast based analysis identified a total of 253 putative *OvWRKY* genes in the

Sainfoin genome. These *OvWRKY* genes were designated as *OvWRKY1* to *OvWRKY253* based on their location (from top to bottom) on the chromosome Chr.1a-7d. The physicochemical properties of the *OvWRKY* gene family and their subcellular localization are summarized in Table S1. These *OvWRKY* genes encode proteins that range from 90 (OvWRKY209) to 762 (OvWRKY131) amino acids in length, with theoretical isoelectric point (pI) ranging from 4.8 (OvWRKY149) to 9.91 (OvWRKY47, OvWRKY64, OvWRKY81, OvWRKY99, and OvWRKY100), with OvWRKY132 designated as a neutral protein (pI = 7). Notably, 57% of OvWRKY members are acidic (pI < 7.0). The instability index of members ranged from 26.23 (OvWRKY211) to 72.71 (OvWRKY166), with 21 stable OvWRKY proteins (instability index < 40), while the aliphatic index varied from 39.89 (OvWRKY209) and 105.05 (OvWRKY89). All identified proteins were hydrophilic except for OvWRKY54, OvWRKY70, OvWRKY88, and OvWRKY89, which exhibited hydrophobic characteristics (GRAVY > 0). Subcellular localization predictions indicated that 234 OvWRKY proteins localize in the nucleus, while the remaining 19 were distributed in chloroplast, plasma membrane, peroxisomal, cytoplasm, and mitochondria (Table S1).

Chromosomal distribution and phylogenetic analysis of the *OvWRKY* gene family

Chromosomal localization analysis revealed that the distribution of 253 *OvWRKY* genes is uneven across 28 chromosomes (Fig. 1). Notably, chromosome 2a-d harbors the highest number (72) of *OvWRKY* genes, while chromosome 3a-d contains the lowest (13). No correlation was observed between chromosome length and the number of *OvWRKY* genes. However, the similarities in gene location and quantity on homologous chromosomes suggest a shared inheritance and genetic variation process throughout evolution.

To investigate the *OvWRKY* gene family members, a phylogenetic tree comprising AtWRKYs and OvWRKYs (protein sequences) was constructed using MEGA11. The 253 *OvWRKY* members were divided into three major groups (I-III); where group II was further subdivided into five subgroups (IIa, IIb, IIc, IId, IIe), containing 16, 34, 62, 25, and 40 members, respectively (Fig. S1). Group I contained 48 *OvWRKY* genes (18.97% members), while group III includes 28 *OvWRKY* genes (10.27% members) (Table S1).

Conserved motifs and exon–intron structures of *OvWRKY* gene family

The *OvWRKY* family members were analyzed for conserved domains and exon–intron architecture (Fig. 2). Our findings indicated that, compared to different groups/subgroups, genes within the same groups/subgroups exhibit identical/similar conserved motifs. The motif 1 (typical WRKY conserved sequence), motif 2, and motif 3 (both represent zinc finger motifs) were identified in nearly all OvWRKY proteins. The motif sequence is shown in supplementary Fig. 2. In addition, group I includes motif 9 as well as motif 6, which is also present in OvWRKY196 (Group IIe members). All group IIa members contained both motif 7 and motif 4, and group IIb harbored motif 5, motif 8, and two repeated motif 4. With the exception of OvWRKY41 and OvWRKY10, the remaining members of group IIb also included motif 7. Furthermore, several group IIc members also harbor motif 5, and group IId members contain motif 10, also observed in most members of group III. There is a lack of fully conserved motifs in group IIe. The results suggest that the differences in motifs between different members of the groups/subgroups may cause functional differences.

For insights into the extent of divergence of *OvWRKY* genes, the UTR-exon–intron organization was compared based on the annotation (Table S2). Among *OvWRKY* genes, the number of exons ranged from one to eight, while the intron counts ranged from zero to seven. Notably, four group IIe genes (*OvWRKY53*, *OvWRKY70*, *OvWRKY87*, and *OvWRKY106*), localized on homologous chromosome 2, possessed a single exon. Only *OvWRKY150* contains eight exons, and *OvWRKY128* contains seven exons, and these two members of group I are located on homologous chromosome 4. The other *OvWRKY* genes showed two to six exons. Genes *OvWRKY236* and *OvWRKY242* contained five exons, compared to other group III members, which contained three exons.

Analysis of gene duplication in the *OvWRKY* gene family

To investigate the underlying duplication events of *OvWRKY* genes, collinearity relationship analysis of *OvWRKY* genes was performed within and between species. After excluding 339 collinear gene pairs between homologous chromosomes, 232 collinear gene pairs were identified between non-homologous chromosomes, indicating that segmental duplication events occur extensively within the *OvWRKY* gene families. Most of these relationships involved one gene interacting with multiple genes, suggesting that gene duplication events occurred within this *OvWRKY* gene family (Fig. 3, Table 1, and Table S3). The high frequency of segmental duplication occurred between chromosomes 1 and 2, which contained 39 collinear gene pairs. There are 120 segmental duplication events between chromosome 1 and chromosomes 2, 4, 5, 6, and 7. Chromosome 3 and 2 showed only

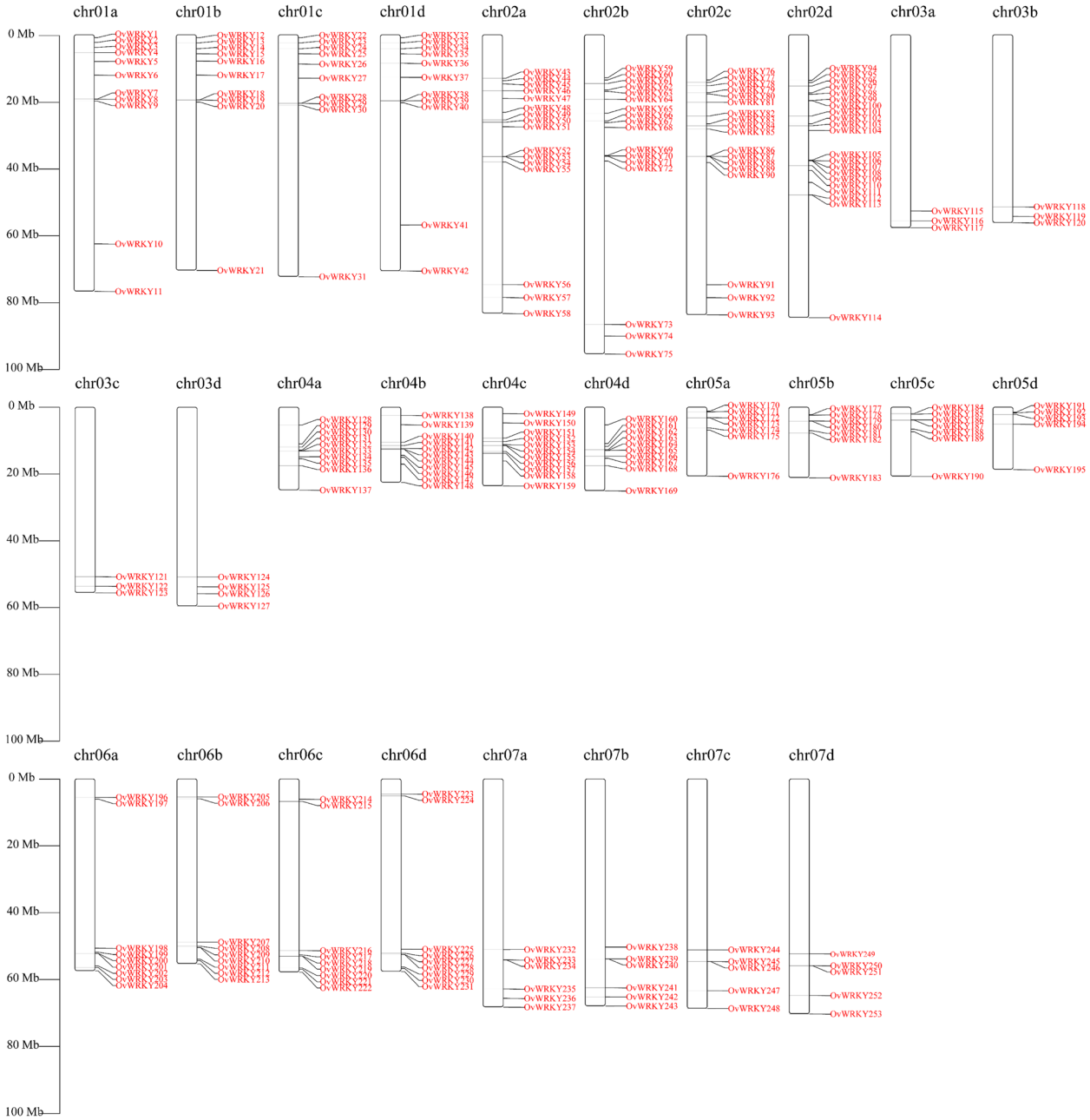


Fig. 1 Chromosomal locations of *OvWRKY* genes. Each chromosome number is shown in black font, the red font represents the putative *WRKY* genes, and the scale on the left is used to show the physical distance between chromosomes

7 segmental duplications. Interestingly, chromosome 3 shared no segmental duplication events with chromosomes 1, 4, 5, 6, or 7, and no segmental duplication events occurred between chromosome 2 and chromosome 6, or between chromosome 4 and chromosome 7. Additionally, only four pairs of tandem repeats were found, which included *OvWRKY88* and *OvWRKY89*, *OvWRKY99* and *OvWRKY100*, *OvWRKY107* and *OvWRKY108*, and *OvWRKY112* and *OvWRKY113*. These results indicate that fragment duplication may play an important role in the diversity and expansion of *WRKY* gene families.

