

Identification of the WRKY gene family and the functional analysis of DoWRKY71 in yam (*Dioscorea opposita* Thunb.)

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Abstract

Background: *Dioscorea opposita* Thunb. is one of the top 10 most important edible tuber and root plants worldwide, have been widely cultivated for nutritional and medicinal purposes. Yam is susceptible to various biological and abiotic stresses that will affect the yield and quality of the tubers. The WRKY gene family are widely distributed in plants growth and respond to abiotic stresses. However, current information on WRKY characteristics in yam is limited.

Results: This study identified 22 *DoWRKY* genes from transcriptome of *Dioscorea spp.*, which were divided into 6 clades based on phylogenetic analysis. Analysis of conserved motifs showed that there were 10 conserved motifs, with lengths ranging between 17 and 50 amino acids. Expression pattern analysis showed *DoWRKY* genes are expressed in different growth and development stages and responded to a variety of abiotic stress. *DoWRKY71* was up-regulated in the late stage of tuber expansion, and it was differentially expressed in cold and ABA stress. Therefore, further study indicated that DoWRKY71 protein was localized in the nucleus. Overexpression of *DoWRKY71* enhanced the cold tolerance of transgenic tobacco, promoted abscisic acid-mediated stomatal closure.

Conclusions: This paper is the first systematic analysis the *WRKY* gene family in *Dioscorea spp.*, and provide a new insight for further studying WRKY transcription factors in *Dioscorea spp.*. The functional study of DoWRKY71 laid a theoretical foundation for further exploring the regulatory function of DoWRKY71 gene in the growth and development related signaling pathway of yam.

Introduction

WRKY transcription factors (TFs) are the 7th largest family of regulatory genes screened from higher plants[1]. The name of the WRKY family is derived from their conserved WRKYGQK amino acid core sequences in the N-terminal. They all regulate gene transcription by binding to the conserved W-box in the promoter region of the target gene. WRKY TFs are categorized into three groups based on the number of WRKY domains (two WRKY domains in Group I and one in Group II and III) and the structure of the zinc fingers (C_2H_2 in Group I and II, and C_2HC in Group III proteins). Group II is further divided into five subgroups (IIa-IIe) according to the phylogeny of the WRKY domain[2–3]. WRKY TFs act separately or in combination with other regulators to activate or suppress transcription[4].

The first *WRKY* gene family member, *SPF1*, was cloned from sweet potato in 1994 by Ishiguro, S. et al., and found that its expression was induced by sucrose and PGA[5]. Subsequently, genes encoding WRKY proteins have been found in many plants, such as 71 members of *Arabidopsis thaliana*[6], 78 members of garlic (*Allium sativum* L.)[7], 174 members of soybean (*Glycine Max*) [8], 104 members of rice (*Oryza sativa*) [9]. The WRKY gene family are widely distributed in plants and play a key role in plant growth and development. For example, Wild Strawberry (*FvWRKY4*, -46, and -48) in group c are involved in fruit development and ripening in *F. vesca* [10]. Overexpression of *WRKY25* in *Arabidopsis* can lead to early flowering and premature senescence phenotypes [11]. Several studies have confirmed that WRKY

transcription factors are involved in seed size[12], growth types[13], and hormone signaling pathways[14]. Simultaneously, WRKY proteins studied thoroughly and reported widely is transcription regulation of response to abiotic and biotic stresses. Studies in bananas found that the *Musa WRKY71* gene was induced by low temperature, salt, abscisic acid(ABA), H₂O₂ and ethylene [15]. Transcriptome analysis showed that of the 100 WRKYs in *Populus*, 61 were induced by Salicylic acid SA, methyl jasmonate (MeJA), injury, cold, and salt stresses[16]. 4°C treatment can up-regulated the expression of the *WRKY71* family genes of *Brassica napus* L., speculated that it positive regulatory in cold tolerance through regulating downstream target genes[17]. Overexpression of *MfWRKY17* can increase the water retention capacity of *Arabidopsis*, maintain chlorophyll content, regulate ABA biosynthesis and the transcription level of related genes, and improve drought resistance[18]. Plant response to environmental stress is regulated by complex signaling pathways and networks which are coordinated by transcriptional factors (TFs).

Chinese yam (*Dioscorea opposita* Thunb.) belong to the genus *Dioscorea* in the *Dioscoreaceae* family is a monocotyledonous vine. As one of the top 10 most important edible tuber and root plants worldwide, various *Dioscorea* species have been domesticated and widely cultivated for nutritional and medicinal purposes[19–20]. Tuber, as the main product organ of Chinese yam, has a complex growth and development process[21]. During the cultivation process, *Dioscorea spp.* is susceptible to various biological and abiotic stresses that will affect the yield and quality of the tubers. Thus, defining the development mechanism of tubers may be important to improvement the yield of yam. With the development of high-throughput sequencing technology, *WRKY* gene family has been identified in *Arabidopsis thaliana*[22], *Oryza sativa*[23], *Triticum aestivum*[24], *Solanum tuberosum*[25], tomato[26]. However, current information on *WRKY* characteristics in *Dioscorea spp.* is limited. In this study, all of the *Dioscorea spp. WRKY* proteins from the Transcriptome database were identified and subjected to phylogenetic analysis, analysis of gene structure, and alignment of conserved domains. In addition, a comprehensive analysis of *WRKY* genes in *Dioscorea spp.* and their expression patterns in different growth and development stages and various biotic and abiotic treatments. Target genes can be initially screened and their functions can be studied, which will lay a theoretical foundation for deeper research on the role of *WRKY* regulatory networks in plant growth and development and stress-related processes.

Results

Identification and sequence analysis of WRKY transcription factor in *Dioscorea spp.*

After manually removing redundant entries through screening (manually delete the genes without ORF/WRKY motif) and validation of the search results, a number of 22 WRKY transcription factors were characterized from *Dioscorea spp.* and named them from *DoWRKY1* to *DoWRKY22*. The characteristics concerning each gene, including gene number, length of coding sequence and amino acid sequence, MW and PI of the proteins, are summarized in Table 1. Subsequent sequence analysis of these 22 DoWRKYs showed that the encoded DoWRKY proteins lengths are ranged from 167 amino acids (aa) (DoWRKY20) to 709 amino acids (DoWRKY21) (average length: 385 amino acids). The calculated molecular weight

(MW) from 19.01 (*DoWRKY20*) to 76.91 (*DoWRKY21*) kDa and isoelectric points (PI) from 4.88 (*DoWRKY18*) to 9.93 (*DoWRKY19*). The subcellular localization prediction showed that 7 of the 22 *DoWRKY*s proteins were extracellular (*DoWRKY4*, 10, 11, 17, 18, 20, 22), and the rest were localized in the nucleus.

Multiple sequence alignment and phylogenetic analysis of *DoWRKY*s

To detect the evolutionary relationships and classification of the WRKY family in *Dioscorea spp.*, unrooted phylogenetic maximum likelihood method trees were constructed with the 22 *DoWRKY* proteins and the known WRKY protein from *Oryza* (Table S2). The results indicated that the 22 *DoWRKY*s were divided into 6 clades (, a, c, d, e,) according to the groups in *Oryza* (Fig. 1). Group I contained the highest WRKY members with 7 *DoWRKY*s, with four proteins in IIa groups, one members in IIc, three in IId, two in IIe, and group five in (Table 1). These results suggest that WRKY members in these groups of *Dioscorea spp.* could have the similar functions with those members in *Oryza*. Group 1 WRKY family members all have two *WRKY* domains and a C₂H₂ type zinc-finger motif (CX₄-CX₂₂₋₂₃-HXH). Group 3 were characterized by a C₂HC type zinc-finger motif, but only the N-terminal WRKY domain of *DoWRKY20* was lost (Fig. 2).

