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Identification of stress-related characteristics of the *WRKY* gene family: A case study of *Dendrobium catenatum*

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Abstract

As one of the largest families of transcription factors (TFs) in plants, the WRKY TF family plays a key role in regulating plant responses to various biotic and abiotic stresses. However, there is no confirmed method to quickly identify stress-responsive members from the *WRKY* gene family. In this study, all reported functional *WRKY* genes were first analyzed, and the amino acid patterns in response to stress were identified in group II-c (T-R/K-S/T-E/Q/D-V/I/L-E/D-I/V/H/N-L/M-D/E-D-G/E-F/Y-K/R-WRKYG-Q/K-K-A/T-VKN-S/N-P), group II-d (VPA-I/V-S-X-K-M/L/V/I-ADIP-P/A/V-D-D/E-Y/F-S-WRKYGQKPIKGSP-H/Y-PRGYYKCS-S/T-V/M-RGCPARKVER), and group II-e (PSD-S/A/L-WAWRKYGQKPIKGSPYPR-G/S-YYRCSSSKGC). *WRKY* genes in *Dendrobium catenatum* were used to validate the accuracy of these patterns. A total of 63 *DcaWRKY* genes were identified, their gene structures, conserved motifs, and gene expression patterns were analyzed, and a phylogenetic tree was constructed. Gene expression patterns were then analyzed under drought stress, and seven *DcaWRKY* genes (*Dca002550*, *Dca002715*, *Dca005648*, *Dca007842*, *Dca010430*, *Dca016437*, and *Dca006787*) were randomly selected to determine their expression levels and verify their expression patterns by quantitative real-time polymerase chain reaction analysis. The identified amino acid patterns were validated by drought-responsive *WRKY* genes in *D. catenatum*, confirming the accuracy of these amino acid patterns and providing valuable insights into further research of the *WRKY* family in *D. catenatum*.

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INTRODUCTION

Transcription factors (TFs) are essential in domesticating plants and are targets for molecular breeding. They play a key role in binding to *cis*-acting regulatory elements[1]. More than 1000 TF genes have been predicted in angiosperm genomes, and these TF genes can be classified into 58 families based on their DNA-binding domains. The *WRKY* gene family is the seventh largest TF family in flowering plants[2], where 74 *WRKY* genes have been identified in dicotyledonous *Arabidopsis*[3], while 109 *WRKY* genes have been identified in monocotyledonous rice[4].

The *WRKY* gene family shares a highly conserved WRKY domain at the N-terminus and a metal chelating zinc finger-like motif at the C-terminus[5]. Some variants of the WRKY domain, such as WRKYGEK, WRKYGKK, WSKYEQK, WRKYSEK, WRRYGQK, WSKYGQK, WVKYGQK, WKKYGQK, WRICGQK, WRMCGQK, WIKYGQK, and WKRYGQK, have been found in various plants[6,7]. Two main types of zinc finger-like motifs C2H2 (C-X4-5-C-X22-23-H-X-H) and C2HC (C-X7-C-X23-H-X1-C) have been identified[5]. All WRKY domains and zinc finger-like motifs are for structural stabilization[5]. According to the phylogenetic analysis, WRKY proteins are classified into three major groups—I, II, and III—based on the number of WRKY domains and pattern features of the zinc finger-like motif[8]. Only group I members have two WRKY domains, whereas those with one WRKY domain belong to group II or III[8]. Group II has five subgroups—IIa, IIb, IIc, IId, and IIe[8]. Those with C2HC (C-X7-C-X23-H-X1-C) zinc finger-like motifs belong to group III, whereas the members of groups I and II have C2H2 (C-X4-5-C-X22-23-H-X-H) zinc finger-like motifs[5,8].

WRKY proteins participate in various plant life activities, including defense against stresses, growth and development, biosynthesis, and regulation of hormone signaling[9,10]. As key regulators in plant immune response to various biotic stresses, WRKYs have been extensively investigated in rice, *Arabidopsis*, soybean, wheat, pepper, and wild tomato[11–23]. In rice, *OsWRKY62.1* and *OsWRKY67* have been established as important regulators against rice pathogens[12,22]. *WRKY8*, *WRKY29*, *WRKY38*, *WRKY48*, *WRKY62*, and *WRKY33* are involved in response to pathogens in *Arabidopsis*[11,13,18–20]. Moreover, *GmWRKY136*, *GmWRKY53*, *GmWRKY86*, *TaWRKY49*, *TaWRKY62*, *CaWRKY27*, *CaWRKY40*, *CaWRKY40b*, *CaWRKY6*, and *SpWRKY1* are involved in defense responses induced by biotic stress[14–17,21,23].

In addition to the response to these biotic stresses, *WRKY* genes can function effectively in most abiotic stress responses or tolerances in various plants. For example, *WRKY25*, *WRKY39*, and *WRKY54* in *Arabidopsis*, *PoWRKY13* in tomato, and *CaWRKY40* in pepper respond to heat stress[24–28]. In contrast, *AtWRKY34*, *VvWRKY24*, *SlWRKY33*, and *VbWRKY32* are associated with cold tolerance[29–32]. In addition, *GhWRKY39-1*, *GbWRKY1*,

GmWRKY13 and *GmWRKY17* are identified as key regulators in response to salt stress[9,33–35].

Among the various abiotic stresses that plants are exposed to during growth and development, drought is one of the most detrimental environmental factors limiting growth, development, distribution range, and especially plant yield[36]. *WRKY* genes can regulate the abscisic acid signaling pathway in response to drought[37]. WRKY can also resist drought stress by enhancing dehydration tolerance, reducing stomatal density, modulating ethylene response factors, and effectively eliminating reactive oxygen species by activating cellular antioxidant systems[2]. Thirty-seven *WRKY* genes that respond to drought stress have been identified in many plant species, including model and non-model plants. In model plants, *WRKY1*, *WRKY1*, *WRKY18*, *WRKY40*, *WRKY46*, *WRKY54*, *WRKY57*, *WRKY60*, *WRKY63*, and *WRKY70* in *Arabidopsis*, and *OsWRKY11*, *OsWRKY45*, *OsWRKY30*, *OsWRKY80*, and *OsWRKY47* in rice, play positive or negative roles in responding to drought stress. Numerous *WRKY* genes regulating drought resistance have also been identified in non-model plants, such as *PbrWRKY53* in *Pyrus betulaefolia*[38], *FvWRKY42* in the diploid woodland strawberry (*Fragaria vesca*)[39], and *FcWRKY70* in *Fortunella crassifolia* to name a few[40].

In addition to stress response, *WRKY* genes play important roles in a range of processes such as root development, senescence regulation, seed germination, and plant nutrient utilization[41–47]. For example, *WRKY42*, *WRKY45*, *WRKY75*, and *WRKY6* in *Arabidopsis*, and *OsWRKY74* and *OsWRKY80* in rice are involved in plant nutrient utilization[42]. *AtWRKY6*, *AtWRKY53*, and *OsWRKY45* can regulate leaf senescence[46]. Moreover, *AtWRKY12*, *AtWRKY13*, *AtWRKY71*, and *OsWRKY11* are associated with floral development in angiosperms[41,44,47].

Based on these important roles, the identification of *WRKY* gene family members in plants is the basis for further functional studies. However, identification is difficult because of the large number of *WRKY* gene family members in most plants. This study examines two pertinent questions to address this difficulty: (1) Is there a sequence pattern for quick identification? and (2) Can this sequence pattern be used for the identification of stress-responsive *WRKY* genes in *Dendrobium catenatum*?

D. catenatum, a perennial herb of *Dendrobium* in Orchidaceae, has important medicinal properties and high ornamental value. In China, *D. catenatum* is found in many provinces with wide differences in both temperature and humidity, including Anhui, Zhejiang, Guangxi, Hunan, Yunnan, and Guizhou. The additional stress-related genes in *D. catenatum* may have contributed to its adaptation to a wide range of environments[48]. Its epiphytic lifestyle and wide distribution range are closely related to its stress tolerance.

Because there may be a common amino acid pattern of *WRKY* genes that respond to stress, a confirmatory study was performed using *D. catenatum* as an example. First, all sequences of reported functional *WRKY* genes were summarized, and the sequence features of genes belonging to groups II-c, II-d, and II-e that responded to stress were completely analyzed, and the common patterns in each of these three subgroups were determined, which might be markers for identifying stress-resistant *WRKY* genes. Second, *D. catenatum* was used to verify the accuracy of these patterns. The *WRKY* gene family members were identified in *D. catenatum* and their sequence characteristics were analyzed. The candidate stress-responsive *WRKY* genes were identified in *D. catenatum* based on these sequence patterns. The consistency of sequence characteristics between candidate *WRKY* members in *D. catenatum* and identified sequence patterns were compared. Finally, the *DcaWRKY* gene expression pattern was analyzed in different tissues under drought stress, and the expression patterns of seven randomly selected genes were confirmed.