Evolutionary and lineage-specific expansion analysis of *WRKY* members in *O. viciifolia*, *A. thaliana*, and *M. truncatula* identified a total of 231 orthologous pairs between *O. viciifolia* and *A. thaliana*, and 343 orthologous pairs between *O. viciifolia* and *M. truncatula* (Fig. 4, Table 1,

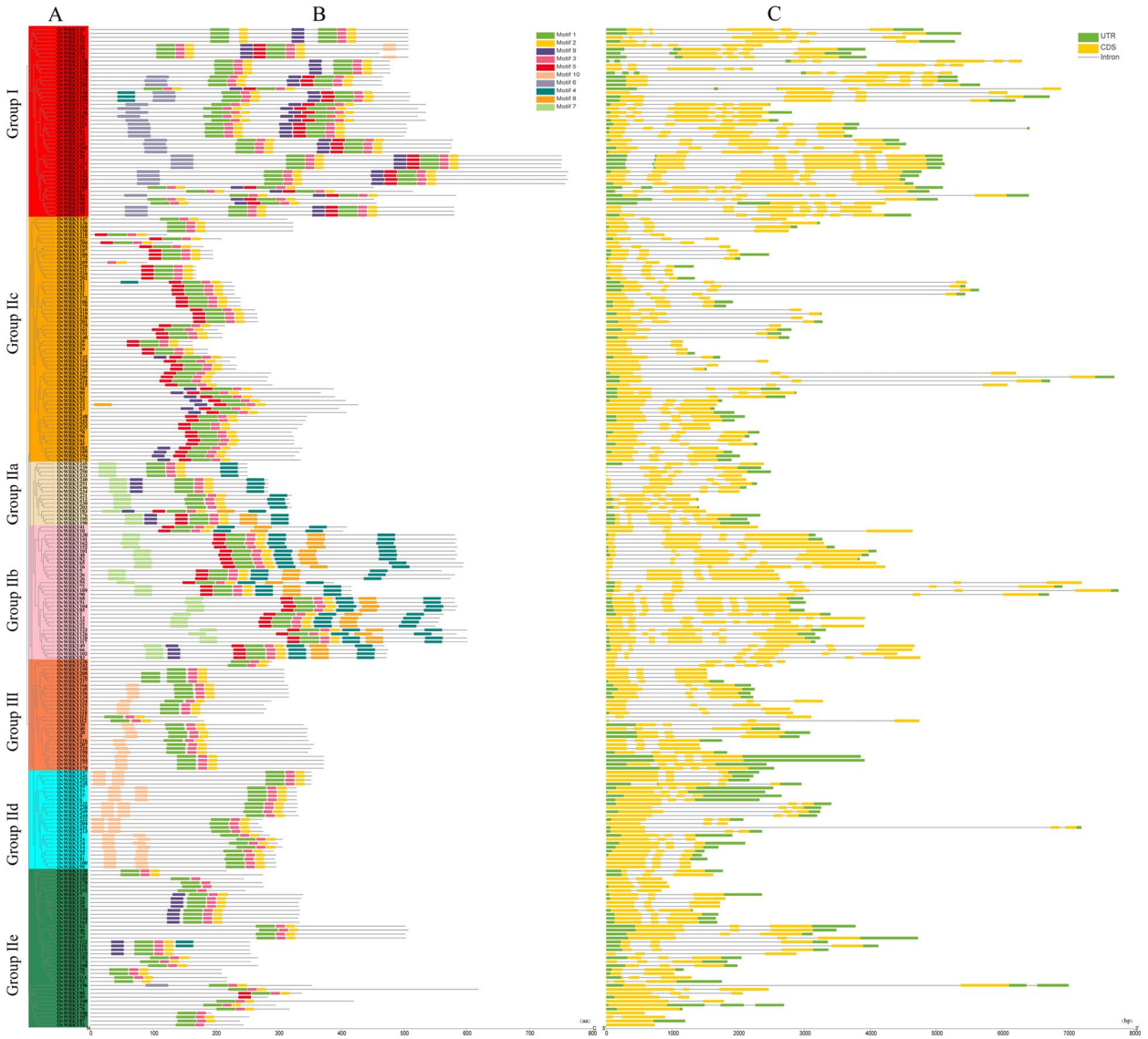


Fig. 2 Phylogenetic tree, conserved motif and gene structure of *OvWRKY* superfamily. (**A**) Evolutionary tree of *OvWRKY* genes family, with different colors representing different subfamilies, whose names are shown on the left. (**B**) Conserved motif visualization according to TBtools, with different colored boxes to indicated motifs 1–10. aa indicate protein length. (**C**) Gene structure visualization, green squares indicate the gene's UTR, yellow squares indicate the gene's exons, black lines indicate introns, bp indicate sequence length

and Table S4). The collinearity analysis indicates a higher similarity between *O. viciifolia* and *M. truncatula* (both belong to the legume family) is compared to *O. viciifolia* and *A. thaliana*. There are only four collinear gene pairs between chromosome 3 of *A. thaliana* and chromosome 3 of *O. viciifolia*, with no collinear gene pairs between chromosome 3 of *O. viciifolia* and chromosomes 1, 2, 4, and 5 of *A. thaliana*. There were 4 and 12 collinear gene pairs between chromosome 3 of *O. viciifolia* and chromosome 4 and 2 of *M. truncatula*, respectively. But no collinear relationship with chromosomes 1, 3, 5, 6, 7, and 8. Interestingly, there are no collinear *WRKY* gene pairs between chromosome 6 of *M. truncatula* and any chromosome of *O. viciifolia*, indicating that the *WRKY* gene is replicated specifically in chromosome 6 of *M. truncatula*, which may be the functional specificity of the *WRKY* gene in *M. truncatula*. These results suggest that *WRKY* genes on these chromosomes play an important role in maintaining the stability and genetic diversity of functions during Sainfoin domestication, which may have influenced traits and adaptations specific to *O. viciifolia*.

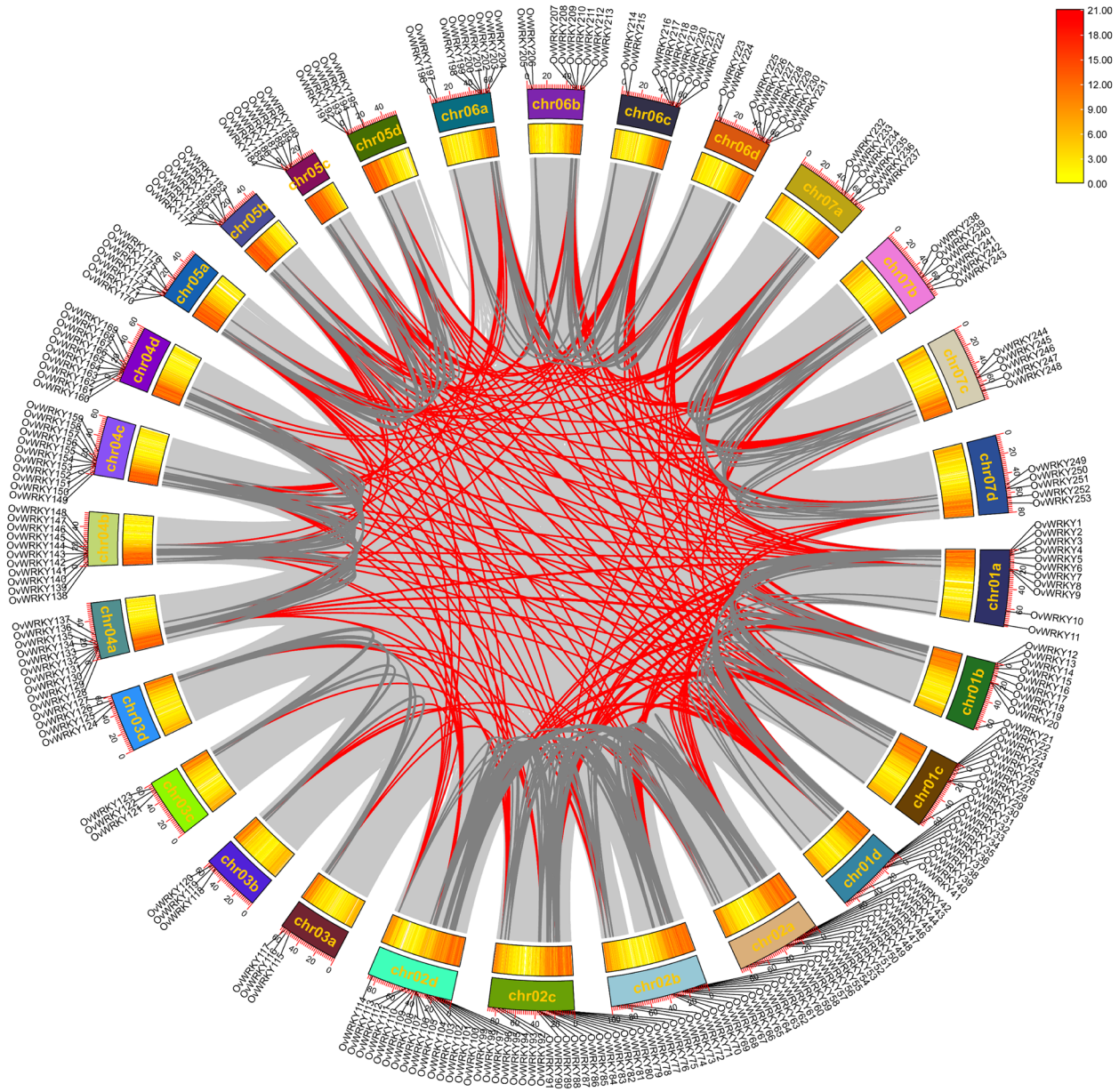


Fig. 3 Duplication events analysis of *OvWRKY* gene family. Around the circle are the gene names, shown in black font. The two boxes represent chromosomes from outside to inside, gene density. The light gray lines in the inner circle represent all collinear gene pairs within the species, the dark gray and red lines represent collinear relationships of *WRKY* genes within homologous chromosomes and between different chromosomes, respectively

Estimation of nonsynonymous (*Ka*) to synonymous (*Ks*) ratios for *WRKY* gene pairs showed that 15 gene pairs had *K*a/*K*s ratios greater than 1 (Table S5, green label font), indicating positive selection acting on these gene pairs. In addition, for another set of 15 gene pairs, *Ka/Ks* ratios were found to be zero (Table S5, red label font), indicating that these gene pairs may have evolved mainly under the influence of neutral selection. 84.41% (482/571) of gene pairs had a *Ka/Ks* ratio less than 1, indicating that most *OvWRKY* genes have undergone purification selection post segmental duplication events, which may be beneficial for maintaining gene stability and function.

