

Genome-wide characterization of WRKYs and function of SpWRKY40 and SpWRKY50 in *Sesuvium portulacastrum*

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Research Article

Keywords: *Sesuvium portulacastrum*, WRKY gene family, Abiotic stress, SpWRKY40, SpWRKY51, Transcription factor

Posted Date: October 20th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7513928/v1>

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Functional & Integrative Genomics on December 22nd, 2025. See the published version at <https://doi.org/10.1007/s10142-025-01769-w>.

1 **Genome-wide characterization of *WRKYs* and function of *SpWRKY40* and**
2 ***SpWRKY50* in *Sesuvium portulacastrum***

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Key message We identified 68 *SpWRKYs* from *S. portulacastrum* genome and revealed that *SpWRKY40* and *SpWRKY51* play crucial roles in adapting to abiotic stress by functioning as transcription factors.

Abstract

WRKYs represent a large family of plant transcription factors characterized by a highly conserved WRKY domain. WRKY transcription factors are important for plant growth, development, and responses to environmental stresses. However, this family has not been previously identified in *Sesuvium portulacastrum*. Here, we comprehensively identified 68 *SpWRKYs* from the genome of *S. portulacastrum* by phylogenetic relationships, chromosomal localization, collinearity analysis, gene structure, duplication events, and evolutionary pressure based on nonsynonymous substitution rate/synonymous substitution rate ratios. Promoter analysis revealed the presence of multiple *cis*-elements associated with abiotic stress signaling, developmental regulation, phytohormone responses, light responsiveness, and tissue-specific expression. We also characterized the physicochemical and structural properties of the *SpWRKY*-encoded proteins, including protein property of primary structure, secondary structures, and predicted three-dimensional models, subcellular localization, conserved motifs, and conserved domains. Furthermore, transcriptome profiling under cadmium, copper, and salt stress conditions demonstrated differential expression patterns of *SpWRKYs*. Among them, *SpWRKY40* and *SpWRKY51* showed significant induced under cadmium treatment and were further validated by quantitative real-time PCR. Subcellular localization and yeast one-hybrid assays both supporting *SpWRKY40* and *SpWRKY51* play as transcription factors. Our study provides a detailed overview of the *SpWRKY* family and their potential involvement in environment tolerance. The findings offer valuable candidate genes for future applications in improving stress tolerance in *S. portulacastrum* and related crop species.

Keywords: *Sesuvium portulacastrum*, WRKY gene family, Abiotic stress, *SpWRKY40*, *SpWRKY51*, Transcription factor

Introduction

Sesuvium portulacastrum, as a typical halophyte, is broadly found in coastal areas [1]. It grows vigorously in saline soils and marshlands due to its excellent ability to tolerance to environment stress [2,3]. It important for stabilizing shorelines, fixing sand, and improving saline-alkaline lands [4]. Its remarkable ability to withstand high salt concentrations results from a combination of structural and genetic adaptations [5]. The plant has specialized salt glands on its epidermis, which help it to actively secrete excess ions, maintaining ionic balance under saline conditions. Additionally, its rapid growth, high rate of vegetative propagation, and strong adaptability to environmental changes make it a promising species for ecological applications [6]. *S. portulacastrum* exhibits strong reproductive ability and adaptability, which strengthens its value for phytoremediation [7]. This species demonstrates notable tolerance and accumulation capacity for multiple heavy metals, particularly cadmium (Cd), copper (Cu) and cesium [1,8,9]. *S. portulacastrum* has been integrated into ecological floating bed systems designed to treat heavy metal-polluted aquatic environments [10]. The plant provides a cost-effective and environmentally sustainable solution for pollution control and ecosystem restoration (11,12).

WRKY Proteins are unique to plants. They are identified by a conserved WRKY domain consisting of 60 amino acids [13]. It contains a WRKYGQK heptapeptide and a zinc-finger motif, usually of the C2H2 or C2HC type, which enables DNA binding and gene regulation [14]. WRKY proteins are classified into three subgroups. Subgroup I has two WRKY domains, subgroup II consists of a single WRKY domain featuring a C2H2-type zinc finger and is further divided into five clades (IIa-IIe), while subgroup III contains only WRKY domain and C2HC-type zinc finger [15].

WRKY transcription factors play a pivotal role in enhancing plant tolerance to salt and heavy metal stresses through various regulatory mechanisms [16]. *AtWRKY2* is involved in regulating seed maturation and flowering time [17]. *AtWRKY46* enhances salt tolerance by promoting the expression of stress-responsive gene [18]. *OsWRKY72* enhances salt tolerance by activating *Na⁺-selective transporter 1*, a key gene involved in sodium transport [19]. *OsWRKY51* contributes to salt resistance by interacting with the abscisic acid (ABA) signaling [20]. *AtWRKY39* contributes to heat stress responses through the combined effects of salicylic acid and jasmonic acid signaling [21]. *ABO3* and *AtWRKY57* play opposing roles in ABA signaling, which affects drought tolerance [22,23]. *AtWRKY18*, *AtWRKY40*, and *AtWRKY60* form a heterotrimeric complex

that regulates ABA signaling for abiotic stress [24]. *AtWRKY13* and *AtWRKY33* improves Cd tolerance by stimulating *PDR8* and *ATL31* expression, respectively (25,26). *AtWRKY6* confers resistance to arsenic stress by suppressing the expression of *arsenate/phosphate transporters*, which reduces arsenate uptake [27]. *AtWRKY45* regulates *PHT1* expression and plays a role in responding to phosphate deficiency [28]. *AtWRKY54* is associated with immune responses [29]. *OsWRKY22* promotes the expression of *OsFRDL4*, enhancing aluminum tolerance [30]. *OsWRKY37* activates *OsCOPT6* expression to regulate copper uptake and its translocation [31]. *OsWRKY72* represses the expression of *OsGLP8-7*, which leads to Cd accumulation. However, *OsWRKY53* interacts with *OsWRKY72*, inhibiting its activity contribute to Cd stress [32]. *oswrky36* promotes lignin accumulation, enhancing resistance to diseases and pests [33]. *OsWRKY70* is involved in cold tolerance, *OsWRKY10* enhances disease resistance through interactions with the valine-glutamine 8 protein [34-36].

In this study, we conducted a comprehensive analysis of 68 *SpWRKYs* identified in *S. portulacastrum*, focusing on protein property of primary structure, secondary structures, and predicted three-dimensional models, intraspecific and interspecific collinearity, and duplication events. We examined the *cis*-elements in *SpWRKYs* promoters associated with abiotic stress signaling and further explored their variations through transcriptome profiling under Cd, Cu, and salt stress, along with quantitative real-time PCR (qRT-PCR). The nuclear localization and DNA binding activity of *SpWRKY40* and *SpWRKY51* support their roles as transcription factors. The findings highlight the involvement of *SpWRKYs* in response to abiotic stress in *S. portulacastrum*, and the functions of *SpWRKY40* and *SpWRKY51* provide strategies for improving crop genetics.

Materials and methods

Comprehensive characterization of *SpWRKYs*

The AtWRKY sequences from *A. thaliana* were retrieved from the TAIR database (<https://www.arabidopsis.org>). The genome sequence data of *S. portulacastrum* is available on PRJNA1234053. The SpWRKYs were identified using the PF03106 domain (<https://www.ebi.ac.uk/interpro/entry/pfam>). Multiple sequence alignments and the construction of a phylogenetic tree were conducted using MEGA11 software. The exon-intron structures were visualized using TBtools. The properties of the SpWRKYs were analyzed by ProtParam program. Conserved motifs were identified through the MEME Suite (<https://meme-suite.org>). Protein secondary structure predictions were carried out using the SOPMA server (https://npsa.lyon.inserm.fr/cgi-bin/npsa_automat.pl), and tertiary structure models were built using the SWISS-MODEL platform (<https://swissmodel.expasy.org>). The subcellular localization of the proteins was predicted using both WoLF PSORT (<https://wolfsort.hgc.jp>) and CELLO v2.5 tools (<https://cello.life.nctu.edu.tw>). Collinearity analysis of *SpWRKYs* were performed using the One Step MCScanX-SupperFast as described previously [37]. For the interspecific collinearity analysis between *S. portulacastrum* and *A. thaliana*, the same One Step MCScanX-SupperFast was utilized according to the methodology described previously [37].