MATERIALS AND METHODS

Basic information and sequence characteristics analysis of reported functional *WRKY* genes

All reported functional *WRKY* sequences were obtained, and their basic information and sequence features were analyzed. The number of amino acids, molecular weight, theoretical isoelectric point (pI), instability index, aliphatic index, and grand average of hydropathicity (GRAVY) of each WRKY protein sequence were calculated by ExPASy protparam (https://web.expasy.org/protparam.html). All amino acid sequences of the *WRKY* gene family from reported plants were aligned by Multiple Alignment using Fast Fourier Transform (MAFFT) with the E-INS-I strategy[49]. The results of multiple alignments were visualized by GeneDoc software (www.softpedia.com/get/Science-CAD/GeneDoc.shtml).

Phylogenetic analysis of the *WRKY* family members from *Arabidopsis*, rice, and other reported species

All *Arabidopsis* and rice genomic data were downloaded from the Phytozome v12.1.6 database (phytozome.jgi.doe.gov) to create a protein dataset. A hidden Markov model (HMM) search was performed against the protein database using the WRKY domain file (PF03106) that was downloaded from the Pfam database to identify *WRKY* family members[50]. To verify the preliminary results, each predicted sequence was analyzed using Pfam, simple modular architecture research tool (SMART), and NCBI CD-Search (www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi)[51]. Sequences with obvious errors were excluded.

All *WRKY* sequences in *Arabidopsis* and rice, as well as reported *WRKY* gene sequences, were used to reconstruct a phylogenetic tree by the maximum likelihood (ML) method using PhyML 3.0 software[52]. Here, 1000 bootstrap replicates were used to test the reliability of the tree. Inspired by a Shimodaira–Hasegawa-like procedure, the approximate likelihood-ratio test branch support was estimated with the Whelan and Goldman model[52].

Identification of stress-related amino acid sequence patterns

To identify the stress response sequence patterns in groups II-c and II-e, sequences of stress-related genes clustered in the same branch in the subgroups were aligned by MAFFT with the E-INS-I strategy[49]. Stress-responsive amino acid sequence patterns in groups II-c and II-e were identified separately by the results of multiple comparisons by GeneDoc software.

Similarly, sequences of *WRKY* members of group II-d clustered in a branch related to abiotic stress were aligned to identify the amino acid sequence patterns in this subgroup.

Identification and characterization of *WRKY* members in *D. catenatum*

All WRKY protein sequences of *D. catenatum* in the two genome versions were downloaded for further analysis[48,53].

The file of the WRKY domain (PF03106) was used to search against the *D. catenatum* genomes by performing hmmsearch using HMMER software[49,50].

Various physicochemical parameters, including theoretical pI, the number of amino acids, aliphatic index, molecular weight, instability index, and GRAVY for DcaWRKY proteins, were computed by the ExPASy protparam online tool.

The *WRKY* family gene structure in *D. catenatum* was analyzed using the Batch SMART plug-in in TBtools[54]. The exons, introns, and untranslated regions of the *DcaWRKY* genes were analyzed and visualized by loading the GFF files of *D. catenatum* to the TBtools software, which was also used for analyzing and searching for conserved motifs.

Multiple sequence alignment and phylogenetic analysis of *WRKY* family members in *D. catenatum*

MAFFT was used to align and manually adjust the protein sequences of all WRKY family members in *D. catenatum* using the E-INS-I strategy[49]. By submitting the multiple sequence results to GeneDoc software, the multiple sequence alignments could be visualized and analyzed.

To study the phylogenetic relationships of DcaWRKY proteins and orthologs in *Arabidopsis* and rice, a phylogenetic tree was reconstructed using the ML method approach with 1000 bootstrap replicates and the Whelan and Goldman model by PhyML[52].

Expression analysis of all WRKY family members in *D. catenatum* under drought stress

Transcriptome data used in this study was from Wang et al.[55]. Briefly, healthy individuals (~12 cm tall) of *D. catenatum* were grown in a greenhouse (12/12 h light/dark, light intensity ~100 μmol·m^{-2}·s^{-1}; 28/22 °C day/night; 60%/70% relative humidity day/night). After first-day irrigation was performed, water was withheld for 10 d to simulate drought stress. The fourth and fifth leaves from the apex and young roots were collected from each plant at 9:00 h when the base material was in a drought and normal growth state. Salmon v0.9.1 was used to estimate gene abundance as reading counts based on alignment patterns[56].

The *DcaWRKY* gene expression patterns in each tissue were analyzed, and heatmaps were generated using TBtools software. The Fragments Per Kilobase of exon model per Million mapped fragments (FPKM) values for each gene in all root and leaf samples were log2 transformed[54,56]. Each sample had three biological replicates. Those with more than 2.0-fold change in expression under drought treatment compared with normal treatment were identified as differentially expressed genes.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of selected *DcaWRKY* genes

Among all the differentially expressed *DcaWRKY* genes in both roots and leaves, four genes with decreased expression and three genes with increased expression when responding to drought stress were randomly selected. These seven genes — *Dca002550*, *Dca002715*, *Dca005648*, *Dca006787*, *Dca007842*, *Dca010430*, and *Dca016437* — were used for further qRT-PCR analysis. Total RNA was extracted using the cetyltrimethylammonium bromide (CTAB) method. Extracted RNA was reverse-transcribed using the Fast Quant RT Kit with gDNase (Tiangen, Beijing, China).

The relative transcription levels of the selected *WRKY* genes during different treatments were analyzed by qRT-PCR. 18S rRNA was used as the internal control. The primer sequences for the selected genes were designed with the Primer3Plus interface (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). All qRT-PCR assays were performed on a Roche 480 real-time PCR system (LightCycler® 480; Roche, Basel, Switzerland) with three biological replicates. The reactions were conducted in a reaction volume of 10 μL with three technical replicates for each sample. The PCR program was as follows: 95 °C for 3 min and 45 cycles of 95 °C for 10 s, 65 °C for 20 s, 72 °C for 30 s, 95 °C for 5 s, and 60 °C for 1 min. The 18S gene was used as an internal control to calculate the relative fold expression levels according to the Ct method. Finally, the TBtools software was used to generate a heatmap of *DcaWRKY* genes in leaves.

RESULTS

Basic information on reported functional *WRKY* genes

All reported functional *WRKY* genes are summarized in Table 1. The majority of genes were from *Arabidopsis*, rice, and soybean, with 27, 16, and nine, respectively. Seven *WRKY* genes were identified in wheat and six *WRKY* genes were identified in land cotton, whereas only one functional *WRKY* gene was reported in *Pyrus*, *Chrysanthemum*, *Tamarix*, and *Macrotyloma*. Numerous functional studies of *WRKY* genes have mostly focused on model plants and crops, whereas reports on ornamentals, especially flowers and medicinal plants, are few.

To analyze the branch specificity of the *WRKY* gene family that responded to stress, a phylogenetic tree was reconstructed from *AtWRKY* genes, *OsWRKY* genes, and all reported functional *WRKY* genes (Fig. 1). All reported functional WRKY proteins were divided into three groups—I, II, and III, with group II being further divided into five subgroups—II-a, II-b, II-c, II-d, and II-e. Most WRKY proteins were widely distributed in all these groups rather than concentrated in the same branch. However, *OsWRKY67*, *SbWRKY50*, *GmWRKY21*, *AtWRKY8*, and *AtWRKY48* play important roles under stress and were clustered in a branch of group II-c. Similarly, as essential stress-related genes, *WRKY80* and *WRKY13* in rice, *WRKY29* in *Arabidopsis*, *CaWRKY27* in pepper, and *ZmWRKY106* in maize clustered in one branch of group II-e. In addition, reported genes responding to abiotic stress, including *ZmWRKY58*, *GhWRKY21*, *GhWRKY17*, *ThWRKY4*, *GmWRKY13*, *GhWRKY39-1*, and *JrWRKY7*, were all located in group II-d.

Therefore, because of these distribution features of reported functional *WRKY* genes, the identification of amino acid sequence patterns in response to stress focused on groups II-c, II-d, and II-e.

Identification of the pattern of stress-related *WRKY* genes

To further characterize the stress-responsive WRKY proteins for identifying the amino acid patterns in different subgroups, the properties of all drought-resistant WRKY proteins (molecular weight, pI, instability index, etc.) were analyzed (Supplemental Table S1). The molecular weight of stress-related WRKY proteins ranged from 16801.82 to 74561.15, PI ranged from 4.86 to 9.96, and the instability index ranged from 37.86 to 88.65, showing a wide range of values.

Table 1. Reported functional *WRKY* genes summarized in this study.