Enrichment analysis of *OvWRKY* gene family

The 253 *OvWRKY* gene family members were further investigated by Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG)

Table 1 Number of collinear gene pairs of WRKY genes between species

Species		***O. viciifolia***							
	chromosome	chr1	chr2	chr3	chr4	chr5	chr6	chr7	total
O. viciifolia	chr1	52	39	0	32	22	15	12	172
	chr2	-	99	7	13	9	0	0	128
	chr3	-	-	12	0	0	0	0	12
	chr4	-	-	-	58	7	5	0	70
	chr5	-	-	-	-	36	29	10	75
	chr6	-	-	-	-	-	51	32	83
	chr7	-	-	-	-	-	-	31	31
									571
M. truncatula	chr1	0	0	0	0	0	12	20	32
	chr2	5	16	12	0	7	2	0	42
	chr3	4	22	0	4	7	28	8	73
	chr4	20	9	4	4	8	3	0	48
	chr5	1	29	0	0	0	8	0	38
	chr6	0	0	0	0	0	0	0	0
	chr7	12	0	0	41	0	2	4	59
	chr8	13	3	0	12	14	9	0	51
									343
A. thaliana	chr1	11	15	0	4	11	9	1	51
	chr2	8	12	0	12	8	20	11	71
	chr3	0	0	4	10	0	1	4	19
	chr4	12	17	0	4	11	8	7	59
	chr5	1	5	0	7	8	7	3	31
									231

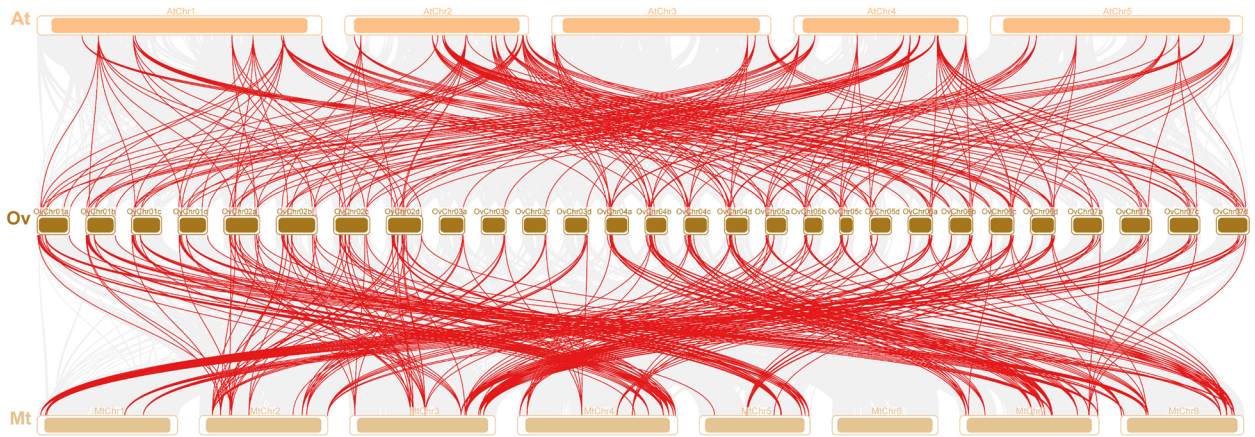


Fig. 4 Collinearity analysis between *O. viciifolia* and *A. thaliana*, and between *O. viciifolia* and *M. truncatula*. Red lines indicate the collinearity relationship of *WRKY* genes across different species

enrichment analysis. *OvWRKY* genes were found to be primarily involved in 18 biological processes, 6 molecular functions, and 3 cellular component pathways (Fig. 5A, Table S6). All *OvWRKY* genes were annotated in relation to the regulation of biological and metabolic processes. Among them, 46 *OvWRKY* genes were enriched in response to stimulus; 20 *OvWRKY* genes were associated with developmental process; while 17 *OvWRKY* genes functioned as negative regulation of biological process. According to KEGG database enrichment classifications, 39 *OvWRKY* genes were found to be involved in the plant pathogen interaction pathway and 27 *OvWRKY*

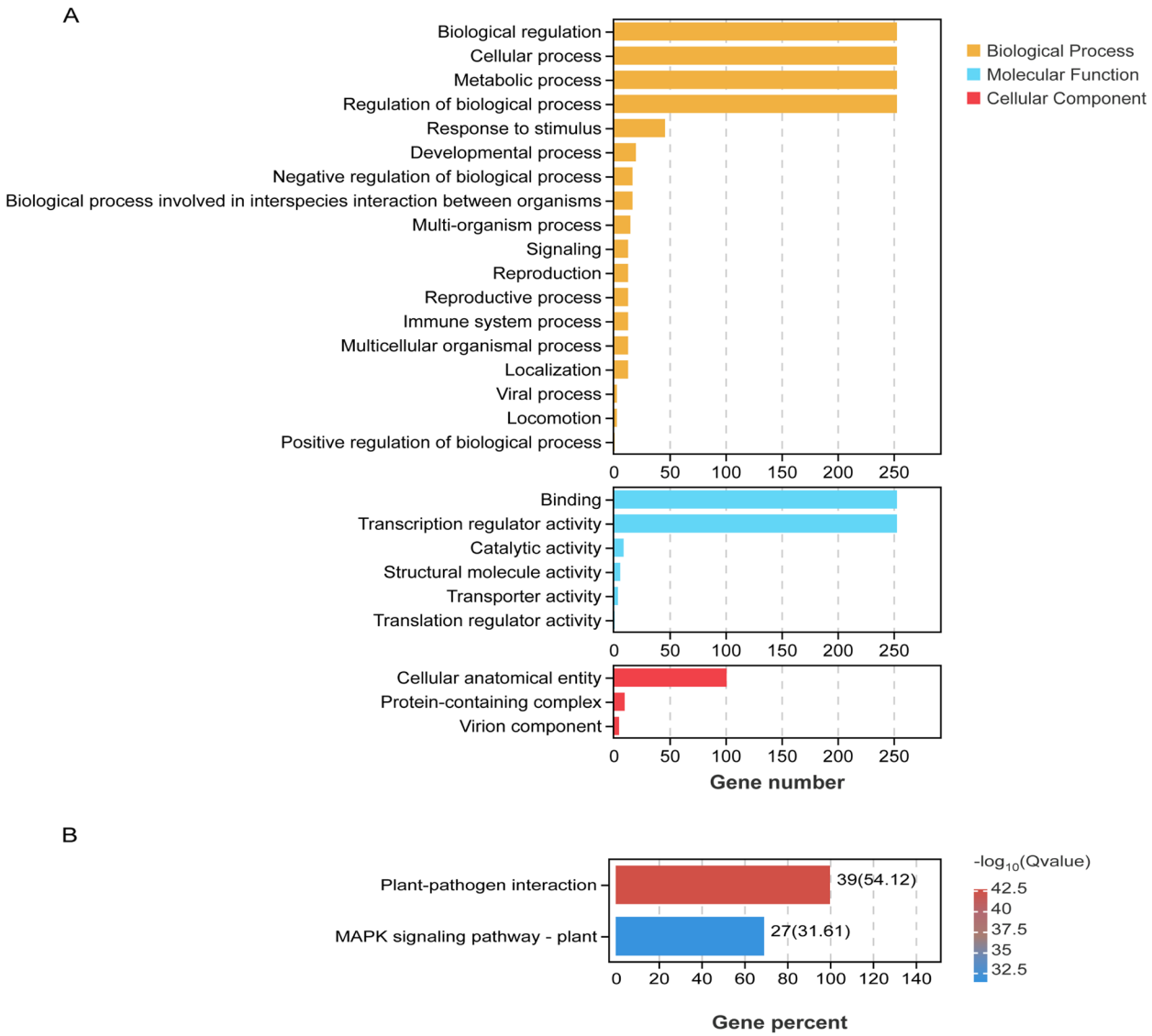


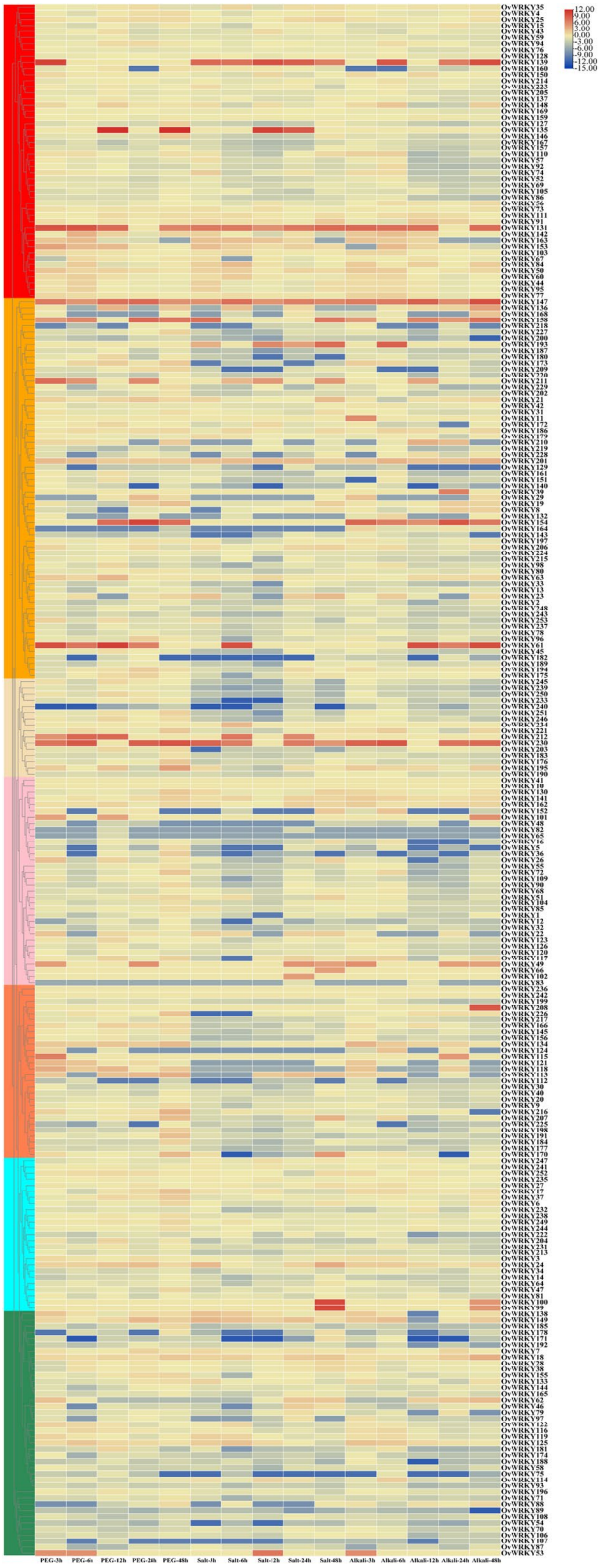
Fig. 5 Enrichment analysis of *OvWRKY* gene family. (**A**) GO enrichment analysis of *OvWRKY* genes. Yellow, blue, and red areas are indicated biological process, molecular function, and cell component, respectively. (**B**) KEGG enrichment analysis of *OvWRKY* genes

genes were involved in the plant MAPK signaling pathway response (Fig. 5B, Table S6).