Analysis of *cis*-elements of *SpWRKYs* promoter

We extracted the 2 kb upstream sequences of *SpWRKYs* to analyze their promoter *cis*-elements. Those sequences were analyzed and visualized using PlantCARE and TBtools.

Analysis the expression pattern of *SpWRKYs*

The expression patterns of *SpWRKYs* were assessed using transcriptome data related to Cd, Cu, and salt stress (Tables S1, S2, S3). The expression levels of *SpWRKY40* and *SpWRKY51* were quantified through qRT-PCR with gene-specific primers (Table S4). The transcript levels of *SpGAPDH* served to normalize the expression levels of the

target genes.

Subcellular localization of SpWRKY40 and SpWRKY51

To investigate the subcellular localization of *SpWRKY40* and *SpWRKY51*, their coding sequences were insert into 35S::GFP, generating the expression constructs 35S::SpWRKY40-GFP and 35S::SpWRKY51-GFP. 35S::TksARF3-mCherry was used as a reference for nuclear localization [37]. These recombinant plasmids were introduced into tobacco cells *via Agrobacterium*-mediated transient transformation. Fluorescence signals from the tagged proteins were observed using a Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Y1H assay

The coding sequences of *SpWRKY40* and *SpWRKY51* were inserted into the pGADT7-rec2 vector using gene-specific primers (Table S4), yielding pGADT7-rec2-SpWRKY40 and pGADT7-rec2-SpWRKY51. The sequences of MA1076 (GTCAA) with triple tandem repeats, MA1076_mutant (GTCAA), MA0589 (TTGAC), and MA0589_mutant (TTGAC) were subcloned into the pHIS2 vector. These recombinant constructs were then transformed into Y187 yeast cells via the polyethylene glycol and lithium acetate transformation protocol. To assess DNA-protein binding, transformed yeast cultures were serially diluted and plated on selective media SD/-Leu/-Trp (DDO) and SD/-His/-Leu/-Trp (TDO) with 50 mM 3-AT for stringent screening.

Results

Phylogenetic analysis of *SpWRKYs*

A total of 68 *SpWRKYs* were identified in the genome of *S. portulacastrum* (Table S5), designated as *SpWRKY1* to *SpWRKY68*. The lengths of the *SpWRKYs* ranged from 107 (SpWRKY13) to 1314 (SpWRKY9) amino acids. The molecular weights varied from 12285.08 (SpWRKY13) to 144736.1 (Da) (SpWRKY9), while the predicted isoelectric points (pI) of these proteins varied between 5.12 (SpWRKY19) and 10.04 (SpWRKY3). Among them, 22 proteins including WRKY2 are basic proteins, and the remaining proteins are all acidic proteins. Additionally, the aliphatic indices ranged from 37.80 (SpWRKY30) to 76.69 (SpWRKY52). Instability indicating that the *SpWRKYs* are predominantly unstable range from 21.50 (SpWRKY13) to 70.01 (SpWRKY57), and hydrophilic with average hydrophilicity values ranging from -1.214 (SpWRKY61) to -0.593 (SpWRKY9). Through subcellular localization prediction, the vast majority of members of the *SpWRKY* gene family are localized in the nucleus, and a few *SpWRKY* members such as SpWRKY2, SpWRKY9, SpWRKY12, SpWRKY20, SpWRKY23, SpWRKY24, SpWRKY33, SpWRKY37, SpWRKY43, SpWRKY44, SpWRKY48, SpWRKY54, SpWRKY55, SpWRKY56, SpWRKY64, SpWRKY67, SpWRKY68 are localized extracellularly. Secondary structure analysis showed that *SpWRKYs* are mainly composed of disordered conformations, and random coils account for a large proportion compared with α -helices and β -sheets (Table S6). Three-dimensional modeling revealed a conserved spatial structure throughout the family (Fig. S1).

Phylogenetic classified the *SpWRKYs* into three subgroups, aligning with the established WRKY classification. Subgroup I comprising 25 *SpWRKYs*, while clades II contained 43, which were subdivided into IIa, IIb, IIc, IId, and IIe. No *SpWRKYs* were assigned to subgroup III (Fig. 1).

Chromosomal localization and covariance of the *SpWRKYs*

The *SpWRKYs* were found to be unevenly distributed across 22 chromosomes (Fig. 2). Chromosome 7 contained the highest number, with nine *SpWRKYs*, while chromosomes 8 and 12 each housed six. Chromosome 14 had five, and chromosomes 10 and 11 each had four *SpWRKYs*. Chromosomes 4 and 13 each contained three. The remaining ten chromosomes carried two *SpWRKYs*, respectively.

We identified 24 segmental duplication events among the *SpWRKYs*. A comparative

collinearity analysis between *S. portulacastrum* and *A.thaliana* indicated 72 orthologous gene pairs (Fig. 3A). Further investigation uncovered 70 duplication events within the *S. portulacastrum* genome, with chromosomes 7, 8, 9, 13, and 14 exhibiting a higher number of duplicated genes. Chromosome 5 contained the fewest *SpWRKYs*, with only two members identified (Fig. 3B). The nonsynonymous substitution rate (K_a) values for duplicated *SpWRKY* gene pairs ranged from 0.01 to 0.7, while synonymous substitution rate (K_s) values varied between 0.4 and 3.1. All gene pairs exhibited K_a/K_s ratios below one (Table S7). Genome-wide analysis of duplication patterns revealed that most *SpWRKYs* were derived from whole-genome or segmental duplication events. *SpWRKY38* was the only member identified as resulting from dispersed duplication (Table S8). Taken together, those results suggested that large-scale and localized duplications were the primary forces driving the expansion of the *SpWRKY* family, and that evolutionary pressure has acted to preserve gene function across most members of this family.

Conserved motif and gene structure analysis of the *SpWRKYs*

A total of ten conserved motifs were identified across the *SpWRKY* protein family (Fig. 4A). Motif 1 was consistently detected in all members, while Motif 2 was present in all except for *SpWRKY15*. Motif 3 was found in a subset of proteins, including *SpWRKY8*, *SpWRKY9*, *SpWRKY22*, *SpWRKY24*, *SpWRKY25*, *SpWRKY26*, *SpWRKY33*, *SpWRKY37*, *SpWRKY38*, *SpWRKY40*, *SpWRKY44*, *SpWRKY49*, *SpWRKY51*, *SpWRKY56*, *SpWRKY57*, *SpWRKY60*, *SpWRKY65*, and *SpWRKY68*. Motif 4 appeared twice in *SpWRKY1*, was absent from *SpWRKY3*, *SpWRKY9*, *SpWRKY13*, *SpWRKY20*, *SpWRKY27*, *SpWRKY28*, *SpWRKY34*, *SpWRKY38*, *SpWRKY39*, *SpWRKY48*, *SpWRKY52*, *SpWRKY53*, and *SpWRKY55*, and was found as a single copy in the remaining proteins. Motifs 5 and 8 were detected in *SpWRKY7*, *SpWRKY15*, *SpWRKY19*, *SpWRKY21*, *SpWRKY23*, *SpWRKY27*, *SpWRKY34*, *SpWRKY35*, *SpWRKY41*, *SpWRKY47*, *SpWRKY50*, *SpWRKY52*, *SpWRKY62*, and *SpWRKY66*. Motifs 6 and 7 were commonly present in *SpWRKY24*, *SpWRKY33*, and *SpWRKY56*. The distribution of other motifs varied among individual *SpWRKY* members. A total of six conserved domains were also identified (Fig. 4B, S2). The WRKY domain was present in all proteins. The Plant_zn_clust domain was observed in *SpWRKY3*, *SpWRKY10*, *SpWRKY16*, *SpWRKY28*, and

SpWRKY39. Both the RplQ and WD40 domains were found in SpWRKY9. The PRK11377 domain was detected in SpWRKY23, while the LGT superfamily domain was identified in SpWRKY37, SpWRKY44, and SpWRKY68.