Gene structure and conserved motifs analysis

To further analyze the characteristics of *DoWRKY*s, the MEME online tool was used to predict the potential conserved motifs. A total of 10 motifs were identified, with lengths ranging between 17 and 50 amino acids. Motif 1 contained the domain present in every WRKY member. The motif information and the multiple alignment information showed that Motif1 was the highly conserved WRKY domain (WRKYGQK) in *WRKY* genes in *Dioscorea spp.*. Excluding *DoWRKY15*, all genes contained at least 2 Motifs. Notably, the members with high similarity in the same group shared a common motif composition. For instance, *DoWRKY7* and *DoWRKY9* were found to contain seven motifs (Fig. 3). It indicates that these two genes may have a similar function.

Expression analysis of *DoWRKY*s in different developmental stages of tuber

By comprehensively considering the protein family classification, subcellular localization, and expression level, we selected 8 genes from six groups to analyze expression level of *Dioscorea spp.*(Table S3). under different growth and development stages. *DoWRKY7* was up-regulated in the whole process and highly expressed in 105d. *DoWRKY4* and *DoWRKY12* showed the same trend and reached the highest value at 120d. Two genes *DoWRKY16* and *18*, downregulation expressed in earlier stage and upregulated expression in mature stage(Fig. 4).

*DoWRKY*s gene expression following abiotic stress by qRT-PCR

To further investigate the role of *DoWRKY*s in abiotic stress responses, we randomly selected 8 genes to made sure their responses to the cold, ABA, and JA treatment.

Following cold treatment (Fig. 5), the expression levels of four genes (*DoWRKY5*, *DoWRKY7*, *DoWRKY14*, *DoWRKY16*) increased by more than twofold, among which the expression of *DoWRKY7* was 15 times and *DoWRKY16* was 9 times higher than that of the control. The expression of two genes (*DoWRKY4* and *DoWRKY11*) decreased significantly at 1h, but their expression were significantly increased and peaked at 24 h.

The expression levels of three genes (*DoWRKY7*, *DoWRKY11*, *DoWRKY16*) were increased by more than twofold following treatment with ABA (Fig. 5). The expression levels of *DoWRKY7* and *DoWRKY16* were 15 fold higher than those of the control, respectively. The expression levels of *DoWRKY18* decreased significantly than control.

For the case of MeJA stress (Fig. 5), four genes (*DoWRKY4*, *DoWRKY5*, *DoWRKY14*, *DoWRKY18*) have the highest level of expression at 24 h. *DoWRKY14* were upregulated in the whole process, but *DoWRKY16* rapidly rising at early time points but with subsequently decreased expression at 24h. In contrast, *DoWRKY7*, *DoWRKY11*, and *DoWRKY12* showed a trend of downregulation (Table S4).

To sum up, *DoWRKY16* was upregulated expression in mature stage and responded to abiotic stresses in cold, ABA, MeJA. we selected it for further study.

Subcellular localization of DoWRKY71

To determine the subcellular localization of the *DoWRKY71*, the recombinant vector CaMV35S::GFP-*DoWRKY71* was transiently expressed in the tobacco leaves. The GFP fluorescence of the CaMV35S::GFP-*DoWRKY71* vector showed a strong fluorescence signal only in the nucleus, while the CaMV35S::GFP vector was relatively uniformly distributed inside the tobacco cells (Fig. 6).

Physiological and biochemical changes and detection of reactive oxygen species in tobacco treated at 4°C

Investigating the cold stress tolerance of *DoWRKY71* in transgenic tobacco. The growth of transgenic tobacco under control conditions was better than that of WT tobacco, and the cold tolerance of transgenic tobacco was better (Fig. 7, A). The chlorophyll content decreased in the whole treatment processes (Fig. 7, B); The soluble protein content decreased firstly and reached the highest level after treatment for 24h, and the transgenic plants was significantly higher than that of WT plants (Fig. 7, C); The activities of SOD and POD both tended to increase, the transgenic plants were significantly higher than WT plants (Fig. 7, D, E).

Low temperature usually induces membranous peroxidation and overproduction of reactive oxygen species, which eventually leads to oxidative stress reaction. NBT and DAB staining was performed on tobacco treated at 4°C (Fig. 7, F), staining was seen in both control WT and transgenic plants, but the O₂⁻ and The H₂O₂ level was lower than that of the WT line. In addition, MDA content of both transgenic and WT plants increased with time when low temperature stress occurred, but decreased slightly at 24h.

MDA content accumulated by transgenic lines in control and experimental groups was significantly lower than that of WT plants (Fig. 7, G) (Table S5). These results indicated that overexpression of *DoWRKY71* regulated reactive oxygen species homeostasis and reduced oxidative damage in tobacco.

Changes of physiological indexes, ABA and GA contents in tobacco under ABA treatment

With the extension of spraying time, the soluble protein content decreased firstly and then increased, the transgenic plants in control and treatment groups was significantly higher than that in WT tobacco (Fig. 8, A). The change trend of SOD and POD activity were basically the same as that under 4°C treatment, and POD activity was significantly higher than that in WT tobacco (Fig. 8, B, C). The GA content showed an overall upward trend and reached the highest value at 12h, the ABA content increased firstly and then decreased. The GA content of the transgenic lines in the control and experimental groups was significantly lower than that of the WT plants (Fig. 8, D), and ABA content is significantly higher than that in WT plants (Fig. 8, E) (Table S6).

Effects of ABA treatment on stomata of tobacco overexpressing *DoWRKY71*

The stomata of *DoWRKY71* transgenic plants and WT plants under ABA treatment were observed under microscope (100×) (Fig. 9, A). Under normal conditions, the stomatal pore size index of T3 and T4 was significantly higher than that of WT (Fig. 9, A). Under 100 μmol/L ABA spraying treatment, the stomatal index of *DoWRKY71* transgenic lines was significantly smaller than that of WT plants. The results indicated that overexpression of *DoWRKY71* could promote ABA mediated stomatal closure in tobacco.

Expression analysis of *DoWRKY71* gene in transgenic tobacco at 4°C and ABA treatment

Under 4°C and ABA treatment time, the expression level of this gene showed a trend of first increase and then decrease. The expression level of transgenic plants was higher than that of untreated plants, and there was a significant difference (Fig. 10) (Table S7).

Discussion

WRKY transcription factors, as a class of regulatory proteins, respond to plant growth and development by regulating the expression changes of numerous downstream genes [35]. They have been investigated and identified at the genome and transcriptome levels in a variety of plants [36], which has become one of the focuses of studies on plant gene function. In this study, 22 *WRKY* gene family members were identified in transcriptome of *Dioscorea spp.*. According to the characteristics of WRKY family and 42 rice protein sequences, WRKY proteins of *Dioscorea spp.* were divided into three groups. The *WRKY* gene family was smaller than those of other plants such as sweetpotato (79 *WRKY* genes)[37] and eggplant (58 *WRKY* genes)[38]. Interestingly, the b subgroup has no *DoWRKY* member. This may be due to the incomplete sequencing of the yam genome and the identification from transcriptome data[39]. The WRKYGQK heptapeptide domain and zinc finger structure are necessary for binding of WRKY transcription factors to the cis-acting element W-box of target genes. Multiple sequence alignment of

DoWRKY protein showed that all proteins contained WRKYGQK conserved sequence. But we found that the N-terminal WRKY domain of DoWRKY20 was lost, which was consistent with barley[40]. It has been reported that the DNA binding activity of the N-terminal WRKY domain is not as strong as that of the C-terminal, and there are more variations in the N-terminal WRKY domain. The conserved motifs of WRKY exist not only in higher plants such as rice [23], but also in lower plants such as algae [41], which also reveals their extensive involvement in different plants. The identification of conserved motifs in WRKY protein of yam showed that: Motif 1 is the core Motif of yam. These results suggest that these traits are conserved in the *WRKY* family of yams. It is worth noting that the members of almost every group cover the same theme. However, studies have shown that members of these families come from the same clade, their functions are quite different[42]. For example, *DoWRKY5*, *DoWRKY13* and *DoWRKY18* belongs to group , but has similar conserved motifs to which group .