Group	Gene ID	Species	Function	References
III	*GhWRKY33*	*Gossypium hirsutum*	Tolerance to drought	[58]
III	*PbrWRKY53*	*Pyrus betulaefolia*	Tolerance to drought	[38]
II C	*GmWRKY54*	*Glycine max*	Tolerance to drought	[59]
II A	*GmWRKY27*	*Glycine max*	Tolerance to drought	[58]
III	*AtWRKY63*	*Arabidopsis thaliana*	Tolerance to drought	[60]
I	*TaWRKY2* and *TaWRKY19*	*Triticum aestivum*	Tolerance to drought	[61]
II C	*OsWRKY11*	*Oryza sativa*	Tolerance to drought	[57]
III	*OsWRKY45*	*Oryza sativa*	Tolerance to drought	[62]
II A	*WRKY18*, *WRKY40* and *WRKY60*	*Arabidopsis thaliana*	Tolerance to drought	[63,64]
I	*WRKY1*	*Arabidopsis thaliana*	Tolerance to drought	[65]
III	*WRKY46*, *WRKY54*, and *WRKY70*	*Arabidopsis thaliana*	Tolerance to drought	[42]
II C	*AtWRKY57*	*Arabidopsis thaliana*	Tolerance to drought	[37]
I A	*OsWRKY30*	*Oryza sativa*	Tolerance to drought	[66]
II	*OsWRKY80*	*Oryza sativa*	Tolerance to drought	[67]
III	*OsWRKY47*	*Oryza sativa*	Tolerance to drought	[68]
I	*FvWRKY42*	*Fragaria vesca*	Tolerance to drought	[39]
II D	*ZmWRKY58*	*Zea mays*	Tolerance to drought	[41]
II E	*ZmWRKY106*	*Zea mays*	Tolerance to drought	[69]
II A	*ZmWRKY40*	*Zea mays*	Tolerance to drought	[69]
II E	*CmWRKY10*	*Chrysanthemum morifolium*	Tolerance to drought	[70]
III and I	*TaWRKY1* and *TaWRKY33*	*Triticum aestivum*	Tolerance to drought	[71]
II D	*ThWRKY4*	*Tamarix hispida*	Tolerance to drought	[72]
I	*MuWRKY3*	*Macrotyloma uniflorum*	Tolerance to drought	[73]
II D	*GhWRKY17*	*Gossypium hirsutum*	Tolerance to drought	[34]
III	*GhWRKY41*	*Gossypium hirsutum*	Tolerance to drought	[74]
I	*TaWRKY44*	*Triticum aestivum*	Tolerance to drought	[75]
III	*FcWRKY70*	*Fortunella crassifolia*	Tolerance to drought	[40]
II C	*GmWRKY12*	*Glycine max*	Tolerance to drought	[76]
III	*ZmWRKY79*	*Zea mays*	Tolerance to drought	[77]
II D	*GhWRKY21*	*Gossypium hirsutum*	Tolerance to drought	[78]
III	*SlWRKY81*	*Solanum lycopersicum*	Tolerance to drought	[79]
I	*SPF1*	*Ipomoea batatas*	Root development	[43]
I and II A	*ABF1* and *ABF2*	*Avena fatua*	Seed germination	[45]
II B	*WRKY42* and *WRKY6*	*Arabidopsis thaliana*	Plant nutrient	[42]
II C	*WRKY45* and *WRKY75*			
III and II E	*WRKY74* and *WRKY80*	*Oryza sativa*	Plant nutrient	[42]
III	*AtWRKY53*	*Arabidopsis thaliana*	Leaf senescense	[46]
II C	*OsWRKY11*	*Oryza sativa*	Floral development	[41]
II C	*AtWRKY12* and *AtWRKY13*	*Arabidopsis thaliana*	Floral development	[44]
II C	*AtWRKY71*	*Arabidopsis thaliana*	Floral development	[47]
I	*AtWRKY2*	*Arabidopsis thaliana*	Reproductive development	[80]
I	*OsWRKY70*	*Oryza sativa*	Defense response	[81]
II C	*FvWRKY48*	*Fragaria vesca*	Pectin degradation	[82]
II E	*OsWRKY13*	*Oryza sativa*	Regulated ABA signaling and tolerance to salt	[83]
II C	*VlWRKY3*	*Vitis vinifera*	Response to *Golovinomyces cichoracearum* and tolerant to salt	[84]
II C	*GhWRKY68*	*Gossypium hirsutum*	Reduced salt tolerance and drought resistance	[85]
I	*GhWRKY25*	*Gossypium hirsutum*	Tolerance to salt	[86]
I	*VvWRKY24*	*Vitis vinifera*	Tolerance to cold	[29]
I	*AtWRKY25* and *AtWRKY33*	*Arabidopsis thaliana*	Tolerance to heat	[25]
I	*AtWRKY34*	*Arabidopsis thaliana*	Negative regulator in cold stress	[31]
III	*AtWRKY53*	*Arabidopsis thaliana*	Reduced drought resistance	[87]
III	*AtWRKY63*	*Arabidopsis thaliana*	Regulated ABA signaling	[60]
III	*AtWRKY54*	*Arabidopsis thaliana*	Response to heat stress	[28]
II C	*OsWRKY72*	*Oryza sativa*	Sensitive to salt, sucrose, and ABA	[88]
III	*OsWRKY74*	*Oryza sativa*	Tolerance to cold and Pi deprivation	[89]
II A	*OsWRKY76*	*Oryza sativa*	Tolerance to cold	[90]
III	*OsWRKY89*	*Oryza sativa*	Tolerance to UV	[91]
II A	*GmWRKY17*	*Glycine max*	Reduced salt tolerance	[34]
III	*BcWRKY46*	*Brassica campestris*	Tolerance to salt	[92]
III	*BhWRKY1*	*Boea hygrometrica*	Tolerance to salt	[93]
III and I	*VpWRKY1* and *VpWRKY2*	*Vitis pseudoreticulata*	Tolerance to salt and cold	[94]
II A	*VpWRKY3*	*Vitis pseudoreticulata*	Tolerance to salt	[95]

(to be continued)

Table 1. (continued)

Group	Gene ID	Species	Function	References
III	*TcWRKY53*	*Thlaspi caerulescens*	Negative regulator in osmotic stress	[96]
I	*NaWRKY3*	*Nicotiana attenuata*	Sensitive to mechanical damage	[97]
I and II D	*JrWRKY2* and *JrWRKY7*	*Juglans regia*	Tolerance to drought and cold	[98]
III	*SbWRKY30*	*Sorghum bicolor*	Tolerance to salt and drought	[99]
II C	*SbWRKY50*	*Sorghum bicolor*	Tolerance to salt	[100]
II A	*MdWRKY30*	*Malus domestica*	Tolerance to salt and osmotic stress	[101]
II C	*GbWRKY1*	*Gossypium barbadense*	Tolerance to salt	[35]
I	*VbWRKY32*	*Verbena bonariensis*	Tolerance to cold	[28]
II C	*OsWRKY67*	*Oryza sativa*	Negative regulator of innate defense response	[22]
II A	*OsWRKY62.1*	*Oryza sativa*	Positive regulator of PTI and ETI against pathogens	[12]
III	*AtWRKY38* and *AtWRKY62*	*Arabidopsis thaliana*	Response to bacterial pathogen	[11]
II A,II C, and II B	*GmWRKY136, GmWRKY53, and GmWRKY86*	*Glycine max*	Tolerance to SCN	[23]
II C and III	*TaWRKY49* and *TaWRKY62*	*Triticum aestivum*	Tolerance to stripe rust	[102]
II A	*CaWRKY40b* and *CaWRKY40*	*Capsicum annuum*	Negative regulation of plant immunity	[17,24]
II B	*CaWRKY6*	*Capsicum annuum*	Tolerance to *R. solanacearum*	[15]
I	*SpWRKY1*	*Solanum pimpinellifolium*	Tolerance to *Phytophthora infestans*	[16]
II D	*ZmWRKY17*	*Zea mays*	Negative regulator of salt stress	[103]
II D	*GhWRKY39-1*	*Gossypium hirsutum*	Tolerance to salt	[33]
II C	*AtWRKY8*	*Arabidopsis thaliana*	Defense response	[13]
II E	*CaWRKY27*	*Capsicum annuum*	Response to *Ralstonia solanacearum* infection	[14]
II C	*AtWRKY48*	*Arabidopsis thaliana*	Tolerance to *P. syringae*	[20]
II E	*AtWRKY29*	*Arabidopsis thaliana*	Resistance to *P. syringe*	[18]
II C	*PoWRKY13*	*Populus tomentosa*	Response to heat stress	[26]
III	*SlWRKY33*	*Solanum lycopersicum*	Tolerance to cold	[30]
II D	*GmWRKY13*	*Glycine max*	Response to salt and mannitol	[9]

From the alignment results, the amino acid patterns responding to stress were found in groups II-c, II-d, and II-e (Fig. 2). In contrast to groups II-d and II-e, where only stress-related *WRKY* genes were reported, group II-c contained *WRKY* genes that play important roles in plant growth and development besides stress-response. All stress-related genes clustered in the group II-e branch had the amino acid pattern 'PSD-S/A/L-WAWRKYGQKPIKGSPYPR-G/S-YYRCSSSKGC'. Similarly, the amino acid pattern 'VPA-I/V-S-X-K-M/L/V/I-ADIP-P/A/V-D-D/E-Y/F-S-WRKYGQKPIKGSP-H/Y-PRGYYKCS-S/T-V/M-RGCPARKVER' was found in the sequences of reported genes responding to abiotic stress clustered in the group II-d branch, which might be closely related to stress. In addition, for group II-c, an amino acid sequence pattern 'T-R/K-S/T-E/Q/D-V/I/L-E/D-I/V/H/N-L/M-D/E-D-G/E-F/Y-K/R-WRKYG-Q/K-K-A/T-VKN-S/N-P' in the stress-related genes clustered in one of the branches. From the alignment results, the sequences of *WRKY* genes involved in plant growth and development belonging to group II-c (*FvWRKY48*, *ATWRKY75*, *ATWRKY45*, *ATWRKY12*, *ATWRKY13*, and *ATWRKY71*) did not match this amino acid sequence pattern, further supporting the accuracy of this amino acid pattern in group II-c (Fig. 2a).