Analyses of the expression levels of *OvWRKY* genes in response to drought, salt, and alkali stresses

We analyzed the expression patterns of the 253 *OvWRKY* genes under drought, salt, and alkali stress conditions using transcriptome data. As illustrated in Fig. 6 and Table S7, this analysis revealed that expression levels for subfamilies group IIc and IIe remained generally low across different treatments. In contrast, highly expressed genes predominantly belonged to groups I, III, IId, IIa, and IIb. Notably, these highly expressed genes, clustered together within the same branch, exhibited similar expression patterns. The transcriptome data also showed that the transcripts of 13, 10, and 9 genes were undetected under drought, salt, and alkali treatments, respectively.

According to the trend analysis, we obtained the four trends with the highest enrichment under different treatments and the trend of continuous up-regulation and down-regulation of expression. Under drought treatment (Fig. S3 A, Table S7), we observed three trends with the highest number of enriched genes containing 35, 33, and 30 genes, respectively; this was consistent with gene expression trends under salt treatment (Fig. S3B), albeit



◀ **Fig. 6** Analyses of the expression levels of *OvWRKY* genes under drought, salt, and alkali stress. The levels of gene expression are displayed by clustering based on subfamily members, with different colors of the phylogenetic tree branches representing different subfamilies. The gene names are shown on the far right, and different treatments are indicated at the bottom. The color scale shows fold-change values, with negative values representing down-regulated expressions in blue and positive values representing up-regulated expressions in red

differing in terms of gene enrichment numbers. At the same time, only the second trend feature showed the same expression trend change under alkali treatment (Fig. S3 C), while the other three trends under alkali treatment did not exhibit the same characteristics under drought and salt treatments, and the trend with the highest enrichment under alkali treatment showed a continuously down-regulated expression condition, containing 81 genes.

Venn analysis revealed 6, 28, and 10 common genes exhibiting more than a two-fold change under drought, salt, and alkali stress conditions, respectively (Fig. 7, Table S8). Among them, only four genes (*OvWRKY147*, *OvWRKY139*, *OvWRKY131*, and *OvWRKY154*) were up-regulated, and the rest were down-regulated. Further, the intergroup analysis of these genes (Fig. S4) identified two differentially expressed genes *OvWRKY89* (down-regulated under three stresses) and *OvWRKY147* (up-regulated under three stresses). Additionally, *OvWRKY240* and *OvWRKY164* were down-regulated under both drought and salt stress, while four genes *OvWRKY36*, *OvWRKY107*, *OvWRKY65*, and *OvWRKY200* were down-regulated under both salt and alkali stress (Table S8). According to GO enrichment analysis (Table S6), it was found that these eight genes were involved in the regulation of biological processes and metabolic processes. Among them, *OvWRKY36* was annotated in the biological process of response to stimulus. To validate the transcriptomic data, we designed specific primers for reverse transcription quantitative PCR (RT-qPCR) analysis. The results indicated that the expression patterns were generally consistent with the transcriptome data across different treatment conditions (Fig. 8).

Cis-acting elements were identified in the promoters of 34 common genes into 25 different functions (Fig. 9, Fig. S5), mainly including abiotic and biotic stresses functions (drought and high-salinity stress responsive, drought inducibility, MYB binding sites, etc.), phytohormone response (abscisic acid responsiveness and auxin responsive, etc.), and plant growth and development (light responsive and meristem expression, etc.). Most genes harbored MYB binding sites, including *OvWRKY89*

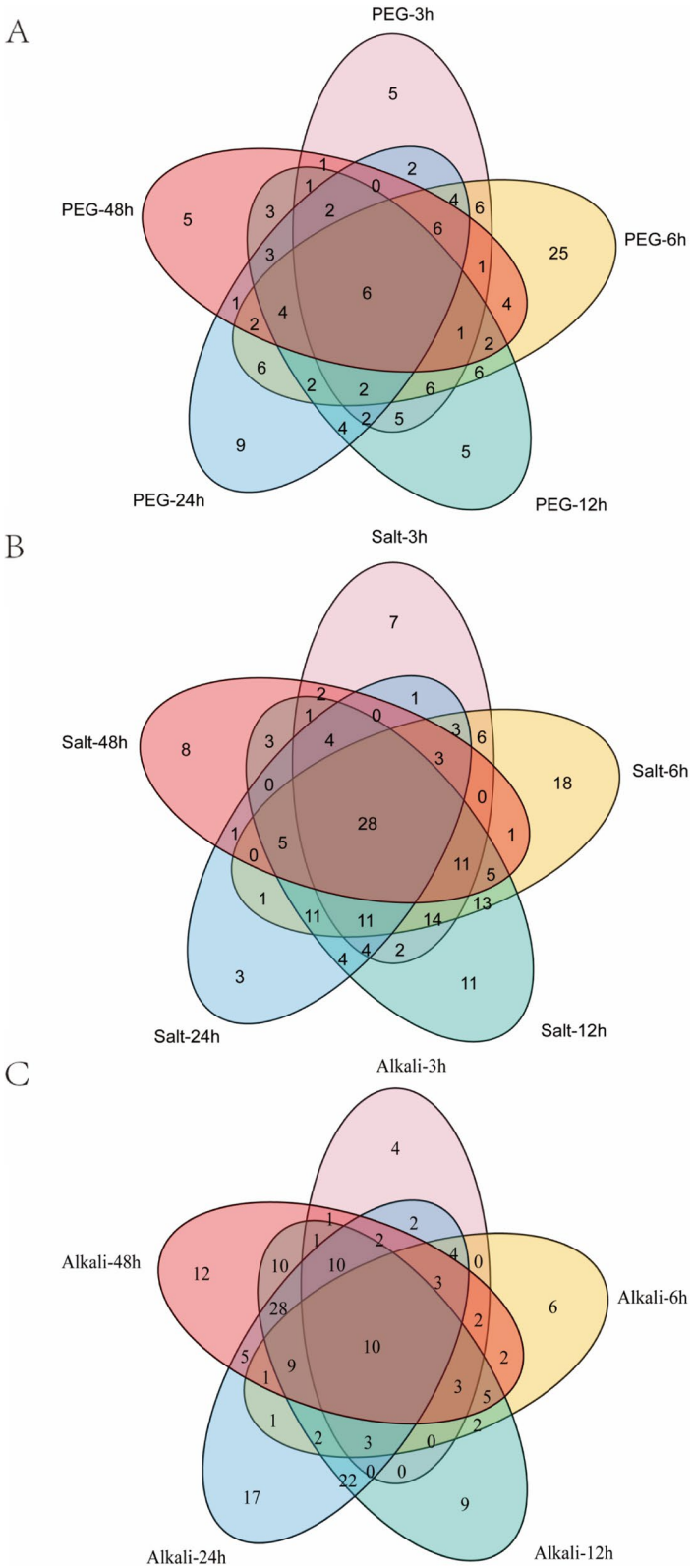


Fig. 7 Venn analyses of stress responsive *OvWRKY* genes. The number of differentially expressed genes under drought (**A**), salt (**B**), and alkali (**C**) treatments, respectively. Different colors represent differentially expressed genes numbers within the treatment groups at 3 h, 6 h, 12 h, 24 h, and 48 h. The overlaps illustrate the number of common and unique genes between different treatment groups

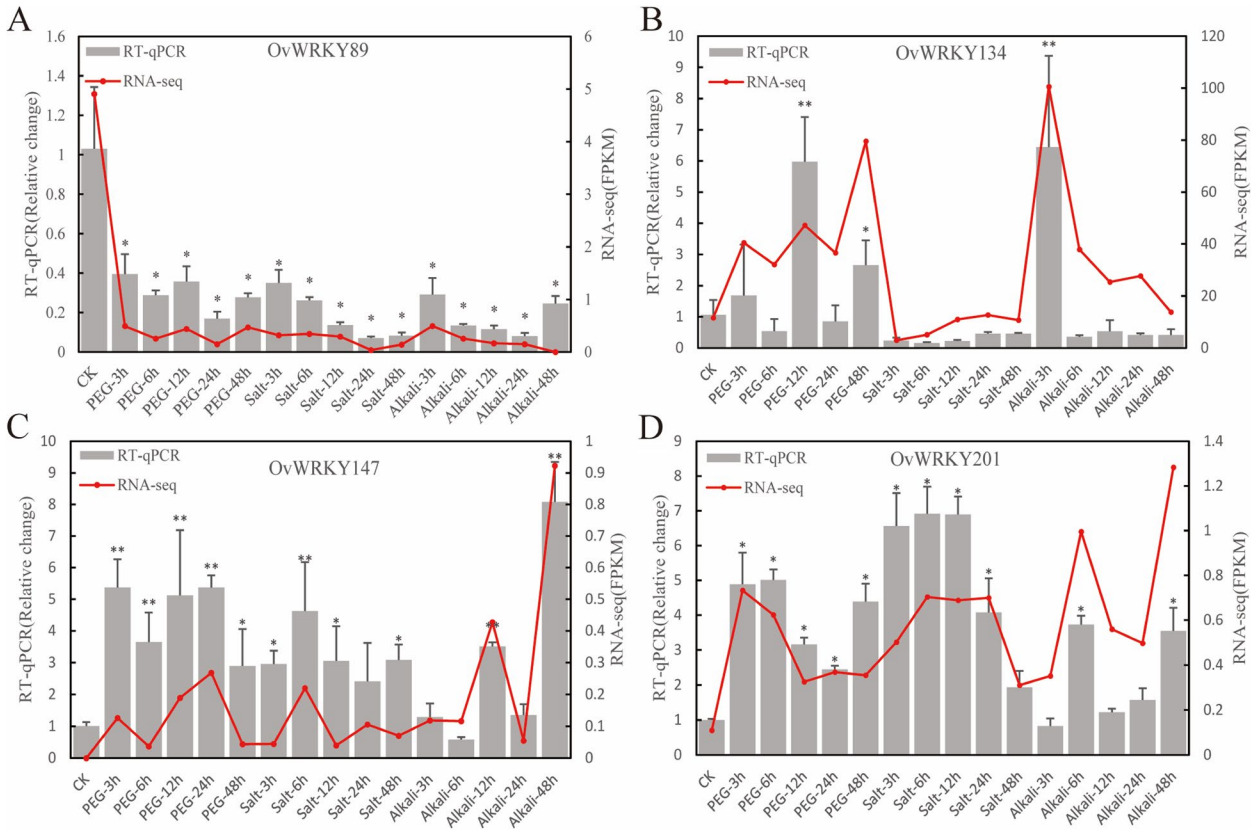


Fig. 8 Expression patterns of *OvWRKY* genes under drought, salt, and alkali stress conditions. The gray bar and red line chart represented RT-qPCR and RNA-seq, respectively. The lines on the bar chart represent the standard error lines, SPSS was used to calculate the significance level of the difference, and asterisks are used to represent (*, $P \leq 0.05$; **, $P \leq 0.01$)