Analysis of exon-intron structure revealed that *SpWRKYs* contained between two and three introns, and between two and eleven exons (Fig. 4C). Among them, *SpWRKY8* had the longest mRNA transcript and the greatest number of exons, totaling eleven. In contrast, *SpWRKY6* exhibited the shortest transcript with only two exons.

Taken together, these structural characteristics suggest that *SpWRKYs* retain a conserved functional core and likely participate in similar transcriptional regulatory processes. However, variations in motif composition, domain architecture, and gene structure imply functional diversification and evolutionary divergence. Some genes, such as SpWRKY8 and SpWRKY38, may represent unique evolutionary variants or serve as potential candidates involved in specialized stress responses.

Cis-element analysis of the *SpWRKYs* promoters

To investigate the regulatory potential of *SpWRKYs*, a comprehensive analysis was performed to predict *cis*-elements in all *SpWRKYs* promoter. Analysis revealed 33 unique *cis*-elements were identified and grouped into six main functional classes according to their biological roles (Fig. 5A). These included elements responsive to phytohormones, abiotic stresses, light signals, as well as those involved in developmental regulation and tissue-specific expression. Among these categories (Fig. 5B), light-responsive elements were present in the promoter regions of all 68 *SpWRKYs* and represented the most abundant class. In contrast, some elements were less commonly distributed. The seed-specific regulatory element was found only once, located in the promoter of *SpWRKY61*. Similarly, the element associated with enhanced transcriptional activity appeared only in *SpWRKY14*. Gibberellin-responsive and additional light-responsive elements were detected in only a subset of the *SpWRKYs*. Collectively, these results indicate that although some regulatory elements are highly conserved and may function in general transcriptional control, others show selective distribution patterns, suggesting possible functional divergence among different members of the *SpWRKY* family.

Expression profiling of *SpWRKYs* under Cd, Cu, and salt stress conditions

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263 To explore the expression of the *SpWRKYs* under various abiotic stress, transcriptome
264 sequencing on *S. portulacastrum* exposed to Cd, copper, and salt treatments. In leaves,
265 *SpWRKY34*, *SpWRKY39*, *SpWRKY41*, *SpWRKY42*, *SpWRKY43*, *SpWRKY44*,
266 *SpWRKY45*, *SpWRKY46*, *SpWRKY47*, *SpWRKY48*, *SpWRKY55*, *SpWRKY59*,
267 *SpWRKY65*, *SpWRKY66*, and *SpWRKY67* exhibited the highest expression levels prior
268 to treatment. A separate group of genes, including *SpWRKY2*, *SpWRKY6*, *SpWRKY8*,
269 *SpWRKY18*, *SpWRKY26*, *SpWRKY29*, *SpWRKY49*, *SpWRKY56*, *SpWRKY58*,
270 *SpWRKY60*, *SpWRKY62*, and *SpWRKY63*, were upregulated at day 7 after Cd treatment.
271 At day 14, genes such as *SpWRKY3*, *SpWRKY6*, *SpWRKY7*, *SpWRKY15*, *SpWRKY16*,
272 *SpWRKY24*, *SpWRKY28*, *SpWRKY40*, *SpWRKY50*, *SpWRKY54*, *SpWRKY57*,
273 *SpWRKY64*, and *SpWRKY65* showed continuously upregulated compared to the earlier
274 time points. By day 21, *SpWRKY4*, *SpWRKY23*, *SpWRKY27*, *SpWRKY32*, and
275 *SpWRKY61* were most highly expressed relative to all prior stages (Fig. 6A). In roots,
276 several genes including *SpWRKY1*, *SpWRKY4*, *SpWRKY8*, *SpWRKY21*, *SpWRKY23*,
277 *SpWRKY25*, *SpWRKY27*, *SpWRKY46*, *SpWRKY47*, *SpWRKY53*, *SpWRKY55*,
278 *SpWRKY60*, and *SpWRKY61* an early increase in expression, followed by a reduction
279 as treatment progressed (Fig. 6A). Among all genes, *SpWRKY40* and *SpWRKY51*
280 consistently demonstrated the highest upregulation in both leaves and roots under Cd
281 treatment.

282 Under copper treatment, several *SpWRKYs* in leaves exhibited the highest expression
283 levels prior to stress exposure. These included *SpWRKY34*, *SpWRKY39*, *SpWRKY41*,
284 *SpWRKY42*, *SpWRKY43*, *SpWRKY44*, *SpWRKY45*, *SpWRKY46*, *SpWRKY47*,
285 *SpWRKY48*, *SpWRKY55*, *SpWRKY59*, *SpWRKY65*, *SpWRKY66*, and *SpWRKY67*. By
286 day 7 of treatment, elevated transcript levels were observed in *SpWRKY2*, *SpWRKY6*,
287 *SpWRKY8*, *SpWRKY18*, *SpWRKY26*, *SpWRKY29*, *SpWRKY49*, *SpWRKY56*,
288 *SpWRKY58*, *SpWRKY60*, *SpWRKY62*, and *SpWRKY63*. At day 14, genes including
289 *SpWRKY3*, *SpWRKY6*, *SpWRKY7*, *SpWRKY15*, *SpWRKY16*, *SpWRKY24*, *SpWRKY28*,
290 *SpWRKY40*, *SpWRKY50*, *SpWRKY54*, *SpWRKY57*, *SpWRKY64*, and *SpWRKY65*
291 reached their highest expression levels. By day 21, peak expression was detected in
292 *SpWRKY4*, *SpWRKY23*, *SpWRKY27*, *SpWRKY32*, and *SpWRKY61* (Fig. 6B). In roots,
293 *SpWRKY1*, *SpWRKY4*, *SpWRKY8*, *SpWRKY21*, *SpWRKY23*, *SpWRKY25*, *SpWRKY27*,
294 *SpWRKY46*, *SpWRKY47*, *SpWRKY53*, *SpWRKY55*, *SpWRKY60*, and *SpWRKY61*
295 displayed a transient increase in expression followed by a decline as copper treatment

progressed (Fig. 6B). Among all genes analyzed, *SpWRKY40* and *SpWRKY51* consistently exhibited the suppress expression in both leaves and roots.

Under salt stress conditions, genes such as *SpWRKY3*, *SpWRKY7*, *SpWRKY8*, *SpWRKY9*, *SpWRKY14*, *SpWRKY16*, *SpWRKY18*, *SpWRKY20*, *SpWRKY22*, *SpWRKY25*, *SpWRKY27*, *SpWRKY28*, *SpWRKY30*, *SpWRKY34*, *SpWRKY35*, *SpWRKY39*, *SpWRKY40*, *SpWRKY49*, *SpWRKY50*, *SpWRKY52*, *SpWRKY57*, *SpWRKY60*, *SpWRKY65*, and *SpWRKY67* exhibited a gradual decrease in transcript abundance with increasing salt concentrations (Fig. 6C). The remaining *SpWRKYs* showed an overall trend of increased expression as salt levels rose. Among all genes analyzed, *SpWRKY40* and *SpWRKY51* consistently exhibited the upregulation under NaCl treatment. Furthermore, qRT-PCR was conducted to varied that *SpWRKY40* and *SpWRKY51* were induced expression in Cd treatment (Fig. 6D). Collectively, these findings indicate that *SpWRKY40* and *SpWRKY51* may function in stress signaling pathways in *S. portulacastrum*.