Identification of all *WRKY* genes in *D. catenatum*

To identify *DcaWRKY* genes, all the potential *WRKY* genes were extracted from two genome versions of *D. catenatum*; an old genome version with 63 *WRKY* genes and an updated genome version with 60 *WRKY* genes[48,53]. After sequences were aligned by MAFFT, all 60 genes from the updated genome version were present in the old genome version, except for three genes (*Dca028175*, *Dca028770*, and *Dca027312*). After verifying the sequence characteristics, *Dca028175* and *Dca027312* had the 'WRKYGQK' domain and a zinc finger motif. *Dca028770* had the variant WRKY domain 'WRKYGKK' but no zinc finger motif. For all three genes containing the WRKY domain, the *WRKY* gene family members could be used for further analysis. In total, 63 candidate *WRKY* gene family members were identified in *D. catenatum*. To validate them, the amino acid sequences of all members were searched against NCBI, Pfam, and SMART databases for the presence of the WRKY domain. The results showed that all sequences contained the WRKY domain (Table 2).

Phylogenetic analysis and characterization of *WRKY* gene family members in *D. catenatum*

WRKYs in *D. catenatum*, *Arabidopsis*, and rice were subjected to phylogenetic analysis to investigate the phylogenetic relationships and potential functions of these *DcaWRKY* genes (Fig. 3). The *WRKY* genes in *D. catenatum* were divided into the three groups I, II, and III based on amino acid sequence similarity, which is the same as the widely accepted classification system for this gene family in *Arabidopsis*[6]. Among all *WRKY* genes in *D. catenatum*, the number of groups I, II, and III was 14, 38, and 11, respectively. There were five subgroups in group II, which were IIa, IIb, IIc, IId, and IIe, and the number of *WRKY* genes in these groups was five, three, 18, four, and eight, respectively. The results indicated that more than half of *WRKY* genes were clustered in group II.

All DcaWRKY proteins were analyzed by ExPASy protparam to calculate the number of amino acids, molecular weight, pI, instability index, aliphatic index, and GRAVY. The molecular weight of DcaWRKY proteins varied from 10,998.29 to 78,355.63, pI ranged from 4.57 to 10.63, and the instability index ranged from 34.57 to 76.36 (Supplemental Table S2).

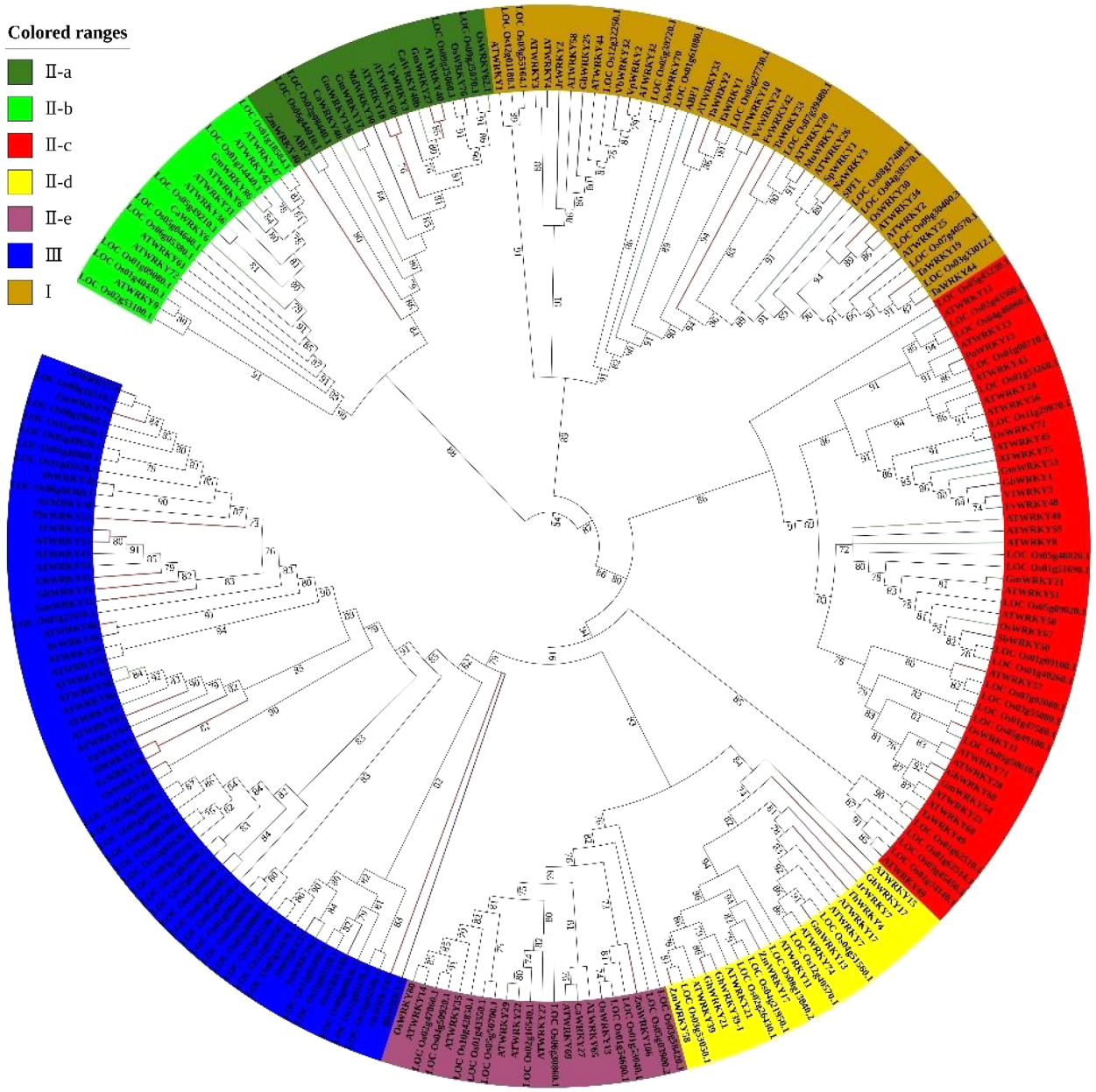

Fig. 1 Phylogenetic analysis of WRKY family proteins in *Arabidopsis*, rice, and other reported species.

Analysis of DcaWRKY proteins indicated that WRKY family members in *D. catenatum* did not show specificity in physicochemical properties, which is the same as reported drought-resistance WRKY proteins.

To further understand the biological functions of *DcWRKY* genes, WRKY domain types, intron/exon distribution, and conserved motifs were analyzed according to their phylogenetic relationships. Of the 77 WRKY domains, 65 contained perfectly conserved WRKYGQK domains, whereas the other domains differed in one or more amino acids in the conserved WRKY signature. In addition, eight conserved motifs in DcaWRKY proteins were found by TBtools. To better understand the phylogenetic relationships and classification of *WRKY* members in *D. catenatum*, the intron/exon distribution was analyzed by TBtools. The number of introns in *DcaWRKY* genes varied from zero to five. Similarity in gene structure and sequence characteristics of *WRKY* members in the same branch were used to validate the reliability of the phylogenetic classification (Fig. 4).

Group I contained two WRKY domains and two C2HC zinc finger motifs, but three DcaWRKY proteins (Dca002197, Dca008967, and Dca008968) contained only one WRKY domain and a C2H2 zinc finger motif. Besides, members of group I had five WRKY domain variant types—WKKYGQK in *Dca002197*, WTKYGQK in *Dca008967*, WNKYGQK in *Dca008968*, and both WRKDGTH and WRKYATN in *Dca002205*. Notably, the variant types WRKDGTH, WRKYATN, WTKYGQK, and WNKYGQK were first found in *D. catenatum* (Table 2). The number of introns in group I members widely varied from one to five. For example, *Dca002205* and *Dca006505* had five introns, whereas *Dca002197* and *Dca008968* had only one intron. Furthermore, all the *DcaWRKY* members of group I contained motifs 1 and 2. Interestingly, motif 7 was unique to group I (Fig. 4).