with a drought induction response element, suggesting its involvement in drought stress response. It was also observed that *OvWRKY124* contains one flavonoid biosynthesis-related element, suggesting a possible involvement in the flavonoid synthesis, but its specific function requires further exploration.

Potential protein interaction analysis of the OvWRKY superfamily

We further analyzed the potential interaction protein network of these common genes (Fig. 10). OvWRKY89 potentially interacts with nine other proteins and OvWRKY129 interacts with one protein. OvWRKY129, OvWRKY233, and OvWRKY239 are all involved in the response to stimulus process. According to annotation files [10], we found that LeOno02bG0229000 and LeOno07aG0383900 are homologous to Arabidopsis TIFY10 A (AT1G19180) and ZAT10 (AT1G27730). Furthermore, LeOno06aG0241700, LeOno07bG0394100, and LeOno07 dG0402700 are homologous to Arabidopsis ZAT6 (AT5G04340) within the C2H2 transcription factor family. The identification of these specific pathways along with their associated genes provides valuable insights into further understanding the functional roles of *WRKY* gene family members.

Discussion

Sainfoin is an important forage crop that has attracted considerable attention due to its effectiveness in reducing the risk of bloat hazards in ruminants [38]. Also, its nutrient-rich profile and feed value are comparable to alfalfa, rendering it highly valuable within agricultural systems [3]. However, drought, salt, and alkali stress remain the primary constraints affecting its growth, biomass yield, and nutritive value potential [14, 39, 40]. In plants, the *WRKY* gene family is known to be important for a number of stress responses [18], however as of now, the role of *OvWRKY* genes has not been studied in Sainfoin. Identification and analysis of stress responsive *WRKY* genes will help to understand their role in molecular mechanisms in Sainfoin under drought, salt, and alkali conditions.

In this study, a total of 253 *WRKY* genes, designated as *OvWRKY1-OvWRKY253* were identified in Sainfoin. The

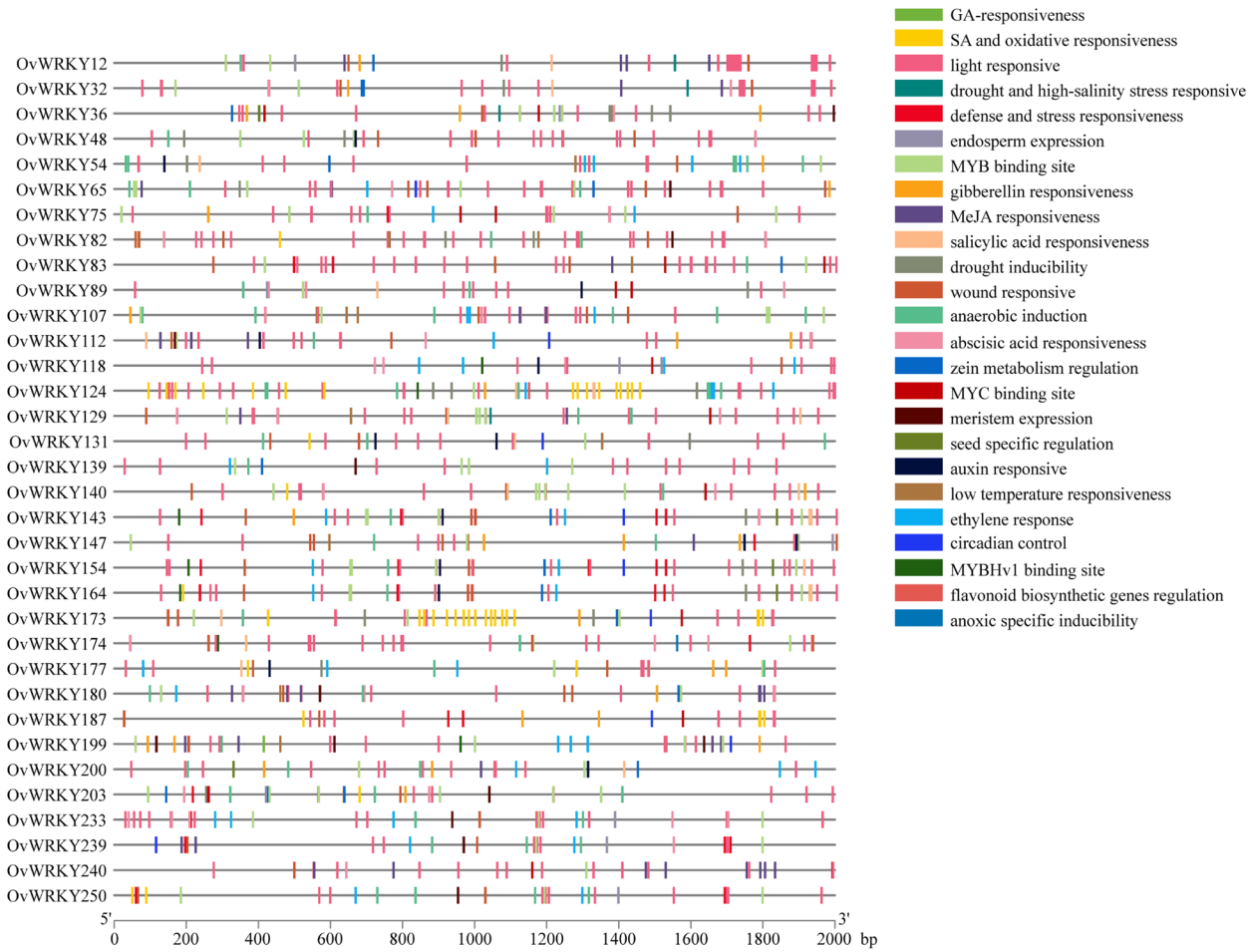


Fig. 9 *Cis*-elements analysis in the promoter region of the *OvWRKY* genes. Different color modules represent different functional annotations

chromosomal distribution of *OvWRKY* genes reflects natural selection and functional constraints, and a trend indicative of more genes on longer chromosomes [41]. Although there seems to be no correlation between the number of *OvWRKY* genes and chromosome length, it is noteworthy that Sainfoin, being an autotetraploid [10], experiences genetic phenomena distinct from diploid species. Polyploids not only undergo gene recombination and loss of homologous genes but also exhibit biased gene expression patterns [42]. This genetic complexity provides a robust foundation for species adaptation to adverse environmental conditions [42–44]. Gene replication is essential for the expansion of gene families and the evolution of genomes [45]. We found that the position and number of *OvWRKY* genes within each homologous chromosomes were remarkably similar (Fig. 1), which may be attributed to evolutionary processes and family expansion. Interestingly, transcriptome data revealed certain genes that are not expressed under treatment conditions; these unexpressed genes occupy very similar positions on different chromosomes within the homologous chromosomes, indicating skewed gene expression in polyploid species. Furthermore, different *OvWRKY* subfamilies showed similarities as well as differences in conserved motifs within and across subfamilies (Fig. 2), indicating their functional diversity. Moreover, their specific distribution contributes to functional differentiation within the *OvWRKY* gene family.

The MAPK signaling cascade, consisting of MAPKKK-MAPKK-MAPK, is an essential mode of activation or inhibition of specific transcription factors (TFs) through phosphorylation, thereby regulating gene expression under drought, salt, and alkali stresses [46–50]. WRKY TFs function downstream of the MAPK signaling pathway. In rice, OsWRKY30 is capable of interacting with OsMPK20-4 and OsMPK20-5, can be phosphorylated by OsMP3, OsMPK7, and OsMPK14 during response to drought [51]. It has also been shown that allogeneic overexpression of *SbMPK14* inhibits the activities of *ERF* and *WRKY* TFs and enhances plant sensitivity to drought

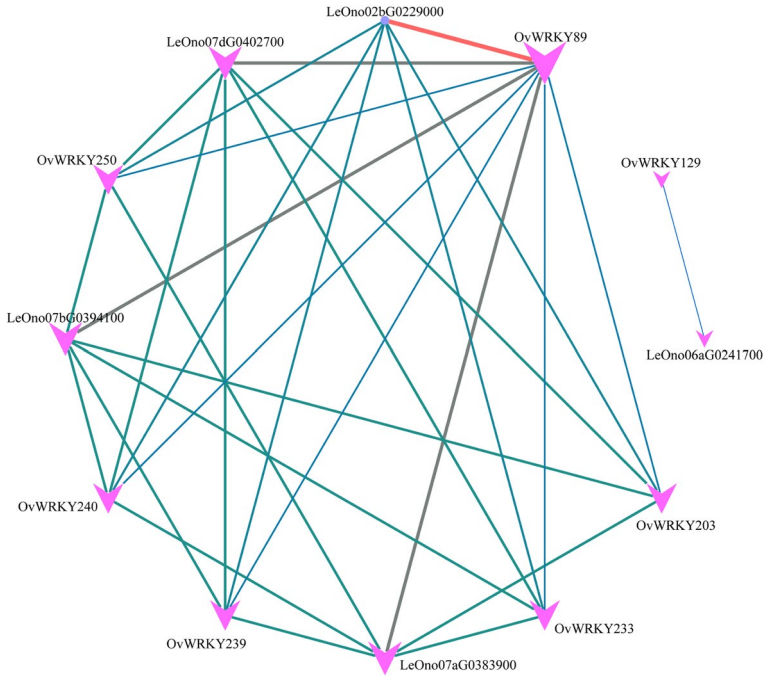


Fig. 10 Protein interaction network analysis of OvWRKY family. Node: gene name (TF in pink, gene in blue). The larger the node, the stronger the connection. The line segment indicates the interaction between proteins

[52]. The GhMEKK3/8/31-GhMAPKK5-GhMAPK11/23 signaling cascade can regulate the function of *WRKY* TFs under drought and salt stress [53]. Downregulation of *OcWRKY22* under salt stress played a role in the MAPK signaling pathway, reducing respiratory cell death [54]. Based on the KEGG database, 27 *OvWRKY* were found to be significantly enriched in the MAPK signaling pathway (Fig. 5B). Interestingly, 14 of these genes belong to the group IIe subfamily and 13 to the group I family. Combined with transcriptome expression pattern analysis, these results suggest that the function of *WRKY* genes in Sainfoin may be mainly reflected in the group IIe and group I subfamilies, which are closely related to plant drought, salt, and alkali resistance.