Functional characterization of *SpWRKY40* and *SpWRKY51* as transcription factors

Subcellular localization predictions indicated that the proteins encoded by *SpWRKY40* and *SpWRKY51* are likely localized in the nucleus (Table S5). Both proteins possess a conserved DNA-binding domain and a putative transcriptional activation region, as identified in domain structure analysis (Fig. 4B). To validate their nuclear localization, *SpWRKY40*-GFP and *SpWRKY51*-GFP fusion constructs were co-expressed with the nuclear marker TksARF3-mCherry in tobacco epidermal cells, respectively. The green fluorescence signal from GFP was observed to overlap with the red fluorescence signal from mCherry, indicating nuclear co-localization (Fig. 7A). To further investigate the transcriptional regulatory potential of *SpWRKY40* and *SpWRKY51*, yeast one-hybrid assays were performed. In these assays, the constructs pHIS2-MA1076 and pGADT7-rec2-*SpWRKY40* were co-transformed into yeast cells. The yeast grew well on both DDO and TDO media supplemented with 50 mM 3-AT, demonstrating successful activation of reporter gene expression. However, the constructs pHIS2-MA1076_mutant and pGADT7-rec2-*SpWRKY40* cannot growth on TDO media even supplemented with 50 mM 3-AT (Fig. 7B, 7C). Similar results were obtained with additional target sequences. Both pHIS2-MA0586 and pGADT7-rec2-*SpWRKY40*, as well as pHIS2-MA1076 and pHIS2-MA0586 co-transformed with *SpWRKY51*,

exhibited growth on selective medium containing 3-AT (Fig. 7B, 7C). These results collectively support the conclusion that SpWRKY40 and SpWRKY51 act as functional transcription factors.

Discussion

WRKY transcription factors play crucial roles as regulators of plant growth, development, and stress responses. It has characterized in *A. thaliana*, *Oryza sativa*, cotton, maize, soybean, sugarcane, and wheat [38-42]. WRKY transcription factors influence heavy metal stress in *Arabidopsis* and regulate salt stress in rice [43]. The present study identified the the property of *SpWRKYs* and its encoded protein and SpWRKY40 and SpWRKY51 may function as transcription factor to response to Cd, Cu, and slat tolerance.

A total of 68 *SpWRKYs* were identified in the *S. portulacastrum* genome through comprehensive genome-wide analysis. These genes were classified into three major subfamilies. The second subfamily was further divided into five subgroups. Functional divergence among these groups was closely associated with differences in domain numbers and types of zinc fingers, which may influence their regulatory roles in stress responses [44,45]. Motif analysis showed that Motif1 and Motif2 were conserved across all WRKY proteins, suggesting their utility as markers for WRKY gene identification. Members of the same subfamily generally shared similar motif composition patterns, while notable variation existed between subfamilies, supporting the functional divergence among them. Structural comparison of homologous gene pairs revealed differences in intron number, such as between SpWRKY10 and SpWRKY16. This variation likely reflects evolutionary intron gain or loss events, which have been linked to accelerated gene evolution through duplication and recombination mechanisms. The distribution of introns and exons also provided clues to phylogenetic relationships within the family. *Cis*-element analysis identified 33 types of *cis*-elements in the promoter regions of the 68 *SpWRKYs*. Those *cis*-elements grouped into hormone-responsive, abiotic stress-related, light-responsive, developmental and tissue-specific, and special function elements. Of which, all *SpWRKYs* promoter contained light-responsive elements, while other elements were distributed with varying specificity across genes. This uneven distribution suggests that individual *SpWRKYs* may participate in specialized regulatory processes during plant development and stress adaptation [46].

Expression profiling under Cd, Cu, and salt stresses revealed that the 68 *SpWRKYs* responded in a stress-specific manner. Under salt stress, many genes showed increasing expression with rising salinity, while others followed a bell-shaped curve, first increasing then decreasing. Under Cd exposure, leaf samples generally exhibited high initial expression levels, while roots samples showed stronger induction during prolonged exposure, indicating greater Cd tolerance in roots. Cu treatment also triggered stronger activation of *SpWRKYs* in roots than in leaves, with many genes showing their highest expression under control conditions, suggesting root-specific adaptation to Cu exposure. Of which, *SpWRKY40* and *SpWRKY51* consistently displayed stable expression patterns across all three stress conditions. This suggests their potential roles in regulating multiple physiological processes. The qRT-PCR validation confirmed the Cd-induced expression patterns of *SpWRKY40* and *SpWRKY51*, consistent with transcriptome results. Their expression also varied significantly under salt and Cd treatments, further highlighting their regulatory importance.

Subcellular localization experiments confirmed that both *SpWRKY40* and *SpWRKY51* are nuclear proteins. Their expression in roots increased and then decreased under Cd treatment, and their consistent differential expression across treatments supports their involvement in stress signaling. These findings align with previous studies demonstrating *WRKY* function in salinity, Cu, and Cd stress responses [47-49]. The functional role of *WRKYs* in abiotic resistance is often attributed to their interaction with VQ proteins via the C-terminal *WRKY* domain, enabling regulation of multiple signaling pathways [50]. As molecular switches, *WRKY* transcription factors modulate diverse downstream signaling events. For example, *OsWRKY13* regulates pathogen resistance by coordinating salicylic acid and jasmonic acid pathways [51]. The roles of *SpWRKY40* and *SpWRKY51* in *S. portulacastrum* suggest they may serve similarly as key integrators of multiple environmental and hormonal signals.

In summary, we characterized the property of the 68 *SpWRKYs*, along with their corresponding protein properties, structural features, and regulatory elements. Of which, *SpWRKY40* and *SpWRKY51* were identified as candidate transcription factors likely involved in regulating responses to Cd, Cu, and salt stress. These two genes exhibited consistent stress-responsive expression patterns and nuclear localization, suggesting functional roles in abiotic stress tolerance. The uncovered function of *SpWRKY40* and *SpWRKY51* provide a potential genetic target for improving abiotic stress resistance in

halophytes and related crop species through molecular breeding strategies.

Conclusion

We identified 68 *SpWRKYs* in *S. portulacastrum* and classified them into three subgroups comparison with *Arabidopsis*. The gene structure, chromosomal distribution, duplication events, and Ka/Ks ratios indicated that both whole-genome and segmental duplications have driven the expansion of *SpWRKYs*. We further characterized the encoded proteins in terms of amino acid composition, molecular weight, isoelectric point, aliphatic index, hydropathicity, instability index, subcellular localization, secondary and tertiary structures, as well as conserved motifs and domains, confirming their potential roles as transcription factors. *Cis*-elements analysis abundant to phytohormone signaling, abiotic stress response, light sensitivity, development, and tissue specificity. Transcriptome analysis under Cd, Cu, and salt stress showed that *SpWRKYs* exhibit distinct expression patterns in response to stress. *SpWRKY40* and *SpWRKY51* were significantly upregulated under Cd treatment. Functional verification further suggested that both *SpWRKY40* and *SpWRKY51* act as transcription factors. Together, these findings provide insight into the regulatory roles of *SpWRKYs* under abiotic stress and offer potential targets for developing stress-tolerant crops.

Declarations

The authors declared that they do not have any commercial or associative interest that represents a conflict of interest in connection with this work.