Previous studies have shown that members of the WRKY family have multiple diverse functions in plant growth and various stress responses, the functions in *WRKY* genes within the same group or subgroup usually retain similar[43–45]. Besides, regulation of root growth is under the control of several hormones and the expression of various transcriptional regulators. Among the expression level of *DoWRKY* genes of *Dioscorea spp.*, *DoWRKY16* and *DoWRKY18*, downregulation expressed in the early stages and upregulated expression in mature stage, they all belonged to Group II. The results showed that many genes can be grouped according to their abundant expression in specific organs, a finding that might reflect their participation in the common developmental processes[45]. Four genes (*DoWRKY5*, -7, -14, -16) significantly upregulated during cold stress, which results are similar to the *TkWRKY* genes that 8 *TkWRKY* genes are induced by cold stress[46]. Under ABA stress, three *DoWRKY* genes (*DoWRKY7*, -11, -16) were significantly induced. Similar results have been reported in *Scutellaria Baicalensis* Georgi[47] and *Gossypium hirsutum*[48]. In addition, the *DoWRKY7*, -11, -12, -14 genes are also sensitive to MeJA treatment. Three *DoWRKY* genes (*DoWRKY7*, -11, and -12) were down-regulated expression during MeJA treatment. Research show that MeJA identified as a transcription activator of *PpPAL* and *Pp4CL* via binding to their W-BOX [49]. The above research confirms that the response mechanisms of *DoWRKYs* members may be complex and varied, including cold, ABA, MeJA, and the selection of candidate genes can provide reference for future investigations.

DoWRKY16, which was unregulated expressed at mature developmental stages and cold, ABA, MeJA stresses, was selected for further study. After preliminary cloning and comparison with NCBI, it was named as *DoWRKY71*. Transcriptional regulation of various effector genes in plants is the key to activating inducible stress responses in plants [15]. The subcellular location assay displayed that the DoWRKY71 protein was localized in the cell nucleus. WRKY71 localized in the cell nucleus and acted as a promoter of flowering a transcriptional regulatory cascade and in wild strawberry[50]. It is consistent with FvWRKY71, suspected that WRKY71 may closely related to the transcriptional regulatory function in the nucleus. In this study, the transgenic *DoWRKY71* plants were treated with cold and ABA stress, indicating that *DoWRKY71* is sensitive to environmental stress. Chlorophyll is an important part of the plant's photosynthetic apparatus for harvesting light chlorophyll content of the leaf is closely related to the photosynthetic function of the plant and represents the physiological state of the plant [51–52]. Under

low temperature treatment, the change of chlorophyll content indicating that transgenic plants can improve their photosynthetic capacity by increasing chlorophyll content.

The antioxidant enzyme content is a main index reflecting the degree of peroxidation of cells in the plant[53]. In this study, under low temperature and ABA treatments, the content of soluble protein first decreased and then increased, the POD and SOD activity showed an overall upward trend, and the SOD activity decreased (Fig. 7 ,8). The antioxidant enzyme system protects the membrane system by scavenging reactive oxygen species, and then alleviating the damage to plants caused by stress [54], but the effect will weaken with time, and the activity of antioxidant enzymes will gradually decline with the production of reactive oxygen species. Prolonged low temperature can lead to excessive production of reactive oxygen species, resulting in damage of plant cell membrane, protein and nucleic acid damage, and even cell death [55]. MDA concentration is a parameter of membrane lipid peroxidation, which can reflect the degree of stress. NBT and DAB staining tests showed that transgenic tobacco suffered lower membrane damage, less accumulation of reactive oxygen species and MDA, and stronger cold tolerance under low temperature stress.

WRKY protein also plays an activation or inhibition the signaling pathway[56], White, C. N. Et al. found that ABA inhibited GA signal in early germination of maize[57]. In this study, the endogenous GA and ABA were relatively antagonistic during ABA spraying. Exogenous ABA could reduce leaf stomatal conductance, water loss and reduce the pressure of osmotic regulation system. The leaf stomatal tension decreased under ABA spraying, which provided a guarantee for plants to prevent water loss in adversity. *DoWRKY71* may regulate stomatal opening and closure in plants through ABA-dependent signaling, which is consistent with *MfWRKY17* reported in *Argyrophylla*[58]. Tolerance in plants can be manifested in two ways by enhancing functional gene expression and protein synthesis. The expression level of transgenic tobacco *DoWRKY71* under both treatments was significantly higher than that of the control, indicating that both low temperature and ABA could trigger the expression of *DoWRKY71* gene in response to various physiological processes. This result is consistent with that of cucumber [59] and orchid [60].

Conclusion

In conclusion, this study provides the first comprehensive and systematic analysis of the 22 *WRKY* genes identified in *Dioscorea spp.* transcriptome. All *DoWRKY* genes can be divided into six groups (I-III), and were found to responding to growth and development process and abiotic stresses. Besides, *DoWRKY71* may be one of the important members of the *WRKY* gene family of yam, with the ability to cross-regulate other genes involved in abiotic stress signaling pathways. However, the molecular mechanism of which pathways *DoWRKY71* and others members participate in the growth and development of yam and how it interacts with downstream proteins still needs to be further studied.

Materials And Methods

Identification of *WRKY* genes in *Dioscorea spp.*

The data used for analysis were downloaded from the whole transcriptome sequences of *Dioscorea spp.* (Nuohezhiyuan Company, Beijing, 2020). The HMM (Hidden Markov Model) profiles based on the *Oryza sativa* WRKY (PFAM ID: PF03106.15) protein downloaded from PFAM protein family database (<http://pfam.xfam.org/>)[27]. BLASTp analysis of *Dioscorea spp.* transcriptome database was performed using HMMER 3.0. The cut-off E-value was set as 0.1. The candidate WRKY transcription factors were obtained subsequently. They were evaluated using the online program PFAM (<http://pfam.xfam.org/search>), Conserved Domains DataBase Search (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and SMART tool (<http://smart.embl-heidelberg.de/>) to confirm the presence of WRKY motif. The biophysical properties of WRKY members, such as, peptide length, molecular weight (MW), isoelectric point (pI) were predicted using the online ExPasy program (<http://www.expasy.org/tools/>)[28]. Protein subcellular localization prediction using online website (<http://linux1.softberry.com>).

Multiple sequence alignment, phylogenetic analysis and conserved motif detection

All 22 DoWRKYs proteins with WRKY domains and selected OsWRKYs proteins were used to generate multiple protein sequence alignments by using the default settings in Clustal Omega. A phylogenetic tree was constructed with the maximum likelihood method with MEGAX software[29]. Branch support for the tree topology was estimated by using a bootstrap analysis with 1,000 replicates. The online ITOL (<http://itol.embl.de/help.cgi>) tool was used to embellish the phylogenetic tree. In addition, the online software MEME (<http://meme.ebi.edu.au/>) was used to identify the conserved motifs among all DoWRKY genes. The parameters in this study were set as follows: zero or one occurrence per sequence; maximum number of motifs: 10; and other optional parameters was set default[30].