Analysis of gene expression patterns and trends is useful for a better understanding of the temporal and spatial rules of gene expression and their synergistic pathways. As shown in Figure S2 A, among these six trends, 15 genes have been implicated in environmental adaptation and signal transduction pathways. Notably, seven genes belong to the group IIe subfamily and the other eight genes belong to the group I family. Gene expression bias exists in polyploid species [42]. This further indicates the conservation, functional stability, and expression bias of the WRKY gene family during the genetic evolution of Sainfoin. Overexpression of *AtWRKY57* significantly enhances drought resistance in *Arabidopsis* [55]. *TpWRKY46* was significantly induced by drought stress [21], while overexpression of *MsWRKY11* enhanced drought resistance in alfalfa [56]. Structure-similar WRKY genes are induced by similar methods, with both *AtWRKY25* and *AtWRKY33* being responsive to salt, drought, and cold stresses [57]. *GmWRKY54* may enhance the drought and salt tolerance of transgenic *A. thaliana* by regulating *DREB2 A* and STZ/*Zat10* [58]. Among the DEGs common to three stresses, *OvWRKY89* was found to be homologous to *AtWRKY33*, which was in the center of the network and regulated cytochrome P450 gene *CYP94B1* through self-activation and phosphorylation of MAP kinase in response to salinity and other abiotic stresses [59, 60]. The specific genes identified in this study provide valuable insights for understanding their functions; however, their regulatory mechanisms need to be further investigated.

Protein–protein Interaction Networks (PPI) are crucial for identifying key interacting partners in multiple stress response pathways and can also reveal functional significance [61–64]. PPI networks have been utilized to screen key antiretroviral factors such as AP2/ERF, WRKY, ZAT, MSI, and VRN in plants like red clover [63], alfalfa [64], and *Vicia sativa* [65]. Through the PPI network, we have screened several potentially interacting TFs, and the function of these TFs in adversity has been reported. *AtTIFY10 A* is involved in plant growth, development, and defense response [66]. Heterologous expression of *TaWRKY* increases salt and osmotic stress tolerance,

accompanied by overexpression of *ZAT10*, and induces the expression of marker gene *AtPR1* in transgenic *A. thaliana* [67]. Overexpression of *AtZAT6* enhances salt stress tolerance via induction of *CPK9* and *CPK25*, and antioxidant enzyme activity [68]. These studies provide strong evidence of our interaction results. At the same time, we found that *OvWRKY233* and *OvWRKY129* might play a role in environmental stress responses, the interaction between these TFs needs to be further verified. These genes screened through the PPI network may play an important role in the functional evolution of the WRKY family, providing a reference for subsequent studies.

Conclusions

In this study, a total of 253 WRKY genes were identified in Sainfoin and analyzed for different characteristics, including phylogenetic analysis. Segmental duplication seems to be the main mode of expansion of the WRKY gene family in Sainfoin. KEGG enrichment and expression pattern analysis revealed that group IIe and group I families dominated the functional evolution in Sainfoin. In addition, two *OvWRKY* genes, *OvWRKY89* and *OvWRKY147,* were down-regulated and up-regulated, respectively, under drought, salt and alkali stresses. OvWRKY89 also showed potential interactions with multiple WRKYs, and other stress-related TFs based on the PPI network. In summary, this study provides valuable insights into the functional characteristics of the *OvWRKY* genes and contributes to the mining of key stress response genes in Sainfoin.

Materials and methods

Sequence retrieval and in silico analysis of characteristics of WRKY genes in Sainfoin

The Sainfoin genome sequence was obtained from literature [10]. To identify *WRKY* genes within the Sainfoin genome, the *Arabidopsis thaliana* WRKY sequences were used as a reference (https://www.arabidopsis.org/browse/gene_family/WRKY). The Hidden Markov Model (HMM) profile of the WRKY domain (PF03106) was retrieved from the Pfam database (https://www.ebi.ac.uk/interpro/entry/pfam/) and subsequently was employed in TBtools to identify OvWRKY family members [69]. Additionally, the native Blastp tool was employed to similarity search *A. thaliana* proteins, with the e-value threshold set at 1e-5 to obtain homologous proteins present in the Sainfoin genome. For further verification CD-search was done (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) to confirm presence of WRKY domain among the OvWRKY members. The identified WRKY proteins underwent an analysis of their physical and chemical properties were analyzed by Protein Paramter Calc function of TBtools [69]. Subcellular localization was predicted on an online website (https://wolfpsort.hgc.jp/). Finally, all identified sequences were systematically named according to their locations on the chromosome, with gene location visualization performed using TBtools [69].

Phylogenetic tree and duplication analysis of OvWRKYs

The Align by ClustalW function in the MEGA11 software [70] was used to analyze multiprotein sequence alignments of WRKY protein from both Sainfoin and Arabidopsis to construct a distance matrix. The optimal model for phylogenetic analysis was selected using the Models function, neighbor-joining (NJ), with a bootstrap value set at 1000 to construct a phylogenetic tree. This phylogenetic tree was visualized using an online website (https://itol.embl.de/login.cgi). The Comparative Genomics function of TBtools software [69] was used to analyze the collinear gene pairs between genomes, and the Advanced Circos function was used to visualize the collinear gene pairs, and the *Ka*/*Ks* ratio between gene pairs was calculated using the Simple *Ka/Ks* Calculator function.

Gene structure and conserved motif analysis of *OvWRKY* genes

The MEME online tools were utilized to identify conserved motifs within full-length protein sequences (https://meme-suite.org/meme/index.html), with parameters set to detect a maximum of ten motifs. Both gene structure and these conserved motifs were subsequently visualized using the Gene Structure View function of TBtools software [69].

Cis-acting elements analysis of *OvWRKY* genes

The 2,000 bp promoter region sequences upstream of the *WRKY* genes were extracted using Fasta Tools function of TBtools software [69] and used for identification of *cis*-acting elements at online PlantCare database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/). R software was utilized for organizing tabular data, while TBtools was employed for visualization. Finally, Adobe Illustrator software was used to enhance the overall presentation.

Expression pattern and enrichment analysis of *OvWRKY* genes

The RNA-seq data of Sainfoin under drought, salt, and alkali stress treatments have been deposited in the National Center for Biotechnology Information database (Project: PRJNA1234881). According to the fold-change values of transcriptome data compared with CK at different times under drought, salt, and alkali treatment, the HeatMap function of TBtools software was used to

map gene expression heat map. By using ShortTime-series Expression Miner (STEM) software, trend analysis was performed according to the expression levels of all differential genes. Additionally, Gene Ontology (GO) enrichment analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment and potential protein interaction network analysis were performed using the Gene Denovo analysis platform (https://www.omicsmart.com/).

Plant materials and treatments

In this study, the seeds of *Onobrychis viciifolia* cv. Gansu were deposited in Chinese Crop Germplasm Resources Information System (No. 17B026028), and they were identified by Professor Lili Nan (Gansu Agricultural University). They were provided by Gansu Agricultural University, China, and were potted in the Institute of Animal Science, Chinese Academy of Agricultural Sciences in Beijing (40°02′N, 116°28′E). After sprouting on moist filter paper, the seeds were cultivated in pots with nutrient-rich soil. For 30 days, the seedlings were cultivated under controlled settings at 25℃ (16 h of light) and 23℃ (8 h of darkness). Following this growth period, the plants were subjected to treatments with −0.8 MPa polyethylene glycol (PEG-6000), 75 mM NaCl, or 75 mM a mixture of sodium bicarbonate to sodium carbonate at a ratio of 2:1 ($NaHCO_3$:Na_2CO_3= 2:1) to simulate drought, salt, and alkali stress, respectively. Leaves were collected at 0 h, 3 h, 6 h, 12 h, 24 h, and 48 h post-treatment, and all samples were collected in three biological replicates.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using an RNA extraction kit (P134) and the first-strand cDNA was synthesized using a cDNA synthesis kit (A234) as described previously [71] with minor modifications. We used the SLAN-965 Real-time PCR system and 2× RealStar Fast SYBR qPCR Mix (Low ROX) kit (A304) for RT-qPCR experiments. All kits are provided by the Genstar, Beijing, China. The *OvActin* gene was used as a housekeeping gene [72]. The primer sequences used in this study are listed in Supplemental Table 9. The $2^{-\Delta\Delta Ct}$ method [72] was used to calculate the relative expression of genes in different samples according to Ct values, using the shiny APP quickQrtPCR package within R software (https://github.com/nongxinshengxin/quickQrtPCR). Data were calculated from biological triplicates with technical triplicates.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11646-2.

Supplementary Material 1.
Supplementary Material 2.
Supplementary Material 3.
Supplementary Material 4.
Supplementary Material 5.
Supplementary Material 6.
Supplementary Material 7.
Supplementary Material 8.
Supplementary Material 9.
Supplementary Material 10.

Collection of plant material

The collection of plant material complies with relevant institutional, national, and international guidelines and legislation.