Data availability

Data will be made available on request.

Author contributions

S. H., Y. L., and F. F. initiated and designed the project, S. H., Y. L., F. F., X. W., T. C., Y. Y., and Z. X. reviewing the referenced articles and preparing the figures and tables, S. H., Y. L., and F. F. writing and revising the manuscript. All authors approved the final version of the text.

Acknowledgments

The authors express their sincere gratitude to Dr. Xuchu Wang in Guizhou University.

Funding

This work was supported by the National Natural Science Foundation of Guizhou University (Guida Tegang Hezi [2025] No. 02), the Guizhou Provincial Basic Research Program (Natural Science) General Program (No. [2025] 660).

Appendix A. Supporting information

Fig. S1 Prediction of the tertiary structure of SpWRKYs.

Fig. S2 The conserved motifs in SpWRKYs.

Table S1. Transcriptome data of *SpWRKYs* under Cd treatment.

Table S2. Transcriptome data of *SpWRKYs* under Cu treatment.

Table S3. Transcriptome data of *SpWRKYs* under salt stress.

Table S4. Primers used in this study.

Table S5. Basic information of *SpWRKYs* identified in *S. portulacastrum*.

Table S6. Prediction of SpWRKYs secondary structure.

Table S7. Ka, Ks, and Ka/Ks values for the homologous gene pair gene 1 and gene 2 in *SpWRKYs*.

Table S8. WGD/segmental duplication event of *SpWRKYs* in *S. portulacastrum*.

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Figures and legends

Fig. 1 Phylogenetic analysis of *WRKYs* in *S. portulacastrum* and *A. thaliana*.

WRKY protein sequences was drawn by neighbor-joining (NJ) method in MEGA7.0. All *WRKYs* are classified into seven branches indicated by specific colors.

Fig. 2 The chromosome distribution of *SpWRKYs*.

The yellow vertical bars represent chromosomes. Chromosome numbers (yellow) are located above each chromosome, and gene numbers are located on the right side of each chromosome (black).

Fig. 3 Collinearity Analysis of *SpWRKYs*.

(A) Duplication events of *SpWRKYs* in *S. portulacastrum*. The red lines respectively represent collinear regions and syntenic *SpWRKYs* pairs. (B) Collinearity analysis of *SpWRKYs* between *S. portulacastrum*. and *A. thaliana*. The red-highlighted lines represent syntenic *SpWRKYs* pairs.

Fig. 4 Conserved motifs and gene structures of *SpWRKYs*.

(A) Distribution of conserved motifs. Colored rectangles distinguish the specific motifs. (B) Conserved structural domains in the 68 *SpWRKYs*. (C) Gene structural coding sequences (CDS) by yellow rectangles, and UTRs by green rectangles.

Fig. 5 Analysis of *cis*-elements in *SpWRKY* promoters.

(A) *Cis*-elements in *SpWRKY* promoters. Different colors and the number of squares indicate the number of different promoter elements. (B) The sum of each type of *cis*-element is shown by histograms of different colors.

Fig. 6 Expression patterns of *SpWRKYs* in *S. portulacastrum* under various abiotic.

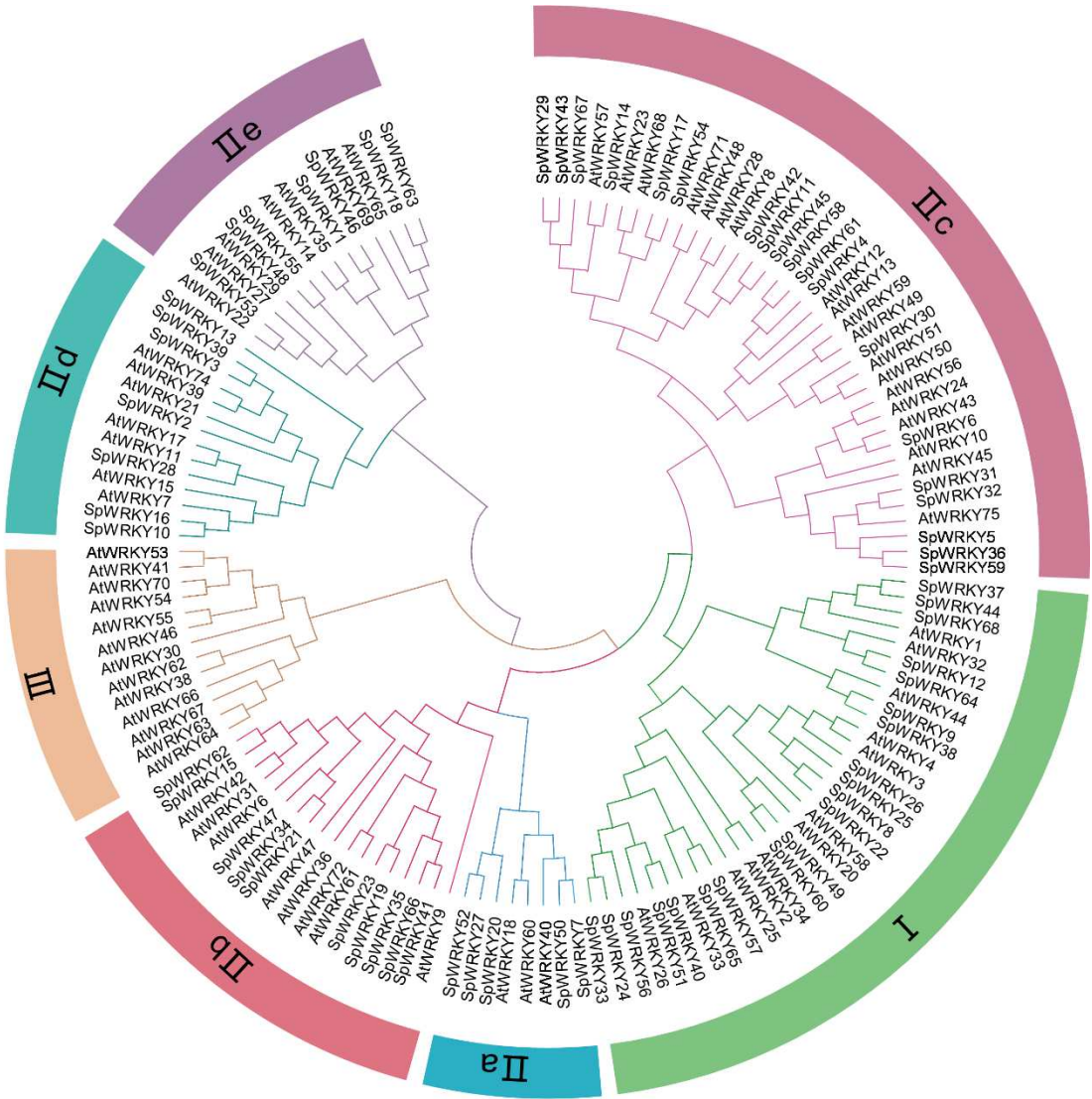
(A) Fold change of *SpWRKYs* under Cd stress. (B) Fold change of *SpWRKYs* under C stress. (C) Fold change of *SpWRKYs* under salt stress. (D) Relative expression patterns of *SpWRKY40* and *SpWRKY51* under Cd treatment analyzed by qRT-PCR. Asterisks indicate a significant difference between CK and Cd treatment as determined by 2-tailed Student's t test at $**P < 0.01$ or $*P < 0.05$.