Plant materials and treatments

Tissue culture seedling of yam (*Dioscorea opposita* Thunb.) was used in present study. The seedling cultured in an artificial climatic chamber kept at 25°C with a 16/8h photoperiod (day/night). 6-week-old seedlings were subjected to different stress conditions: 100µM ABA, cold(4°C), 100µM MeJA and cultured for a total of 24 h, leaves were collected after 0, 1, 6, 12 and 24 h. In order to analyze the expression of *WRKY* genes in different growth and development stages, we collected yam(Dahechangyu) tuber in 90,105,120,135,150,165d for RNA preparation. All of the samples were immediately frozen in liquid nitrogen and stored at -80°C for subsequent total RNA extraction. All samples were tested with three technical replicates and three independent biological replicates.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using RNA extraction kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Reverse transcription PCR (RT-PCR) was performed using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). First-strand cDNA synthesis was accomplished

using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen Biotech, Beijing, China). Quantitative Real-time PCR (qRT-PCR) was performed using TB Green Premix Ex Taq II (6210A, TaKaRa, Dalian, China) on an FTC-3000P system (Funglyn Biotech, Toronto, Canada) with the primers listed in Table S1. The PCR conditions consisted of denaturation at: 95°C for 30s, followed by 40 cycles of 5s at 95°C, and 30s at 60°C, and 4°C to finish. All samples were tested with three technical replicates and three independent biological replicates. The relative expression level was calculated while using the $2^{-\Delta\Delta CT}$ method. The ribosomal RNA (18S) of yam as the internal control[31].

Subcellular localization analysis

DoWRKY71 were cloned into the transient expression vector CaMV35S::GFP to generate CaMV35S::GFP-*DoWRKY71* recombinant vector, restriction sites are BamH I and Kpn I. Then, *Agrobacterium tumefaciens* strain LBA4404 (Weidi Biotechnology, Shanghai,China) was cotransformed with the recombinant plasmid, and transiently expressed in *Nicotiana benthamiana* leaves. CaMV35S::GFP were used as the positive control individually. The results were observed by laser scanning confocal microscope (C2-ER, Nikon, Tokyo, Japan) after 2–3 days [32].

Treatment and functional analysis of overexpressed *DoWRKY71* in tobacco

The successfully transformed tissue culture seedlings and wild-type tobacco (WT) were rooted and then transplanted into plug trays to grow for 1 month. Plants with basically the same growth activity were selected and treated with ABA at 100μmol/L and 4°C, and their leaves were taken at 0, 1, 6, 12 and 24 h after treatment for the determination of the following physiological indicators: Chlorophyll content, soluble protein and malondialdehyde (MDA) content, peroxidase (POD), superoxide dismutase (SOD) content [33], respectively; Gibberellin (GA) and ABA content were using the kit(Shanghai Best Biotechnology Co., Ltd. kit); The production rate of superoxide anion radical ($O_2^{\cdot-}$) and the content of hydrogen peroxide (H_2O_2) were detected by staining of Nitroblue Tetrazolium (NBT) and Diaminobenzine (DAB) [34]. The experiment was repeated at least three times, with three biological replicates for each sample.

Tobacco leaves grown in plug trays for 1 month were collected, and the stomatal openings of wild-type tobacco (WT) and transgenic tobacco leaves under ABA treatment were observed and photographed with an electron microscope (C2-ER, Nikon, Tokyo, Japan). Digimizer was used to calculate the index of pore size. The relative expression levels of *DoWRKY71* gene in transgenic tobacco were determined at different time in 4°C and ABA treatment.

Statistical Analysis

SPSS (Ver. 25.0, SPSS Inc., Chicago, IL, USA) software was used for significance analysis, and significance levels of $P < 0.05$ or $P < 0.01$. Excel 2019 was used for data analysis and mapping.

Declarations

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Availability of data and materials

All data analyzed during this study are included in the supplementary information fles.

Ethics approval and consent to participate

All plant materials were used following national and international standards and local laws and regulations. Using all plant materials does not pose any

risk to other species in nature. No specifc permission is required to collect all samples described in this study. The planting area of yam(*Dioscorea opposita* Thunb.) samples is the base of our laboratory, and the field collection does not involve endangered or protected species.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Authors' contributions

Linan Xing conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft. Yanfang Zhang and Xiuwen Huo conceived and designed the experiments,authored or reviewed drafts of the paper, and approved the final draft. Mingran Ge and Lingmin Zhao analyzed the data, prepared figures and/or tables.

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Table 1

Table 1 is available in the Supplementary Files section.

Figures

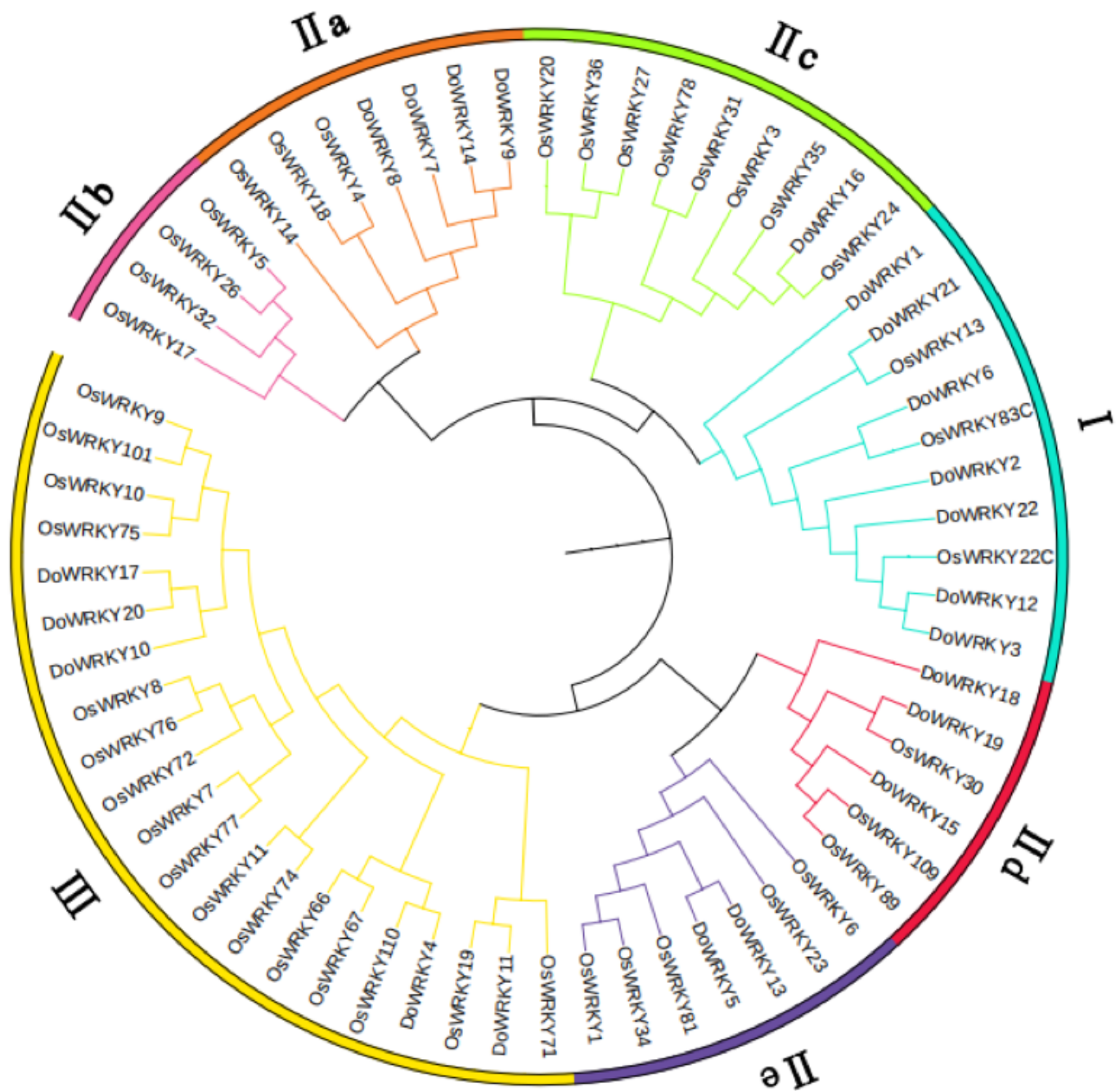


Figure 1

Phylogenetic tree analysis of the *WRKY* genes in *Dioscorea* spp. and *Oryza*. The clusters were designated as group I-III and indicated in a specific color .