Authors' contributions

W.L., L.N. and W.J. conceived the study. W.L., Y.Y., Y.L. and S.Y. conducted experiments. W.L., Y.Y., Y.L. and M.X. carried out the data analysis. W.L., D.Y., J.L. and W.J. contributed to the data visualization. W.L., Y.L., F.Y., Y.Z., L.N. and W.J. contributed to interpretation of the results. W.L., L.N. and W.J. wrote and edited the manuscript. Y.L. and F.Y. reviewed the manuscript critically. All authors read and approved the manuscript.

Funding

This work was supported by National Center of Pratacultural Technology Innovation (under way) Special fund for innovation platform construction (CCPTZX2023W01), the National Natural Science Foundation of China (32360339), Ministry of Finance and Ministry of Agriculture and Rural Development: The National Modern Agricultural Industrial Technology System (CARS-34), University graduate students innovation star of Gansu Province (2025CXZX-839), and the National Center of Technology Innovation for Comprehensive Utilization of Saline-Alkali Land.

Data availability

All data supporting the results are included within the article and its Additional files. The transcriptome sequencing raw data has been deposited in National Center for Biotechnology Information (Project: PRJNA1234881).

Declarations

Ethical approval and consent to participate

The study was approved by Institute of Animal Science, Chinese Academy of Agricultural Sciences.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, China. [2]Key Laboratory of Grassland Ecosystem of Ministry of Education, College of Pratacultural Science, Gansu Agricultural University, Lanzhou 730070, China. [3]Key Laboratory of Herbage & Endemic Crop Biology, Ministry of Education, School of Life Sciences, Inner Mongolia University, Hohhot 010021, China. [4]College of Grassland, Resources and Environment, Key Laboratory of Grassland Resources, Ministry of Education, Inner Mongolia Agricultural University, Hohhot 010018, China. [5]Key Laboratory of Grassland Resources and Ecology of Western Arid Region, College of Grassland Science, Xinjiang Agricultural University, Urumqi 833400, China. [6]Inner Mongolia Pratacultural Technology Innovation Center Co. Ltd, Hohhot 010070, China. [7]The National Center of Technology Innovation for Comprehensive Utilization of Saline-Alkali Land, Dongying 257300, Shandong Province, China.

Received: 15 January 2025 Accepted: 28 April 2025
Published online: 02 May 2025

References

1. Mora-Ortiz M, Smith LMJ. Onobrychis viciifolia: a comprehensive literature review of its history, etymology, taxonomy, genetics, agronomy and botany. Plant Genetic Resources-Characterization and Utilization. 2018;16(5):403–18.
2. Yang XY, Lu MQ, Wang YF, Wang YR, Liu ZJ, Chen S: Response Mechanism of Plants to Drought Stress. Horticulturae 2021, 7(3).
3. Sheppard SC, Cattani DJ, Ominski KH, Biligetu B, Bittman S, McGeough EJ. Sainfoin production in western Canada: A review of agronomic potential and environmental benefits. Grass Forage Sci. 2019;74(1):6–18.
4. Kozlova ZV, Matais LN, Glushkova OA: Influence of sainfoin on soil fertility and agro-economic indicators of fodder crop rotations under conditions of East Siberia. In: Multifunctional adaptive feed production. vol. 23 Federal Williams Research Center of Forage Production and Agroecology; 2020: 67–72.
5. Sebastià MT, Banagar F, Palero N, Ibáñez M, Plaixats J: Quality Production of Sainfoin Swards Challenged by Global Change in Mountain Areas in the Western Mediterranean. Agronomy-Basel 2024, 14(1).
6. Zarrabian M, Majidi MM, Ehtemam MH. Genetic Diversity in a Worldwide Collection of Sainfoin Using Morphological, Anatomical, and Molecular Markers. Crop Sci. 2013;53(6):2483–96.
7. Sayar MS, Hang Y, Basbag M: Forage yield and forage quality traits of sainfoin (Onobrychis viciifolia SCOP.) genotypes and evaluation with biplot analysis. Fresenius Environmental Bulletin 2022, 31(4):4009–4017.
8. Avci MA, Ozkose A, Tamkoc A. Study of Genotype x Environment Interaction on Agricultural and Quality in Sainfoin (Onobrychis sativa) Genotypes. J Anim Vet Adv. 2013;12(4):428–30.
9. Mohajer S, Jafari AA, Taha RM, Ahmed ABA: Evaluation of Yield and Morphology Traits in 72 Genotypes of Sainfoin (Onobrychis viciifolia Scop) Through Factor Analysis Legume Research 2012, 35(2):132–137.
10. He JY, Tian DY, Li X, Wang XM, Wang TT, Wang ZY, Zang H, He XF, Zhang TJ, Yun QZ et al: A chromosome-level genome assembly for Onobrychis viciifolia reveals gene copy number gain underlying enhanced proanthocyanidin biosynthesis. Communications Biology 2024, 7(1).
11. Jin ZZY, Jiang WB, Yi DX, Pang YZ. The complete chloroplast genome sequence of Sainfoin (Onobrychis viciifolia). Mitochondrial DNA Part B-Resources. 2021;6(2):496–8.
12. Mora-Ortiz M, Swain MT, Vickers MJ, Hegarty MJ, Kelly R, Smith LMJ, Skot L: De-novo transcriptome assembly for gene identification, analysis, annotation, and molecular marker discovery in Onobrychis viciifolia. BMC Genomics 2016, 17.
13. Shen SS, Chai XT, Zhou Q, Luo D, Wang YR, Liu ZP: Development of polymorphic EST-SSR markers and characterization of the autotetraploid genome of sainfoin (Onobrychis viciifolia). Peerj 2019, 7.
14. Abebe H, Tu Y. Impact of Salt and Alkali Stress on Forage Biomass Yield, Nutritive Value, and Animal Growth Performance: A Comprehensive Review. Grasses. 2024;3(4):355–68.
15. Agudelo A, Carvajal M, Martinez-Ballesta MD: Halophytes of the Mediterranean Basin-Underutilized Species with the Potential to Be Nutritious Crops in the Scenario of the Climate Change. Foods 2021, 10(1).
16. Zhang H, Zhu J, Gong Z, Zhu JK. Abiotic stress responses in plants. Nat Rev Genet. 2022;23(2):104–19.
17. Shinozaki K, Yamaguchi-Shinozaki K. Gene networks involved in drought stress response and tolerance. J Exp Bot. 2007;58(2):221–7.
18. Khoso MA, Hussain A, Ritonga FN, Ali Q, Channa MM, Alshegaihi RM, Meng QL, Ali M, Zaman W, Brohi RD et al: WRKY transcription factors (TFs): Molecular switches to regulate drought, temperature, and salinity stresses in plants. Frontiers in Plant Science 2022, 13.
19. Mao C, Zhang J, Zhang Y, Wang B, Li W, Wang X, Huang L. Genome-wide analysis of the WRKY gene family and their response to low-temperature stress in elephant grass. BMC Genomics. 2024;25(1):947.
20. Guo XQ, Ullah A, Siuta D, Kukfisz B, Iqbal S: Role of WRKY Transcription Factors in Regulation of Abiotic Stress Responses in Cotton. Life-Basel 2022, 12(9).
21. Yuan GX, Zhang NJ, Zou YM, Hao YQ, Pan JH, Liu YZ, Zhang WG, Li BB: Genome-wide identification and expression analysis of WRKY gene family members in red clover (Trifolium pratense L.). Frontiers in Plant Science 2023, 14.
22. Li MM, Zhang XQ, Zhang TX, Bai Y, Chen C, Guo DL, Guo CH, Shu YJ: Genome-wide analysis of the WRKY genes and their important roles during cold stress in white clover. Peerj 2023, 11.
23. Wen WW, Wang RY, Su LT, Lv AM, Zhou P, An Y: MsWRKY11,activated by MsWRKY22, functions in drought tolerance and modulates lignin biosynthesis in alfalfa (Medicago sativa L.). Environmental and Experimental Botany 2021, 184.
24. Mao P, Jin XY, Bao QY, Mei C, Zhou Q, Min XY, Liu ZP: WRKY Transcription Factors in Medicago sativa L.: Genome-Wide Identification and Expression Analysis Under Abiotic Stress. DNA and Cell Biology 2020, 39(12):2212–2225.
25. Song H, Nan Z. Genome-wide identification and analysis of WRKY transcription factors in Medicago truncatula. Hereditas (Beijing). 2014;36(2):152–68.
26. Tang LL, Cai H, Zhai H, Luo X, Wang ZY, Cui L, Bai X: Overexpression of Glycine soja WRKY20 enhances both drought and salt tolerance in transgenic alfalfa (Medicago sativa L.). Plant Cell Tissue and Organ Culture 2014, 118(1):77–86.
27. Li KY, Liu XJ, He F, Chen SS, Zhou GY, Wang YH, Li LH, Zhang SQ, Ren MJ, Yuan YY: Genome-wide analysis of the Tritipyrum WRKY gene family and the response of TtWRKY256 in salt-tolerance. Frontiers in Plant Science 2022, 13.
28. Sun SL, Liu X, Zhang TL, Yang H, Yu BJ: Functional Characterisation of the Transcription Factor GsWRKY23 Gene from Glycine soja in Overexpressed Soybean Composite Plants and Arabidopsis under Salt Stress. Plants-Basel 2023, 12(17).
29. Fan SG, Chen JM, Yang RZ: Candidate Genes for Salt Tolerance in Forage Sorghum under Saline Conditions from Germination to Harvest Maturity. Genes 2023, 14(2).
30. Cheng LQ, Peng XJ, Su M, Li XX, Gao Q, Ma XY: Cloning and Function Research of Sheepgrass (Leymus chinensis) Genes. Sheepgrass (Leymus Chinensis): An Environmentally Friendly Native Grass for Animals 2019:247–268.
31. Xiong X, James VA, Zhang HN, Altpeter F. Constitutive expression of the barley HvWRKY38 transcription factor enhances drought tolerance in turf and forage grass (Paspalum notatum Flugge). Mol Breeding. 2010;25(3):419–32.
32. He X, Li JJ, Chen Y, Yang JQ, Chen XY. Genome-wide analysis of the WRKY gene family and its response to abiotic stress in buckwheat (Fagopyrum tataricum). Open Life Sciences. 2019;14(1):80–96.
33. Ren JC, Hu JL, Zhang AL, Ren SP, Jing TT, Wang XS, Sun M, Huang LK, Zeng B: The whole-genome and expression profile analysis of WRKY and RGAs in Dactylis glomerata showed that DG6C02319.1 and DgWRKYs may cooperate in the immunity against rust. Peerj 2021, 9.
34. Cheng SB, Yang XZ, Zou L, Wu DD, Lu JL, Cheng YR, Wang Y, Zeng J, Kang HY, Sha LN et al: Comparative physiological and root transcriptome analysis of two annual ryegrass cultivars under drought stress. Journal of Plant Physiology 2022, 277.
35. Jia XJ, Xiong Y, Xiong YL, Li DX, Yu QQ, Lei X, You MH, Bai SQ, Zhang JB, Ma X: Identification and Validation of Reference Genes for RT-qPCR Analysis in Reed Canary Grass during Abiotic Stress. Genes 2023, 14(9).
36. Ma QB, Xia ZL, Cai ZD, Li L, Cheng YB, Liu J, Nian H: GmWRKY16 Enhances Drought and Salt Tolerance Through an ABA-Mediated Pathway in Arabidopsis thaliana. Frontiers in Plant Science 2019, 9.
37. Ma L, Li X, Zhang JJ, Yi DX, Li F, Wen HY, Liu WH, Wang XM: MsWRKY33 increases alfalfa (Medicago sativa L.) salt stress tolerance through altering the ROS scavenger via activating MsERF5 transcription. Plant Cell and Environment 2023, 46(12):3887–3901.
38. Asma AF, Theodoridou K, Halbwirth H, J A, Stich K: Effect of different conservation methods and maturity stages on peroxidase activity in sainfoin (Onobrychis viciifolia). Agricultural and Food Sciences 2009.
39. Yao Y, Nan L, Wang K, Xia J, Ma B, Cheng J. Integrative leaf anatomy structure, physiology, and metabolome analyses revealed the response to drought stress in sainfoin at the seedling stage. Phytochem Anal. 2024;35(5):1174–85.
40. Gao ZW, Zhu H, Gao JC, Yang CW, Mu CS, Wang DL: Germination responses of Alfalfa (Medicago sativa L.) seeds to various salt-alkaline mixed stress. African Journal of Agricultural Research 2011, 6(16):3793–3803.