Fig. 7 Transcriptional activation assay of *SpWRKY40* and *SpWRKY51*. (A)

641 Subcellular localization of SpWRKY40 and SpWRKY51 in tobacco leaves. *Taraxacum*
642 *kok-saghyz* ARF3 protein fused to mCherry was used as a nucleus marker. Scale bars
643 correspond to 20 μ m. (B) Schematic diagram of *cis*-element of SpWRKY40 and
644 SpWRKY51 binding site. (C) Analysis of the transcriptional activity of SpWRKY40
645 and SpWRKY51 in yeast cell.
646

647 Figures

648 Fig.1

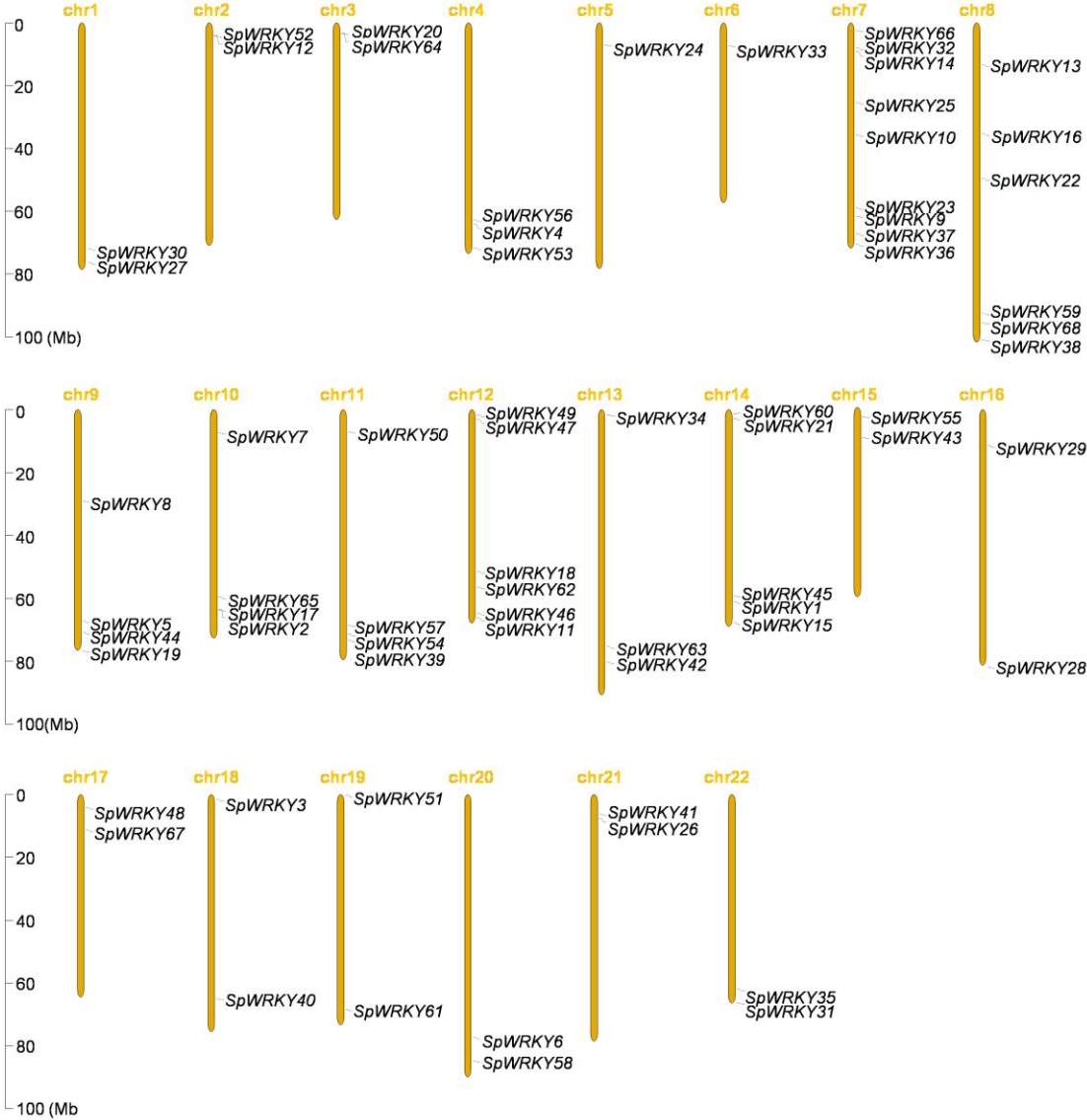


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Fig. 2

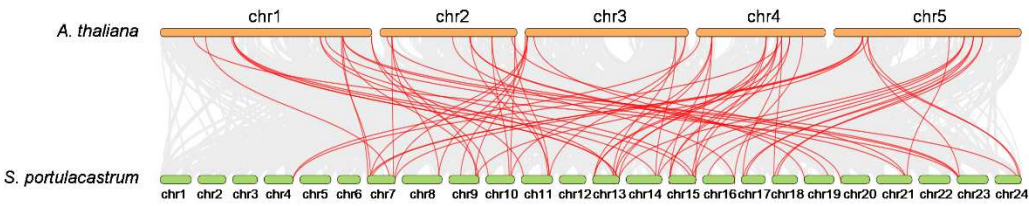


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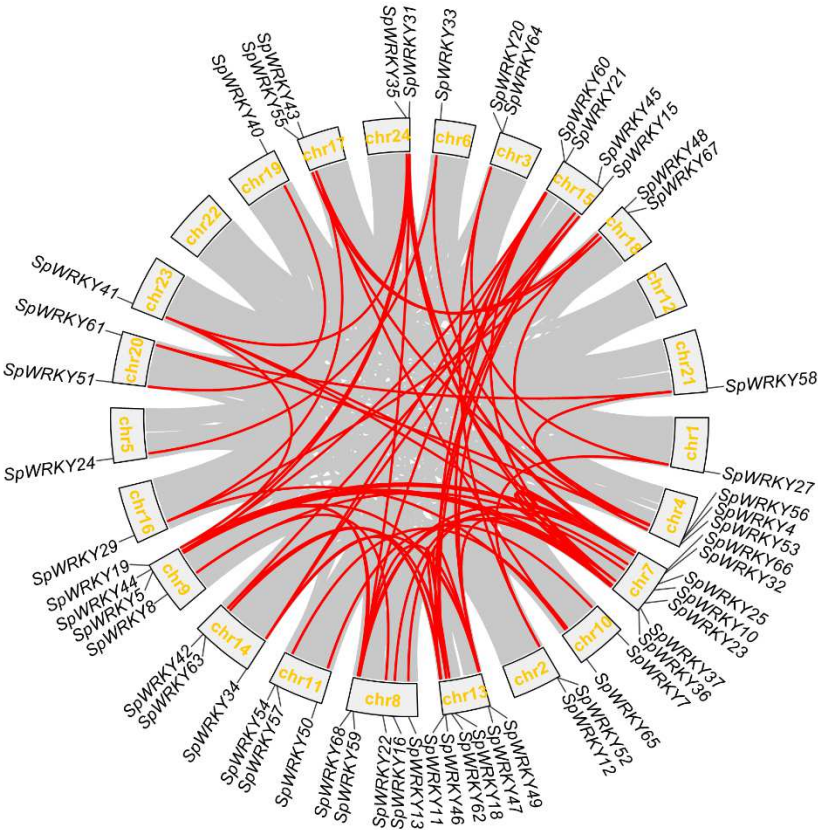
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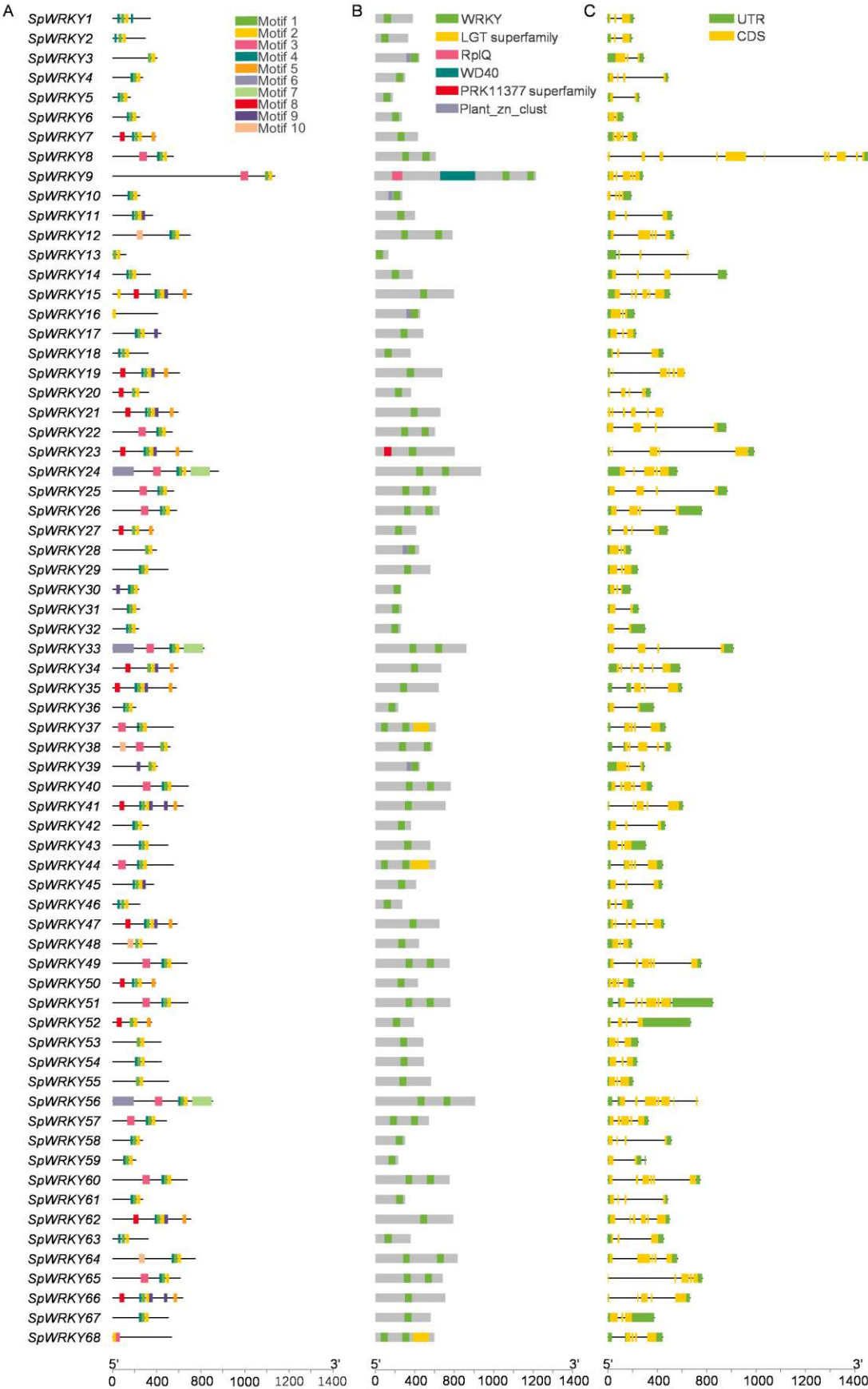
Fig. 3