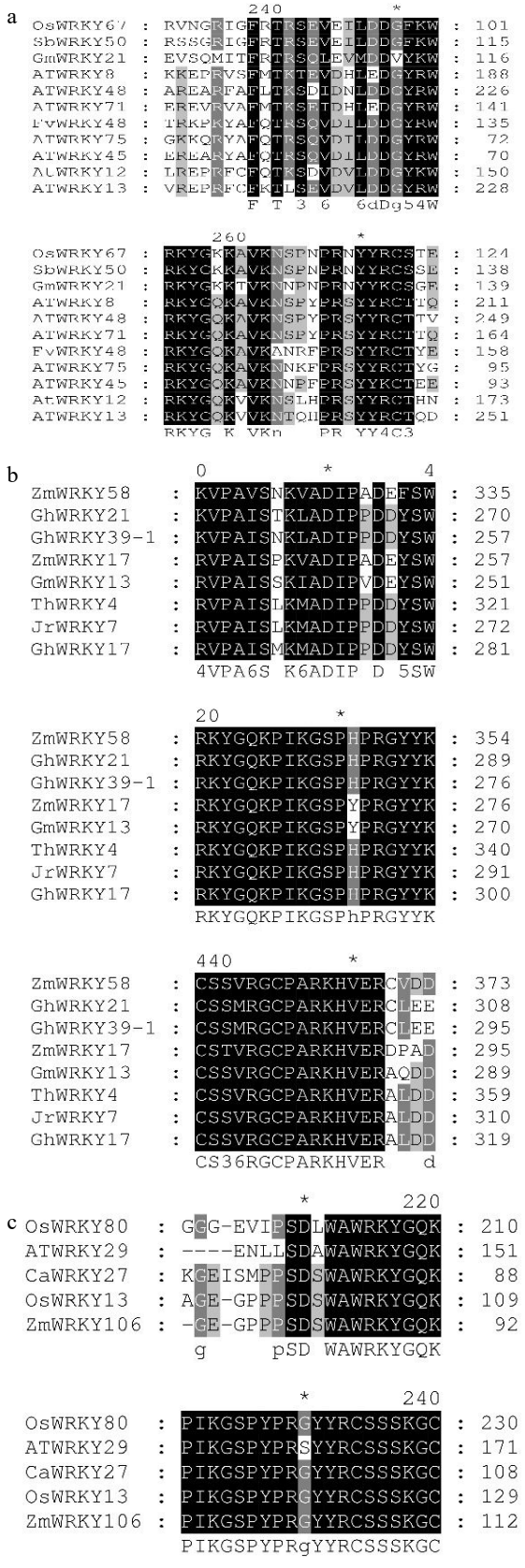

Fig. 2 Multiple sequence alignment of reported WRKY family proteins. (a) Multiple sequence alignment of reported WRKY proteins in group II-c. (b) Multiple sequence alignment of reported WRKY proteins in group II-d. (c) Multiple sequence alignment of reported WRKY proteins in group II-e.

Group II proteins were close to group I proteins in the phylogenetic tree, but they contained only one WRKY domain, except for *Dca011499* and *Dca015914*, which had two domains and two C2H2 zinc finger motifs. Besides, the only WRKY variant type WRKYGKK in group II was found in group II-c members *Dca015639*, *Dca019840*, and *Dca028770* (Table 2). Similar to group I, the number of introns in group II varied from one to four except for *Dca028770*, which lacked introns. However, the number of introns was more focused in each subgroup of group II compared with group I. For example, groups II-a, II-b, and II-d contain three, four, and two introns, respectively. Among the three subgroups, the distribution and quantity of the motifs in all genes of the same subgroup were the same. Motifs 6, 1, and 2 were dispersed in group II-d. All members of group II-b had motifs 5, 1, 4, and 2, whereas *DcaWRKY* members of group II-a had motifs 5 and 2. Besides, motif 5 was uniquely dispersed in group II (Fig. 4).

All members of group III had one WRKYGQK domain, except for *Dca021638*, which had two WRKYGEK domains. Unlike groups I and II, the zinc finger motif of most WRKY proteins in group III was replaced by a C2HC zinc finger motif. For *Dca0021638*, variants of WRKYGEK/WRKYGEK or loss of a C2HC zinc finger motif might contribute to its classification in group III rather than group I (Table 2). Besides, all group III members had two exons except for *Dca005048* (one), *Dca014563* (one), and *Dca021638* (four). The motif analysis results indicated that all members of group III contained motifs 1, 2, 6, and 8, and motif 8 was only dispersed in group III (Fig. 4).

Identification of the potential stress-responsive members of groups II-c, II-d, and II-e in *D. catenatum*

The identified stress-related amino acid pattern 'PSD-S/A/L-WAWRKYGQKPIKGSPYPR-G/S-YYRCSSSKGC' was used to identify *WRKY* genes of group II-e in *D. catenatum*. After multiple sequence alignment, *Dca006787*, *Dca002550*, *Dca012410*, and *Dca019656* shared amino acid patterns and were identified as potential stress response genes (Fig. 5). Likewise, *Dca019840*, *Dca005648*, and *Dca028770* of group II-c were also identified using the sequence pattern 'T-R/K-S/T-E/Q/D-V/I/L-E/D-I/V/H/N-L/M-D/E-D-G/E-F/Y-K/R-WRKYG-Q/K-K-A/T-VKN-S/N-P' (Fig. 5). In addition, using the 'VPA-I/V-S-X-K-M/L/V/I-ADIP-P/A/V-D-D/E-Y/F-S-WRKYGQKPIKGSP-H/Y-PRGYYKCS-S/T-V/M-RGCPARKVER' amino acid pattern, one potential gene, *Dca023070*, of group II-d in *D. catenatum* was identified as responsive to abiotic stress (Fig. 5).

Expression pattern analysis of *DcaWRKY* genes

Different *WRKY* genes have different tissue-specific expression patterns. To identify the accuracy of these patterns, the expression of all *WRKY* genes in roots and leaves of *D. catenatum* was separately analyzed under drought stress (Fig. 6, Supplemental Fig. S1).

A total of 29 differentially expressed *WRKY* genes in roots and leaves of *D. catenatum* were identified by the data. In roots, the expression of 22 *DcaWRKY* genes (*Dca000627*, *Dca002550*, *Dca002715*, *Dca003067*, *Dca005648*, *Dca006787*, *Dca007842*, *Dca010430*, *Dca011499*, *Dca011569*, *Dca011914*, *Dca015914*, *Dca016437*, *Dca016988*, *Dca018137*, *Dca019840*, *Dca023070*, *Dca024256*, *Dca026708*, *Dca027312*, *Dca019656*, and *Dca028770*) showed a change in drought-treated plants compared with untreated plants. Among these, most genes showed a decrease in expression, especially the *Dca010430* gene, which induced a

Table 2. Characteristics of *WRKY* genes in *D. catenatum*.