41. Seno S, Takenaka Y, Kai C, Kawai J, Carninci P, Hayashizaki Y, Matsuda H. A method for similarity search of genomic positional expression using CAGE. PLoS Genet. 2006;2(4):578–86.
42. Shimizu-Inatsugi R, Terada A, Hirose K, Kudoh H, Sese J, Shimizu KK. Plant adaptive radiation mediated by polyploid plasticity in transcriptomes. Mol Ecol. 2017;26(1):193–207.
43. Wang MY, Li ZJ, Zhang YE, Zhang YY, Xie YL, Ye LH, Zhuang YL, Lin KD, Zhao F, Guo JY, et al. An atlas of wheat epigenetic regulatory elements reveals subgenome divergence in the regulation of development and stress responses. Plant Cell. 2021;33(4):865–81.
44. Arrigo N, Barker MS. Rarely successful polyploids and their legacy in plant genomes. Curr Opin Plant Biol. 2012;15(2):140–6.
45. Lynch M, Conery JS. The evolutionary fate and consequences of duplicate genes. Science. 2000;290(5494):1151–5.
46. Shanker AK, Maheswari M, Yadav SK, Desai S, Bhanu D, Attal NB, Venkateswarlu B. Drought stress responses in crops. Funct Integr Genomics. 2014;14(1):11–22.
47. Sheoran S, Kaur Y, Kumar S, Shukla S, Rakshit S, Kumar R: Recent Advances for Drought Stress Tolerance in Maize (Zea mays L.): Present Status and Future Prospects. Front Plant Sci 2022, 13:872566.
48. Wu J, Liang X, Lin M, Lan Y, Xiang Y, Yan H. Comprehensive analysis of MAPK gene family in Populus trichocarpa and physiological characterization of PtMAPK3-1 in response to MeJA induction. Physiol Plant. 2023;175(1): e13869.
49. Li JL, Ma MS, Sun YM, Lu P, Shi HF, Guo ZF, Zhu HF: Comparative Physiological and Transcriptome Profiles Uncover Salt Tolerance Mechanisms in Alfalfa. Frontiers in Plant Science 2022, 13.
50. Awan SA, Khan I, Tariq R, Rizwan M, Wang XS, Zhang XQ, Huang LK: Genome-Wide Expression and Physiological Profiling of Pearl Millet Genotype Reveal the Biological Pathways and Various Gene Clusters Underlying Salt Resistance. Frontiers in Plant Science 2022, 13.
51. Shen HS, Liu CT, Zhang Y, Meng XP, Zhou X, Chu CC, Wang XP. OsWRKY30 is activated by MAP kinases to confer drought tolerance in rice. Plant Mol Biol. 2012;80(3):241–53.
52. Zhou M, Zhao B, Li H, Ren W, Zhang Q, Liu Y, Zhao J: Comprehensive analysis of MAPK cascade genes in sorghum (Sorghum bicolor L.) reveals SbMPK14 as a potential target for drought sensitivity regulation. Genomics 2022, 114(2):110311.
53. Ding R, Li J, Wang J, Li Y, Ye W, Yan G, Yin Z. Molecular traits of MAPK kinases and the regulatory mechanism of GhMAPKK5 alleviating drought/salt stress in cotton. Plant Physiol. 2024;196(3):2030–47.
54. Tasnim A, Jahan I, Azim T, Karmoker D, Seraj ZI: Paired growth of cultivated and halophytic wild rice under salt stress induces bacterial endophytes and gene expression responses. Frontiers in Plant Science 2023, 14.
55. Jiang Y, Liang G, Yu D. Activated expression of WRKY57 confers drought tolerance in Arabidopsis. Mol Plant. 2012;5(6):1375–88.
56. Wen WW, Wang RY, Su LT, Lv AM, Zhou P, An Y: MsWRKY11, activated by MsWRKY22, functions in drought tolerance and modulates lignin biosynthesis in alfalfa (Medicago sativa L.). Environmental and Experimental Botany 2021, 184.
57. Jiang YQ, Deyholos MK. Functional characterization of Arabidopsis NaCl-inducible WRKY25 and WRKY33 transcription factors in abiotic stresses. Plant Mol Biol. 2009;69(1–2):91–105.
58. Zhou QY, Tian AG, Zou HF, Xie ZM, Lei G, Huang J, Wang CM, Wang HW, Zhang JS, Chen SY. Soybean WRKY-type transcription factor genes, GmWRKY13, GmWRKY21, and GmWRKY54, confer differential tolerance to abiotic stresses in transgenic Arabidopsis plants. Plant Biotechnol J. 2008;6(5):486–503.
59. Chen YH, Zhang J: Multiple functions and regulatory networks of WRKY33 and its orthologs. Gene 2024, 931.
60. Rachowka J, Anielska-Mazur A, Bucholc M, Stephenson K, Kulik A: SnRK2.10 kinase differentially modulates expression of hub WRKY transcription factors genes under salinity and oxidative stress in Arabidopsis thaliana. Frontiers in Plant Science 2023, 14.
61. Kataria R, Duhan N, Kaundal R: Computational Systems Biology of Alfalfa - Bacterial Blight Host-Pathogen Interactions: Uncovering the Complex Molecular Networks for Developing Durable Disease Resistant Crop. Frontiers in Plant Science 2022, 12.
62. Menéndez AB, Ruiz OA: Stress-regulated elements in Lotus spp., as a possible starting point to understand signalling networks and stress adaptation in legumes. Peerj 2021, 9.
63. Mo F, Li HB, Li YH, Chen X, Wang MS, Li Z, Deng NC, Yang Y, Huang X, Zhang R et al: Physiological, biochemical, and transcriptional regulation in a leguminous forage Trifolium pratense L. responding to silver ions. Plant Physiology and Biochemistry 2021, 162:531–546.
64. Zhao YY, Zhang JC, Sun ZM, Tang YX, Wu YM: Genome-Wide Identification and Analysis of the Polycomb Group Family in Medicago truncatula. International Journal of Molecular Sciences 2021, 22(14).
65. Wei J, Luo B, Kong SY, Liu WX, Zhang CJ, Wei ZW, Min XY: Screening and identification of multiple abiotic stress responsive candidate genes based on hybrid-sequencing in Vicia sativa. Heliyon 2023, 9(2).
66. Liu B, Seong K, Pang SA, Song JQ, Gao H, Wang CL, Zhai JQ, Zhang Y, Gao S, Li XD, et al. Functional specificity, diversity, and redundancy of Arabidopsis JAZ family repressors in jasmonate and COI1-regulated growth, development, and defense. New Phytol. 2021;231(4):1525–45.
67. Kuki Y, Ohno R, Yoshida K, Takumi S. Heterologous expression of wheat WRKY transcription factor genes transcriptionally activated in hybrid necrosis strains alters abiotic and biotic stress tolerance in transgenic Arabidopsis. Plant Physiol Biochem. 2020;150:71–9.
68. Tang W, Luo C. Overexpression of Zinc Finger Transcription Factor ZAT6 Enhances Salt Tolerance. Open Life Sciences. 2018;13(1):431–45.
69. Chen CJ, Wu Y, Li JW, Wang X, Zeng ZH, Xu J, Liu YL, Feng JT, Chen H, He YH, et al. TBtools-II: A "one for all, all for one"bioinformatics platform for biological big-data mining. Mol Plant. 2023;16(11):1733–42.
70. Tamura K, Stecher G, Kumar S. MEGA11 Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol. 2021;38(7):3022–7.
71. Jiang W, Yin Q, Liu J, Su X, Han X, Li Q, Zhang J, Pang Y: The APETALA2–MYBL2 module represses proanthocyanidin biosynthesis by affecting formation of the MBW complex in seeds of Arabidopsis thaliana. Plant Communications 2024, 5(3).
72. Jin ZZY, Jiang WB, Luo YJ, Huang HJ, Yi DX, Pang YZ: Analyses on Flavonoids and Transcriptome Reveals Key MYB Gene for Proanthocyanidins Regulation in Onobrychis Viciifolia. Frontiers in Plant Science 2022, 13.

Publisher's Note