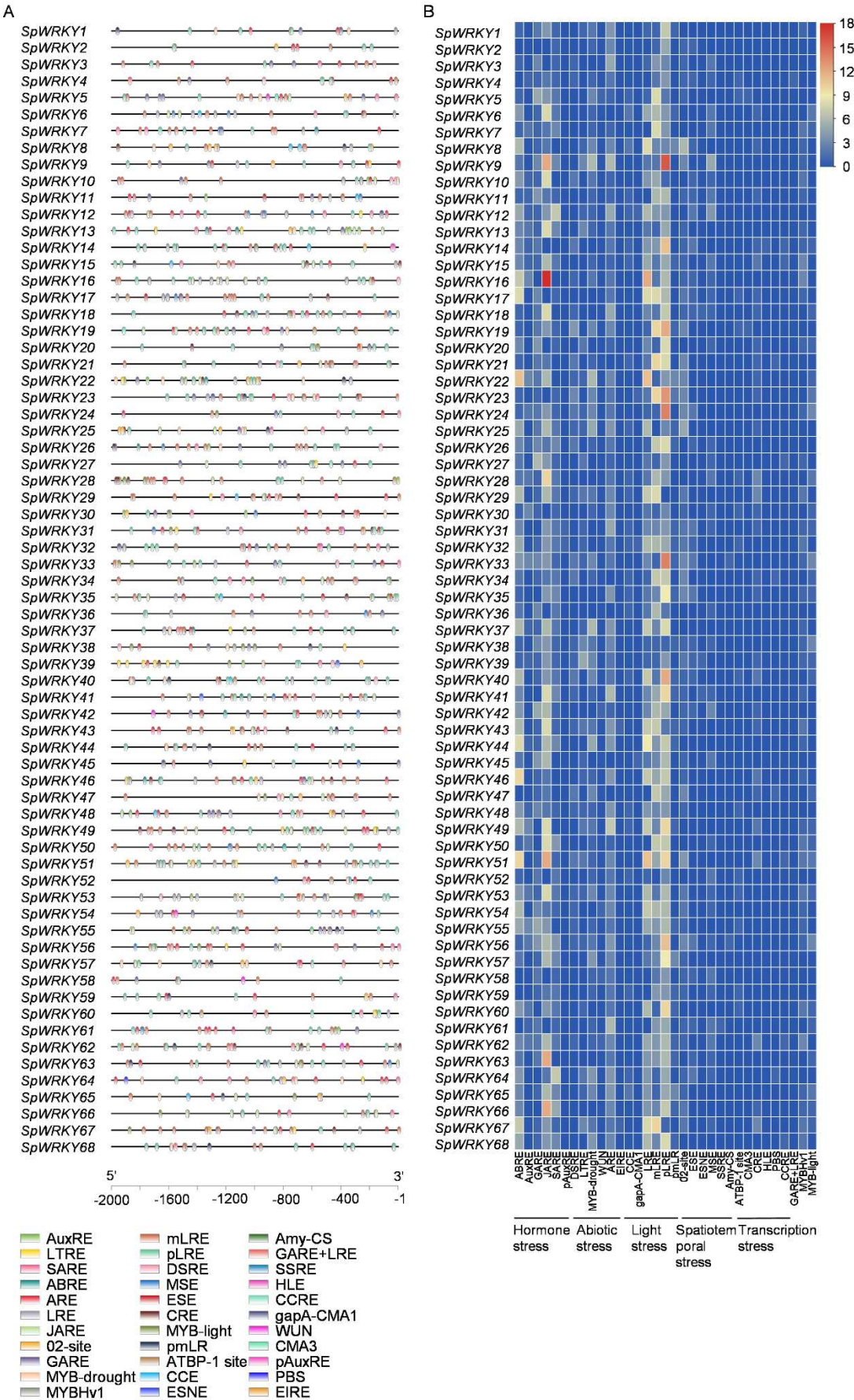
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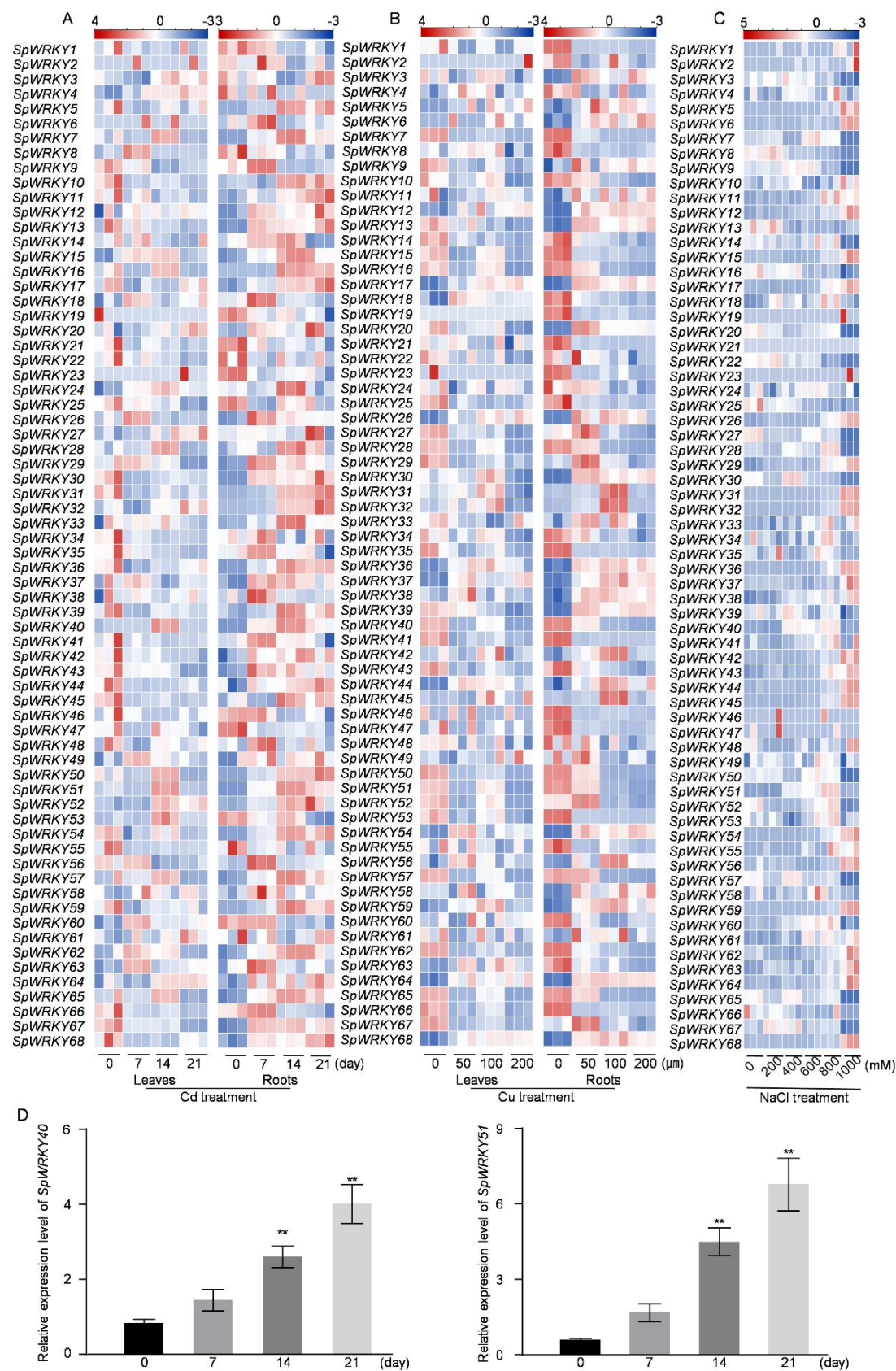
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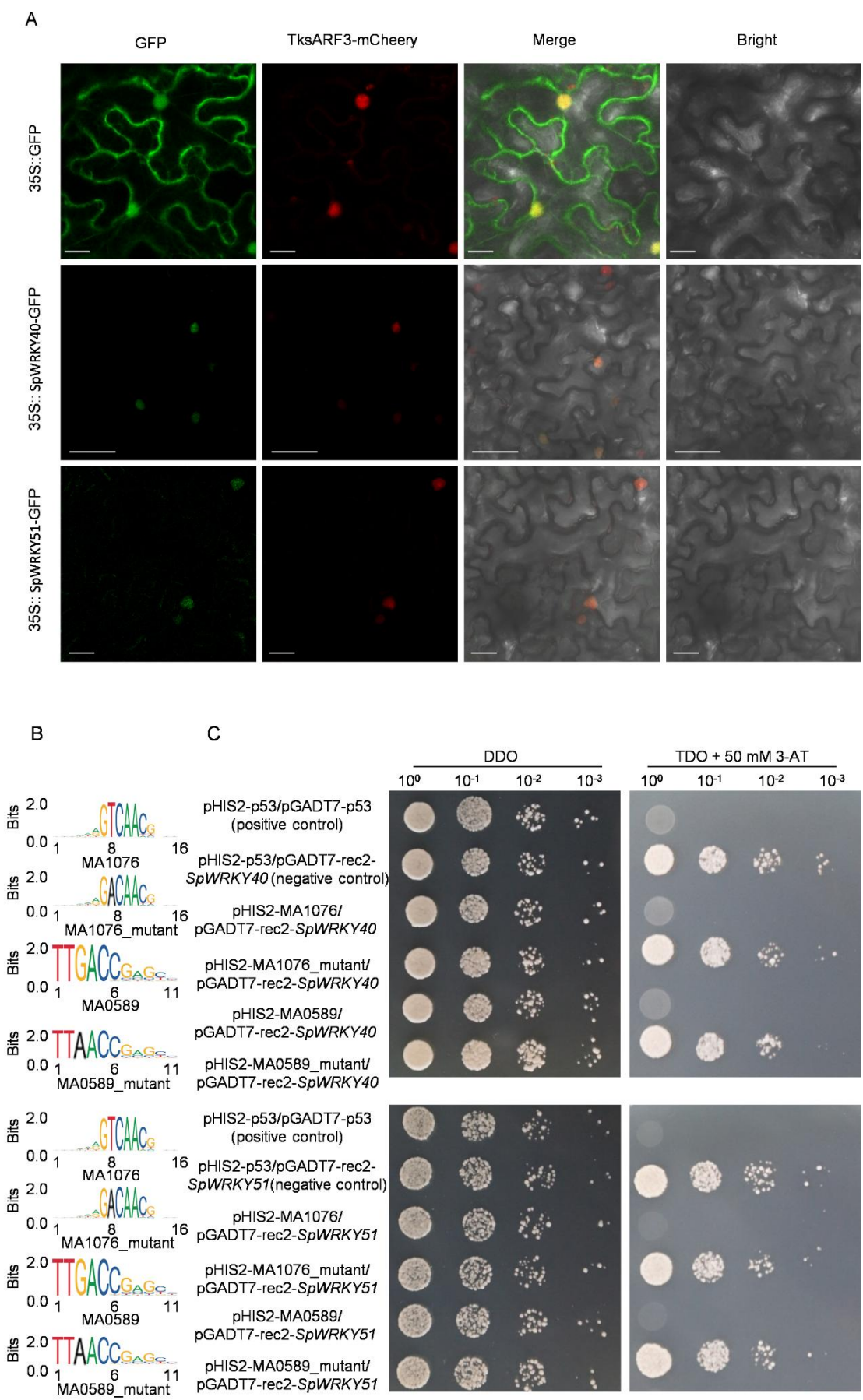


661 Fig. 6



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Supplementary Files

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- [TableS1.TranscriptomedataofSpWRKYsunderCdtreatment.xlsx](#)
- [TableS2.TranscriptomedataofSpWRKYsunderCutreatment.xlsx](#)
- [TableS3.TranscriptomedataofSpWRKYsundersaltstress.xlsx](#)
- [LiYXTable.docx](#)