Gene ID	WRKY domain			No. of domains	No. of exons	No. of introns	Group
	Conserved heptapeptide	Zinc finger	Zinc finger type				
Dca000627	WRKYGQK	–	–	1	3	2	II E
Dca000637	WRKYGQK	C2H2	CX4CX23HXH	1	2	1	II C
Dca000671	WRKYGQK/WRKYGQK	C2H2/C2H2	CX4CX22HXH/CX4CX23HXH	2	5	4	I
Dca000699	WRKYGQK	C2H2	CX4CX23HXH	1	2	1	II C
Dca000873	WRKYGQK	C2H2	CX5CX23HXH	1	5	4	II B
Dca002197	WKKYGQK	C2H2	CX4CX23HXH	1	2	1	I
Dca002205	WRKDGTH/WRKYATN	C2H2/C2H2	CX4CX23HXH/CX4CX23HXH	2	6	5	I
Dca002550	WRKYGQK	C2H2	CX5CX23HXH	1	3	2	II E
Dca002715	WRKYGQK	C2H2	CX4CX23HXH	1	3	2	II C
Dca003067	WRKYGQK	C2HC	CX7CX23HXC	1	3	2	III
Dca003180	WRKYGQK	C2H2	CX5CX23HXH	1	3	2	II D
Dca003732	WRKYGQK	C2H2	CX4CX23HXH	1	2	1	II C
Dca004998	WRKYGQK/WRKYGQK	C2H2/C2H2	CX4CX22HXH/CX4CX23HXH	2	4	3	I
Dca005043	WRKYGQK	C2HC	CX7CX23HXC	1	3	2	III
Dca005048	WRKYGEK	C2HC	CX7CX23HXC	1	2	1	III
Dca005648	WRKYGQK	C2H2	CX4CX23HXH	1	3	2	II C
Dca005780	WRKYGQK/WRKYGQK	C2H2/C2H2	CX4CX22HXH/CX4CX23HXH	2	5	4	I
Dca006278	WRKYGQK	C2H2	CX5CX23HXH	1	3	2	II D
Dca006505	WRKYGQK/WRKYGQK	C2H2/C2H2	CX4CX22HXH/CX4CX23HXH	2	6	5	I
Dca006646	WRKYGQK	C2HC	CX7CX23HXC	1	3	2	III
Dca006787	WRKYGQK	C2H2	CX5CX23HXH	1	3	2	II E
Dca007186	WRKYGQK/WRKYGQK	C2H2/C2H2	CX4CX22HXH/CX4CX23HXH	2	5	4	I
Dca007842	WRKYGQK	C2H2	CX5CX23HXH	1	5	4	II B
Dca008357	WRKYGQK	C2H2	CX5CX23HXH	1	4	3	II A
Dca008967	WTKYGQK	C2H2	CX4CX23HXH	1	3	2	I
Dca008968	WNKYGQK	C2H2	CX4CX23HXH	1	2	1	I
Dca008985	WRKYGQK/WRKYGQK	C2H2/C2H2	CX4CX22HXH/CX4CX23HXH	2	4	3	I
Dca009368	WRKYGQK	C2H2	CX4CX23HXH	1	3	2	II C
Dca010430	WRKYGQK/WRKYGQK	C2H2/C2H2	CX4CX22HXH/CX4CX23HXH	2	5	4	I
Dca010993	WRKYGQK	C2H2	CX4CX23HXH	1	2	1	II C
Dca011499	WRKYGQK/WRKYGQK	C2H2/C2H2	CX4CX22HXH/CX4CX23HXH	2	3	2	II C
Dca011569	WRKYGQK	C2H2	CX5CX23HXH	1	5	4	II B
Dca011912	WRKYGQK	C2H2	CX5CX23HXH	1	3	2	II A
Dca011914	WRKYGQK	C2H2	CX5CX23HXH	1	4	3	II A
Dca012410	WRKYGQK	C2H2	CX5CX23HXH	1	3	2	II E
Dca012846	WRKYGQK	C2H2	CX4CX23HXH	1	3	2	II C
Dca013146	WRKYGQK	C2HC	CX7CX23HXC	1	3	2	III
Dca013149	WRKYGQK	C2HC	CX7CX23HXC	1	3	2	III
Dca013150	WRKYGEK	C2HC	CX7CX23HXC	1	3	2	III
Dca014563	WRKYGQK	C2HC	CX7CX23HXC	1	2	1	III
Dca015482	WRKYGQK	C2H2	CX5CX23HXH	1	3	2	II D
Dca015639	WRKYGKK	C2H2	CX4CX23HXH	1	3	2	II C
Dca015848	WRKYGQK	C2H2	CX4CX23HXH	1	2	1	II C
Dca015914	WRKYGQK/WRKYGQK	C2H2/C2H2	CX4CX22HXH/CX4CX23HXH	2	3	2	II C
Dca016437	WRKYGQK	C2H2	CX4CX23HXH	1	2	1	II C
Dca016988	WRKYGQK	C2H2	CX5CX23HXH	1	2	1	II E
Dca017113	WRKYGQK	C2H2	CX4CX23HXH	1	3	2	II C
Dca018137	WRKYGQK/WRKYGQK	C2H2/C2H2	CX4CX22HXH/CX4CX23HXH	2	4	3	I
Dca018897	WRKYGQK	C2H2	CX5CX23HXH	1	4	3	II A
Dca019319	WRKYGQK	C2HC	CX7CX23HXC	1	3	2	III
Dca019656	WRKYGQK	C2H2	CX5CX23HXH	1	3	2	II E
Dca019840	WRKYGKK	C2H2	CX4CX23HXH	1	3	2	II C
Dca020159	WRKYGQK	C2H2	CX4CX23HXH	1	2	1	II C
Dca020342	WRKYGQK/WRKYGQK	C2H2/C2H2	CX4CX22HXH/CX4CX23HXH	2	5	4	I
Dca020473	WRKYGQK	C2H2	CX5CX23HXH	1	3	2	II E
Dca021638	WRKYGEK/WRKYGEK	—/C2HC	—/CX7CX23HXC	2	5	4	III
Dca023070	WRKYGQK	C2H2	CX5CX23HXH	1	3	2	II D
Dca024256	WRKYGQK	C2H2	CX5CX23HXH	1	2	1	II E
Dca024393	WRKYGQK/WRKYGQK	C2H2/C2H2	CX4CX22HXH/CX4CX23HXH	2	4	3	I
Dca026708	WRKYGQK	C2H2	CX4CX23HXH	1	4	3	II A
Dca027312	WRKYGQK	C2HC	CX7CX23HXC	1	3	2	III
Dca028175	WRKYGQK	C2H2	CX4CX23HXH	1	3	2	II C
Dca028770	WRKYGKK	-	-	1	1	0	II C

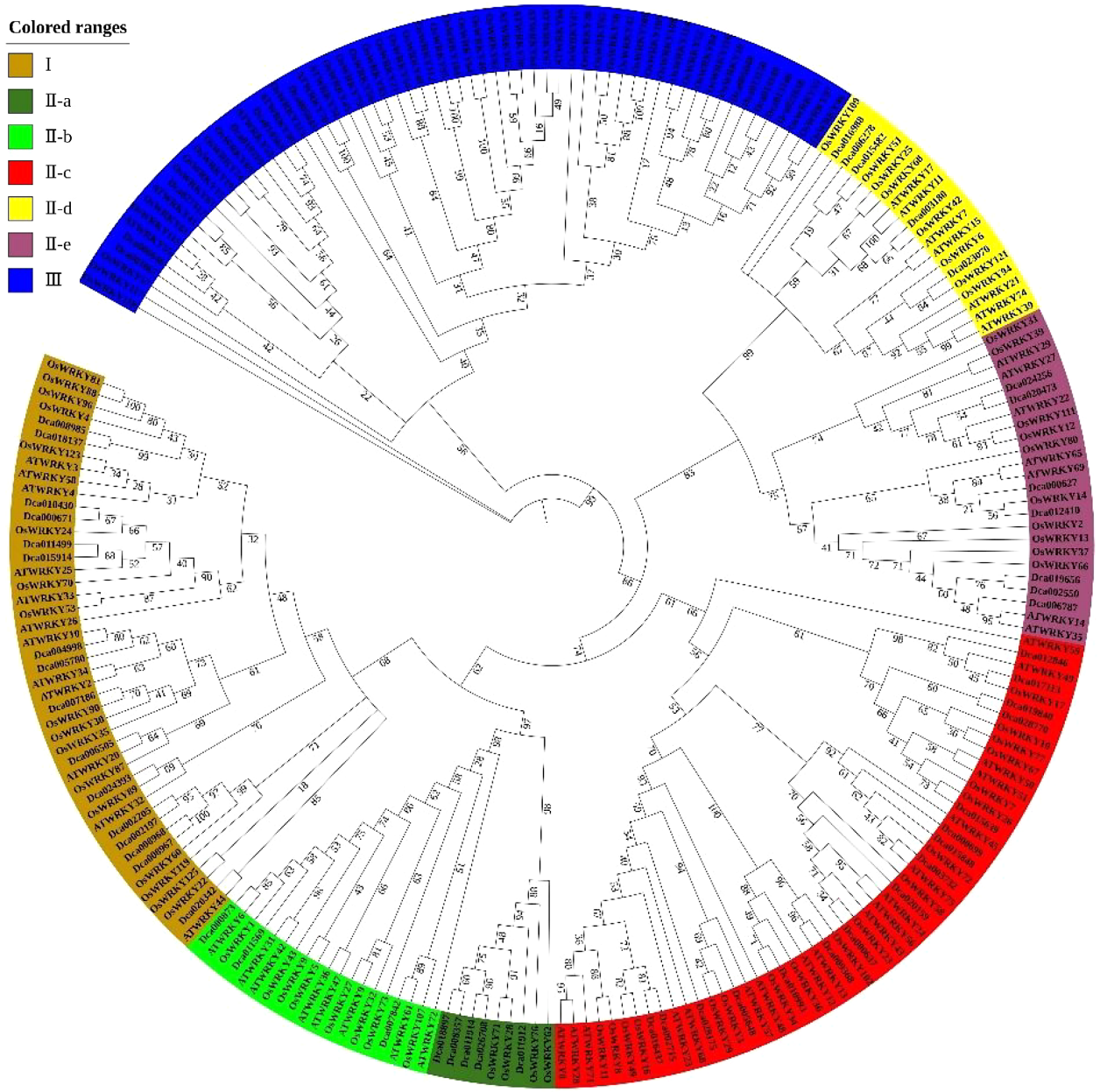

Fig. 3 Phylogenetic analysis of WRKY family proteins in *D. catenatum*.