DoWRKY1 DGYRWRKYGQKIVKGNPNFRSYYRCTHGG...CFVRKHVERALEDAKAMVITYEGKHNHDLF
 DoWRKY2 DGYRWRKYGQKVVKGNPNFRSYYKCTNAG...CFVRKHVERASHD PKAV ITTYEGKHNHDP
 DoWRKY3 DGYRWRKYGQKVVKGNPNFRSYYKCTTVG...CFVRKHVERASHDLRAV ITTYEGKHNHDP
 DoWRKY4 DGHWRKYGQKIDILGAKHPRGYRCTRNRARGCLATKQVQRSDSDPTIFDVVYRGEHSCSCE
 DoWRKY5 DMWAWRKYGQKPIKGSFYPRGYRCSSSKG...CQARKQVERSRDPGVLIITYTGEHNHFPF
 DoWRKY6 DGYRWRKYGQKVVKGNPNFRSYYKCTYSG...CNVRKHVERASTD PKAV ITTYEGKHNHDP
 DoWRKY7 DGYQWRKYGQKVTRDNPFCPRAYFRCSFAPT...CQVKKKVQRSAENQSIIVEVITYEGEHTHNAF
 DoWRKY8 DGYQWRKYGQKVTRDNPFCPRAYFRCAFAPA...CQVKKKVQRSAEDQSILVATYEGDHTHGQP
 DoWRKY9 DGYQWRKYGQKVTRDNPFCPRAYFRCSFAPT...CQVKKKVQRSAENQSIIVEVITYEGEHTHNAF
 DoWRKY10 DGHQWRKYGQKNLQKSNLARSYYRCTYKDE...GCQATKHVQKDNNDPPLFLVITYEQTCK
 DoWRKY11 DGFWRKYGQKEIFGSTAFRSYFRCTHKHNRNCQATQVQKSEEDPSMYVITYMGEHTCKDF
 DoWRKY12 DGYRWRKYGQKVVKGNPNFRSYYKCTTVG...CFVRKHVERASHDLRAV ITTYEGKHNHDP
 DoWRKY13 DVWAWRKYGQKPIKGSFYPRGYRCSSSKG...CMARKQVERSRD PGVLVVITYAEHNHPPF
 DoWRKY14 DGYQWRKYGQKVTRDNPFCPRAYFRCSFAPT...CQVKKKVQRSAENQSIIVEVITYEGEHTHNAF
 DoWRKY15 DDYSWRKYGQKPIKGSFYPRGYRCSSSKG...CPARKHVERCFQEPSMLIVITYEGEHNHTD
 DoWRKY16 DGYRWRKYGQKAVKNSFYPRSYRCTTQK...CTVKKRVERSYQDPTIV ITTYEGQHTHQS
 DoWRKY17 DGYQWRKYGQKNIQKSKSSRSYYRCTYRDE...GCRATKHVEEKDCNDPHLFSVITYENHTCR
 DoWRKY18 DSWAWRKYGQKPIKGSFYPRGYRCSSSKG...CPARKQVERSRMDPTMLVVITYSSDHNHPPF
 DoWRKY19 DEYSWRKYGQKPIKGSFYPRGYRCSVRG...CPARKHVERAPDDPAMLIVITYEWEHRHSLD
 DoWRKY20 DGYQWRKYGQKNIQKSKSSRFVWITFVFC...KDRVPSQFLFFLF.....
 DoWRKY21 DGYRWRKYGQKVVKGNPNFRSYYKCTNPG...CTVRKHVERASHDLKSV ITTYEGKHNHDP
 DoWRKY22 DGYRWRKYGQKVVKGNPNFRSYYKCTNPG...CSVRKHVERASHDLKSV ITTYEGKHNHEVP

d wrkygqk r

Figure 2
 Multiple sequence alignment of *WRKY* gene in *Dioscorea* spp. .

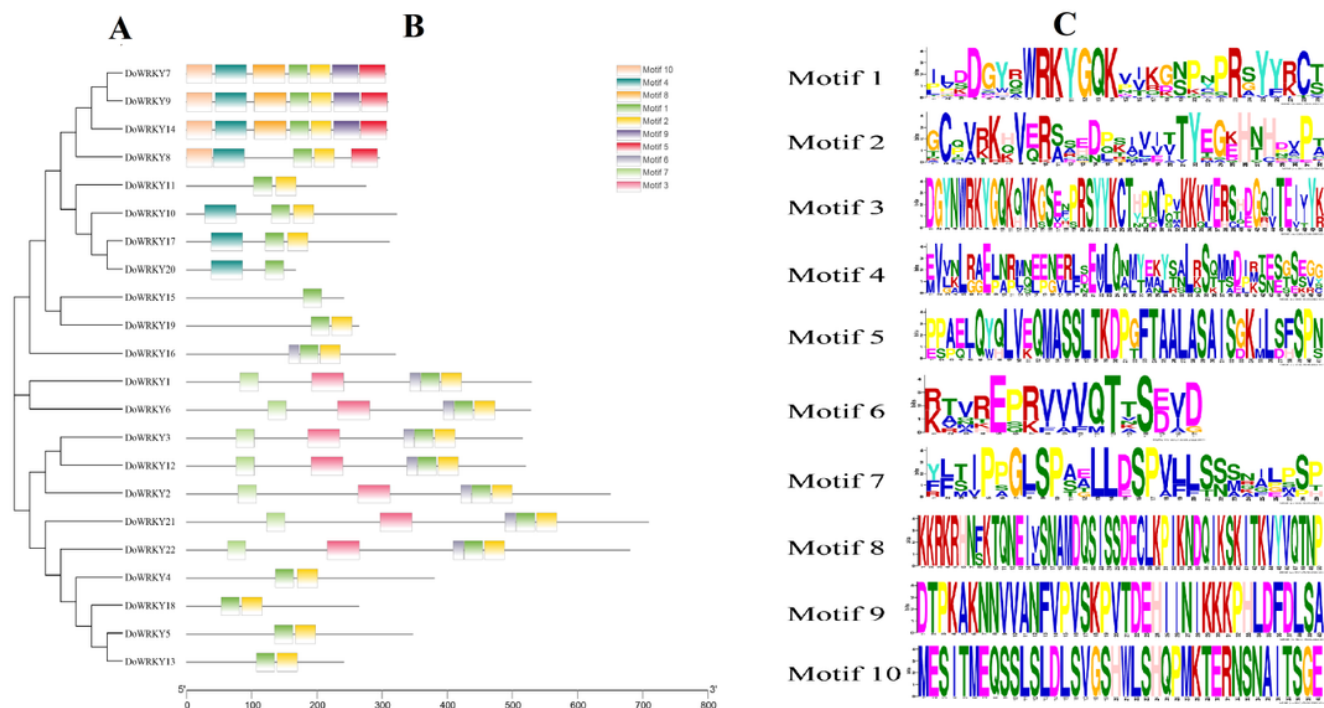


Figure 3

Phylogenetic tree and conserved motifs in DoWRKYs. A: Phylogenetic relationships. B: Conserved motifs of the DoWRKYs. Each motif is represented by a number in colored box. C: Motif model.