> 8.0-fold decrease in response to drought treatment. In contrast, *Dca003067* and *Dca018137* were upregulated and showed an opposite expression pattern (Fig. 7). In leaves, 22 *WRKY* members that were differentially expressed when responding to drought were considered drought-responsive genes; these included *Dca000627*, *Dca000671*, *Dca002550*, *Dca002715*, *Dca003067*, *Dca003180*, *Dca005043*, *Dca005648*, *Dca006787*, *Dca007842*, *Dca008968*, *Dca010430*, *Dca011569*, *Dca012410*, *Dca013149*, *Dca015914*, *Dca016437*, *Dca017113*, *Dca024256*, *Dca019656*, *Dca026708*, and *Dca028770*. All of these showed a decrease in their expression levels, except for *Dca002550*, *Dca005648*, and *Dca010430*, whose expression levels showed 2.0- to 11.0-fold increase in drought-treated plants compared with untreated plants (Fig. 8).

A total of 15 genes in both roots and leaves showed a significant difference in expression levels when responding to drought stress, including *Dca000627*, *Dca002550*, *Dca006787*, *Dca002715*, *Dca005648*, *Dca007842*, *Dca010430*, *Dca011569*, *Dca015914*, *Dca016434*, *Dca024256*, *Dca019656*, *Dca026708*, *Dca028770*, and *Dca003067*. Interestingly, the identified drought-responsive gene *Dca003067* showed the opposite expression patterns in the leaves and roots. Drought treatment led to a significant decrease in the expression level in leaves but an increased expression in roots.

In leaves, seven drought-responsive genes, including three genes whose expression increased and four genes whose expression decreased, were randomly selected for validation by qRT-PCR. These genes were *Dca002550*, *Dca002715*, *Dca005648*, *Dca007842*, *Dca010430*, *Dca016437*, and *Dca006787* (Table 3, Fig. 9). All of them were differentially expressed in drought-treated plants compared with untreated plants. Thus, the expression patterns of these seven genes obtained by qRT-PCR

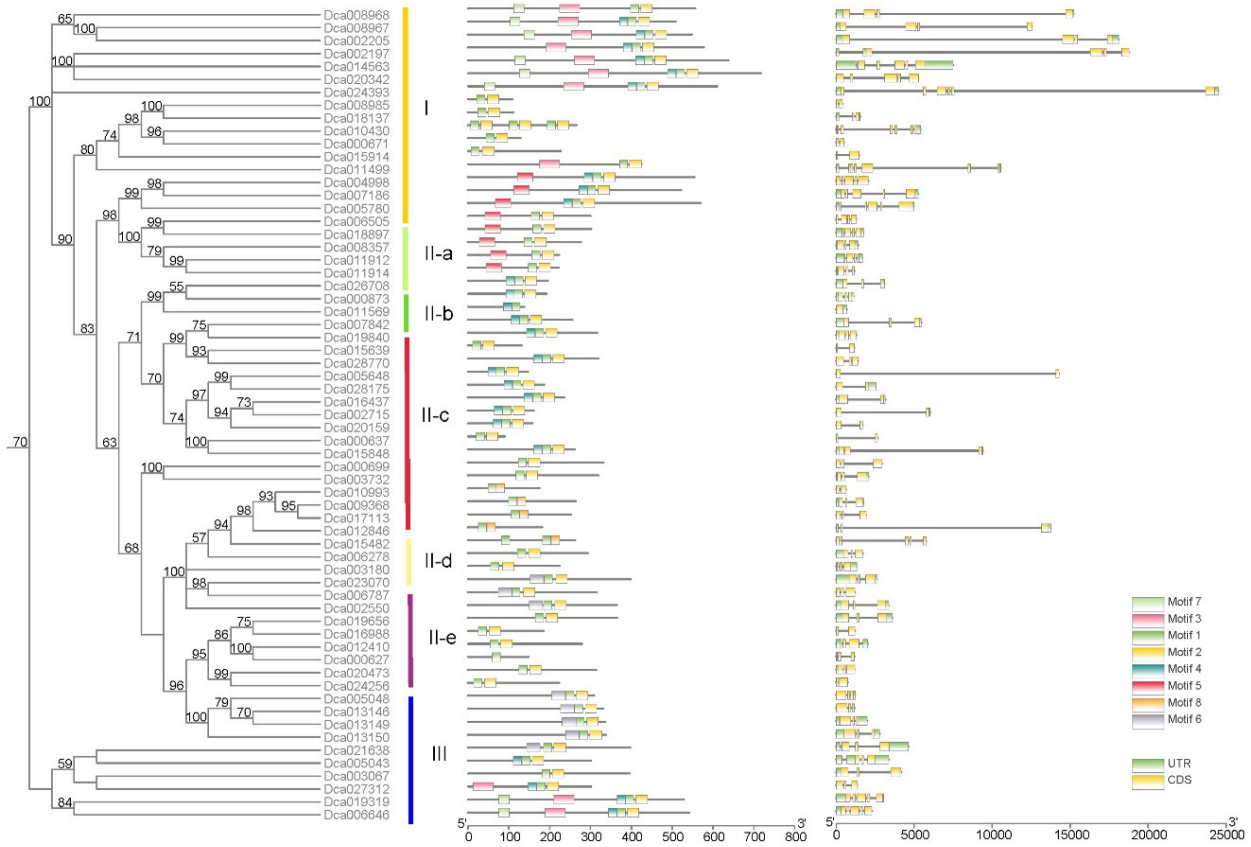

Fig. 4 Conserved motifs and gene structure of *DcaWRKY* genes according to phylogenetic relationships.

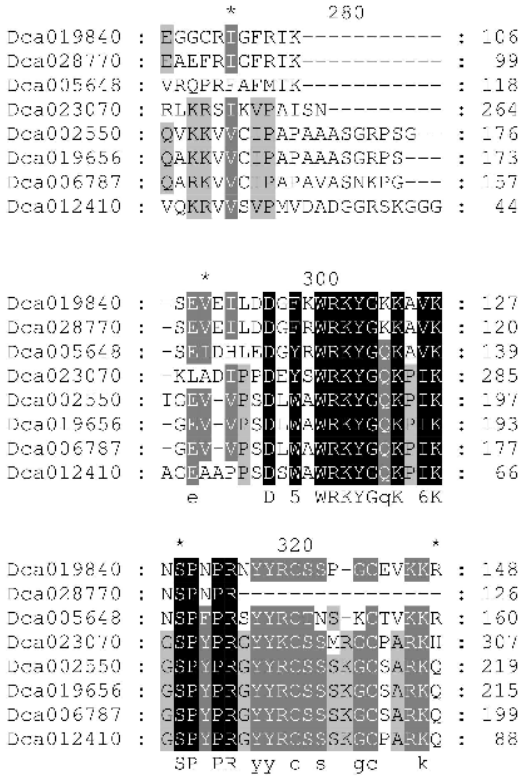

Fig. 5 Multiple sequence alignment of identified DcaWRKY proteins.

were consistent with the transcriptome analysis results, confirming the reliability of the transcriptome data.

DISCUSSION

Validation of identified amino acid patterns using *D. catenatum* as a case

After a comprehensive analysis of reported functional *WRKY* gene sequences, gene structure, and phylogenetic analysis, the amino acid patterns 'T-R/K-S/T-E/Q/D-V/I/L-E/D-I/V/H/N-L/M-D/E-D-G/E-F/Y-K/R-WRKYG-Q/K-K-A/T-VKN-S/N-P', 'VPA-I/V-S-X-K-M/L/V/I-ADIP-P/A/V-D-D/E-Y/F-S-WRKYGQKPIKGSP-H/Y-PRGYYKCS-S/T-V/M-RGCPARKVER', and 'PSD-S/A/L-WAWRKYGQKPIKGSPYPR-G/S-YYRCSSSKGC' were found in groups II-c, II-d, and II-e, respectively, which might be valid features of genes responding to stress in these three subgroups. Then, *D. catenatum* was used to validate these amino acid patterns. Four *WRKY* genes in group II-e (*Dca006787*, *Dca002550*, *Dca012410*, and *Dca019656*), three genes in group II-c (*Dca019840*, *Dca005648*, and *Dca028770*), and one gene in group II-d (*Dca023070*) were identified by homology searches. The expression of the identified *DcaWRKY* genes in roots and leaves was further analyzed under drought stress using qRT-PCR data. The expression levels of all potential genes identified as responsive to abiotic stress (stress under the drought treatment) were found to significantly change compared with untreated plants, suggesting that these identified amino acid patterns were valid and feasible for identifying abiotic stress in *D. catenatum*.