Different stages

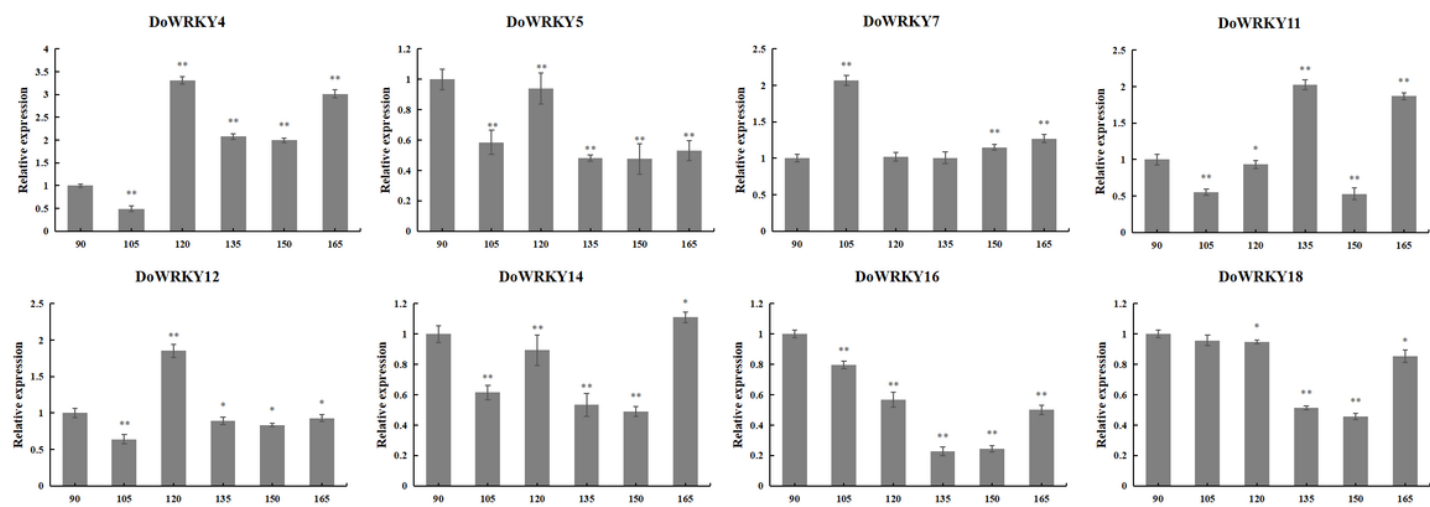


Figure 4

Expression analysis of the *WRKY* genes in *Dioscorea spp.*. Data are presented as mean $\pm$ SD ($n = 3$). ** indicate a significant difference at $P < 0.05$ and $P < 0.01$ compared with WT, respectively.

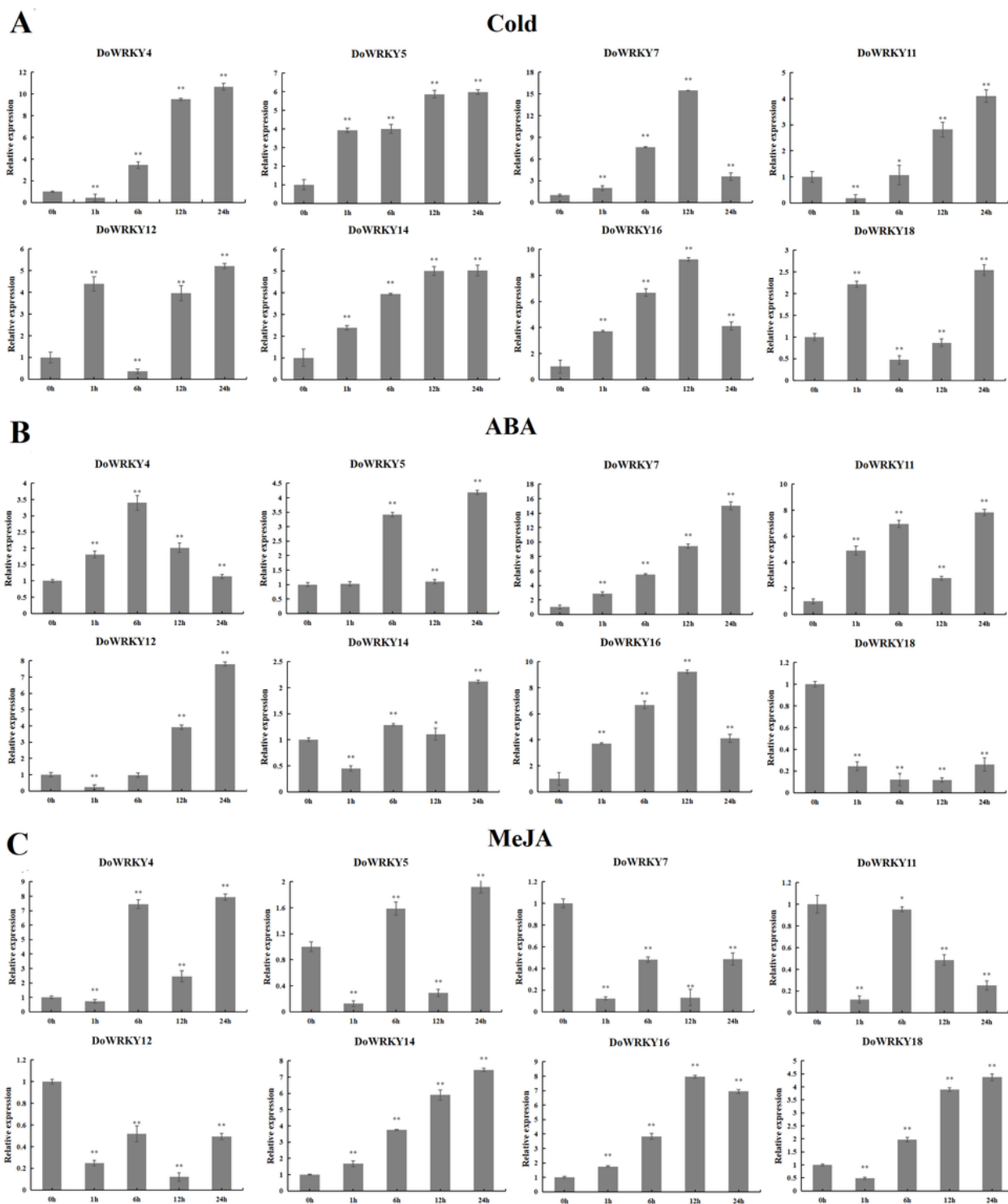


Figure 5

qRT-PCR of *WRKY* genes in cold, ABA, MeJA treatment. Data are presented as mean $\pm$ SD ($n = 3$). ** indicate a significant difference at $P < 0.05$ and $P < 0.01$ compared with WT, respectively.

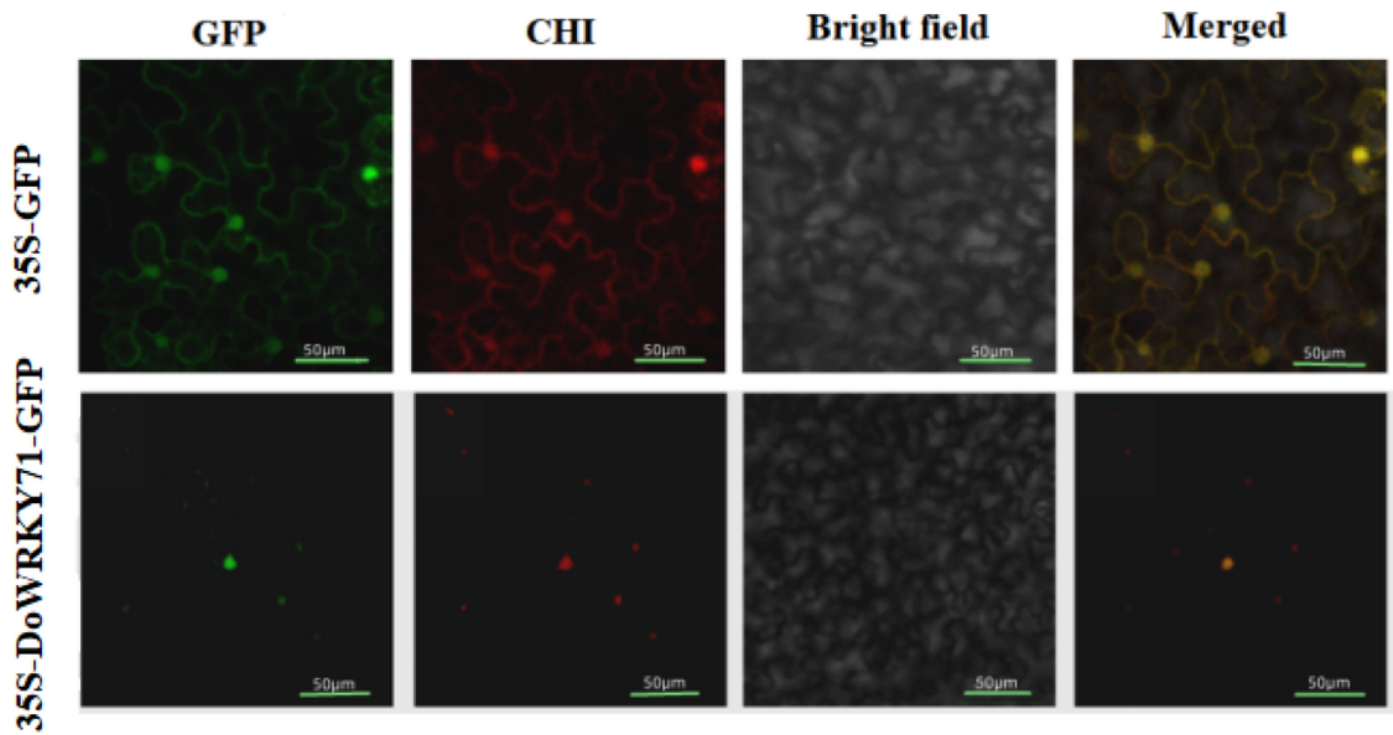


Figure 6

DoWRKY71 subcellular localization. The excitation wavelength of confocal laser microscope was set as: GFP 488nm; Chloroplast 561nm; Bright field:DIC; Merge: superimposed field; Bar=50 µm.

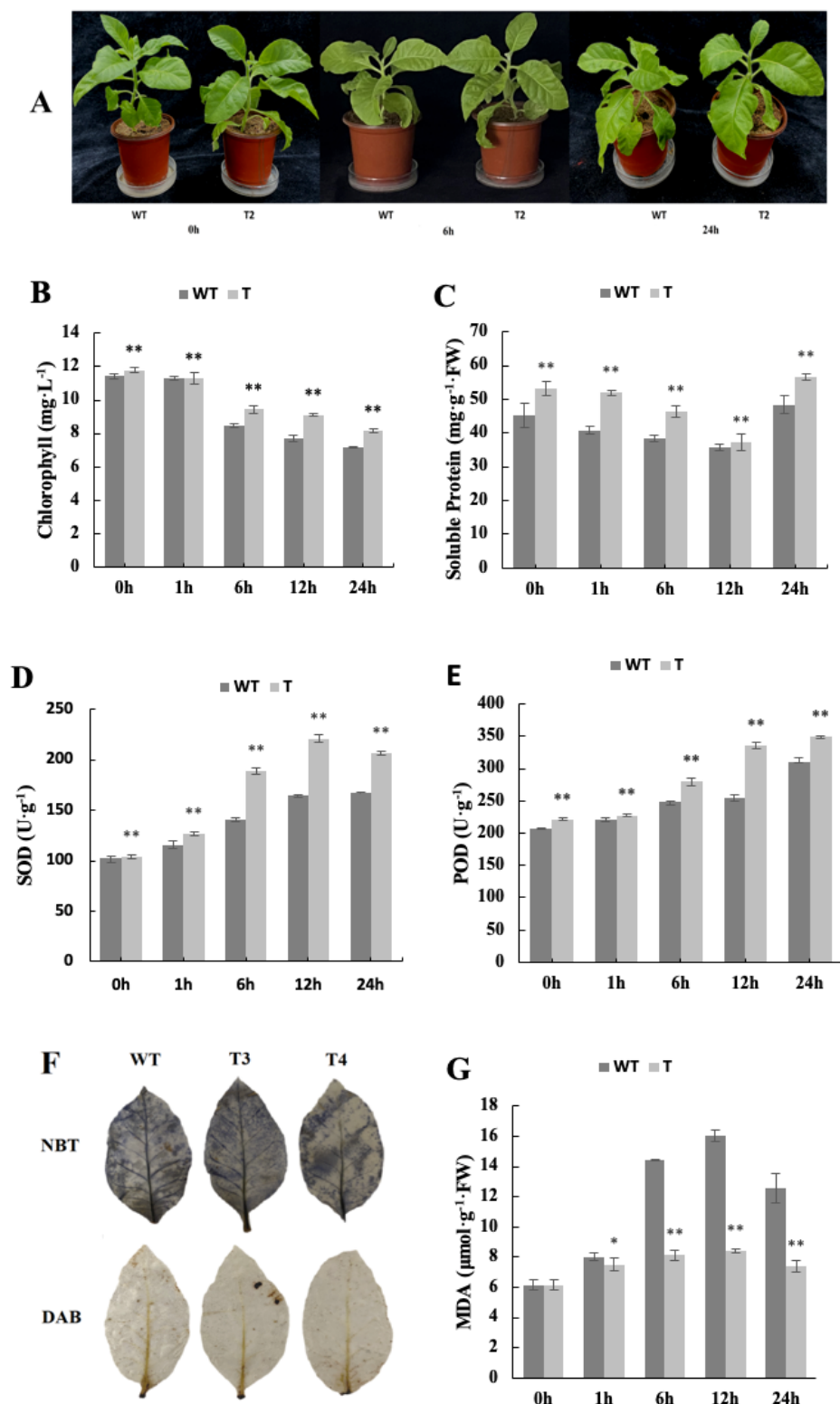


Figure 7

Growth, physiological and biochemical indexes of overexpressing *DoWRKY71* under 4°C low temperature in tobacco. A: 0h:(CK), 6 h: 6 h after treatment, 24h :24h after treatment. WT: wild-type tobacco; T2: transgenic tobacco. T3: transgenic tobacco. T4: transgenic tobacco. B-E: Chlorophyll, Soluble protein, SOD, POD. F:NBT and DAB dye. G: MDA. Data are presented as mean $\pm$ SD (n = 3). ** indicate a significant difference at $P < 0.05$ and $P < 0.01$ compared with WT, respectively.