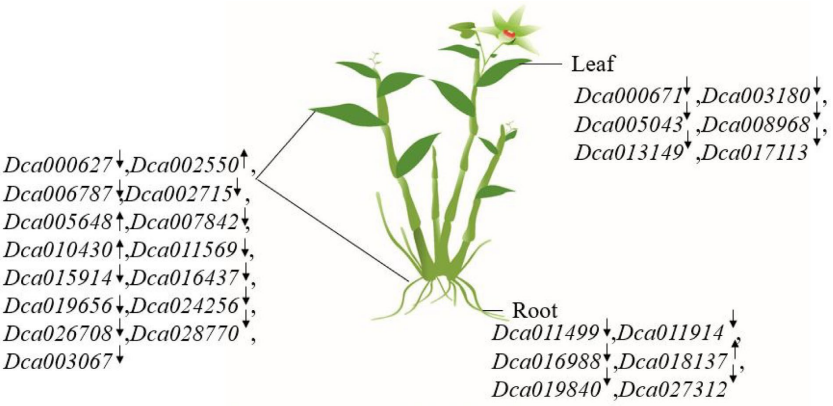

Fig. 6 The summarized figure of *DcaWRKY* genes expression in roots and leaves. '↑' indicates that gene expression increased under drought treatment. '↓' indicates decreased expression under drought treatment. *Dca000671*, *Dca003180*, *Dca005043*, *Dca008968*, *Dca013149*, and *Dca017113* were differently expressed in leaves under drought treatment; similarly, *Dca011499*, *Dca011914*, *Dca016988*, *Dca018137*, *Dca019840*, and *Dca027312* were differently expressed in roots. *Dca000627*, *Dca002550*, *Dca006787*, *Dca002715*, *Dca005648*, *Dca007842*, *Dca010430*, *Dca011569*, *Dca015914*, *Dca016437*, *Dca019656*, *Dca024256*, *Dca026708*, *Dca028770*, and *Dca003067* were expressed both in leaves and roots under drought treatment.

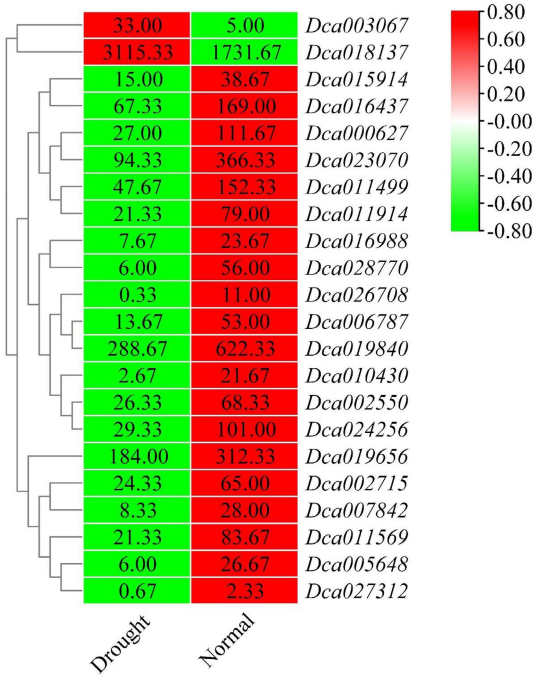

Fig. 7 Heatmap of differentially expressed *DcaWRKY* genes under drought stress in roots. The color scale shows increasing expression levels from green to red, which represents log2-transformed FPKM.

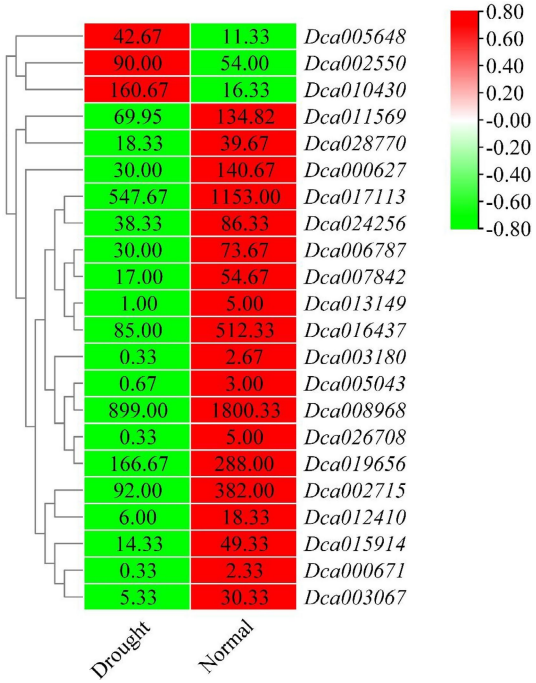

Fig. 8 Heatmap of differentially expressed *DcaWRKY* genes under drought stress in leaves. The color scale shows increasing expression levels from green to red, which represents log2-transformed FPKM.

Although further studies are needed, the amino acid patterns identified in response to stress not only provide an ideal method for quickly identifying stress-related genes in *D. catenatum* but also offer a new perspective for the identification of functional genes in other species.

Diverse expression patterns of *DcaWRKY* genes in different tissues

In this study, *DcaWRKY* gene expression and the phylogeny of *WRKY* genes were analyzed. Various expression mechanisms of *WRKY* genes in *D. catenatum* were found under drought stress.

All differentially expressed *WRKY* genes in roots and leaves were comprehensively analyzed. The expression changes in *WRKY* genes in roots and leaves were analyzed first. Among all 29 differentially expressed *WRKY* genes, the expression levels of 14 *DcaWRKY* genes were changed only in roots or leaves when responding to drought stress. Among these 14 *WRKY* genes, seven were differentially expressed only in roots, including *Dca011499*, *Dca011914*, *Dca016988*, *Dca018137*, *Dca019840*, and *Dca027312*. Similarly, *Dca000671*, *Dca003180*, *Dca005043*, *Dca008968*, *Dca013149*, and *Dca017113* were differentially expressed only in leaves. Some *DcaWRKY* genes might be differentially expressed only in specific tissues when responding to drought stress. Among the 14 *WRKY* genes, the expression levels of *Dca002550*, *Dca005648*, and *Dca010430* were downregulated in leaves but were upregulated in roots in drought-

Table 3. qRT-PCR primers of *DcaWRKYs*.

Primer name	Sequence (5'-3')
Dca002550-F	GTGTTCGAGCTCAACCATCA
Dca002550-R	TGATCGTGATCTCCCATGAA
Dca005648-F	GGCCGATTCACCGAATAATA
Dca005648-R	TTTCAACACGCTTCTTCACG
Dca006787-F	GCGATCTCTTTGCCTCAAAC
Dca006787-R	TTCCTTGCTGAGCATCCTTT
Dca007842-F	GCTCCTCTACCACCCATTCA
Dca007842-R	GTGAGGTCGAGGGTGATTGT
Dca010430-F	AGGAAGTCTGACGACGGCTA
Dca010430-R	CGAGTGGACTGAGGCTTAGG
Dca016437-F	ATCGTTGCACCACACAGAAG
Dca016437-R	AAGTCATGGTGGAAGCTTGG

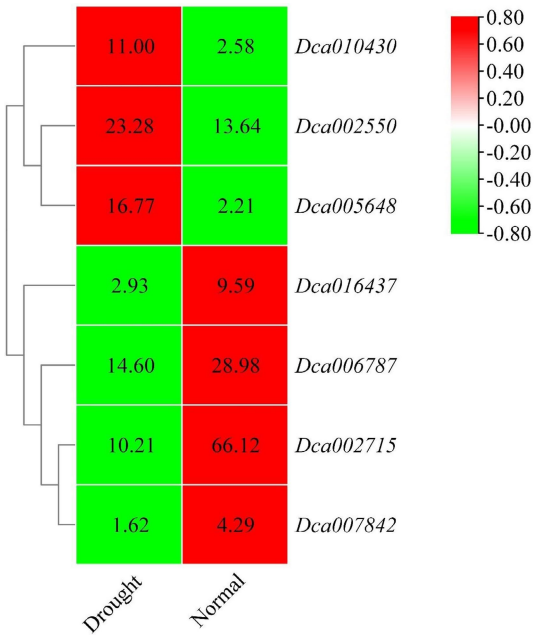

Fig. 9 Heatmap of the selected *DcaWRKY* genes in leaves. The color scale shows increasing expression levels from green to red, which represents log2-transformed FPKM.

treated plants compared with untreated plants. In contrast, under drought stress, *Dca003067* expression was highly upregulated in leaves and downregulated in roots. Except for these three genes, all 11 genes among the 14 *WRKY* genes showed the same trend of expression in roots and leaves when responding to drought stress. Of the 11 *DcaWRKY* genes, only the expression of *Dca003067*, clustered in group III, was upregulated in drought-treated plants, whereas other genes were clustered in group II, and their expression levels were downregulated compared with the control. Genes with expression patterns consistent with phylogenetic branching correlations were clustered in group II; thus, we presume that the downregulation of expression levels is associated with the characteristics of group II. The molecular mechanisms of drought responses in different tissues of *D. catenatum* might be different.

WRKY phylogeny was systematically analyzed in *Arabidopsis*, rice, and *D. catenatum*. According to the phylogenetic tree, the members that belong to the same branch might possess a conserved function because of their common evolutionary processes. A close relationship was found between *Dca016437* and *Dca002715* and *OsWRKY11*, which was identified as an anti-drought gene in rice by the phylogenetic tree. These two genes might have the same response mechanism as *OsWRKY11*, whose ectopic expression resulted in constitutive expression of defense-associated genes to enhance tolerance to drought stress in rice[57].

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Conflict of interest

The authors declare that they have no conflict of interest.

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