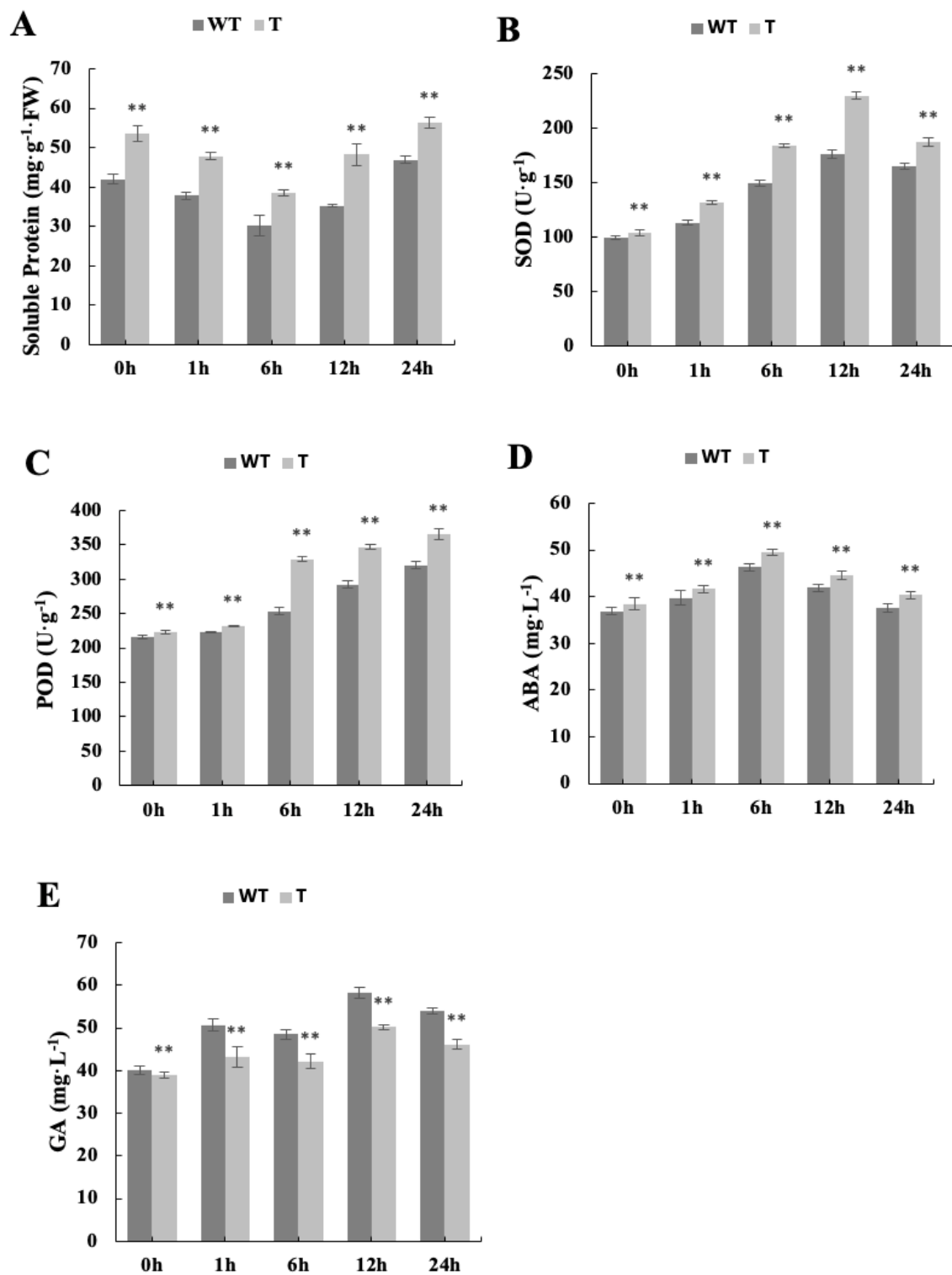


Figure 8

Changes of physiological and biochemical indexes endogenous and hormone content under ABA treatment. A: Soluble protein. B: SOD. C: POD. D: ABA content. E: GA content. Data are presented as mean $\pm$ SD (n = 3). ** indicate a significant difference at $P < 0.05$ and $P < 0.01$ compared with WT, respectively.

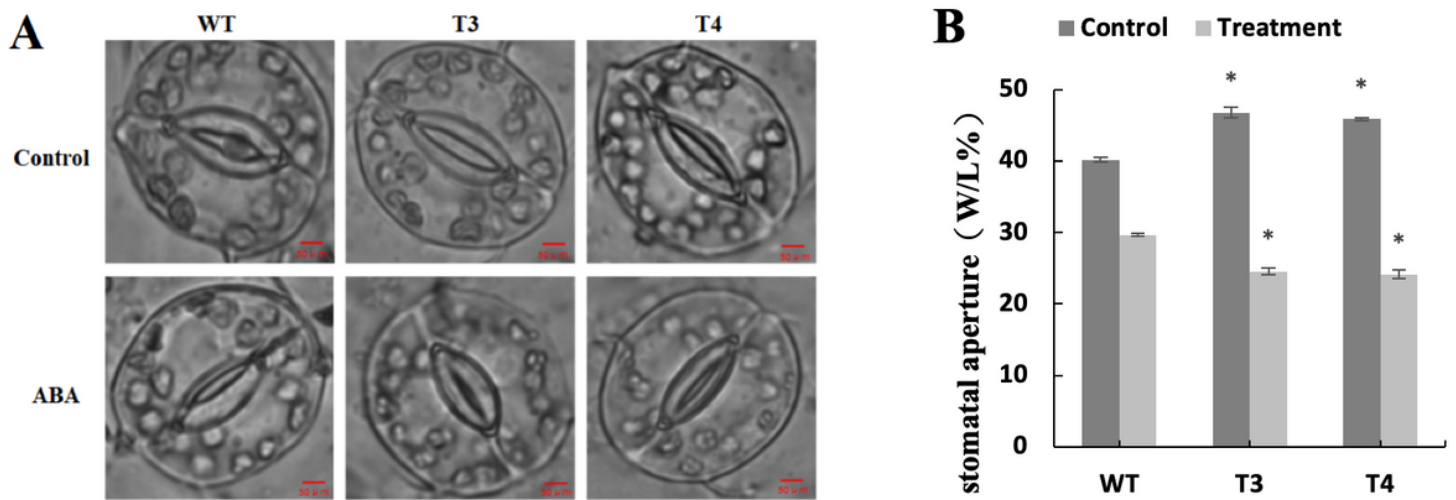


Figure 9

stomatal aperture of WT and *DoWRKY71* transgenic lines under ABA treatment. Data are presented as mean $\pm$ SD ($n = 3$). ** indicate a significant difference at $P < 0.05$ and $P < 0.01$ compared with WT, respectively.

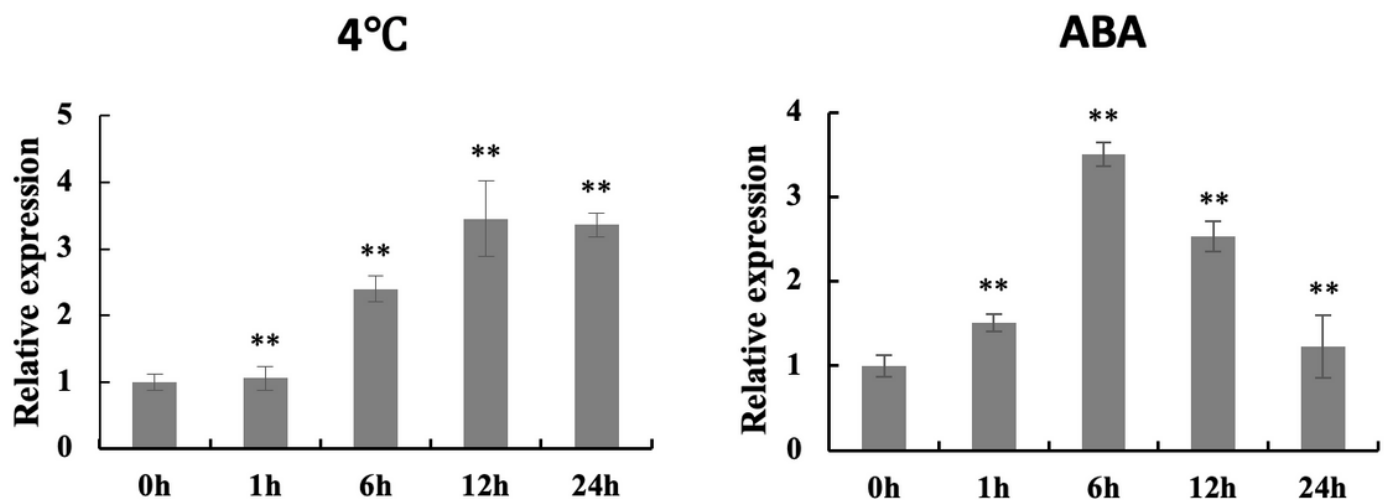


Figure 10

The relative expression of *DoWRKY71* gene in tobacco leaves under 4°C and ABA treatments. Data are presented as mean $\pm$ SD ($n = 3$). ** indicate a significant difference at $P < 0.05$ and $P < 0.01$ compared with WT, respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.png](#)
- [TableS1RelatedprimersequencesofqRTPCR.xlsx](#)
- [TableS2TheWRKYproteinwithDioscoreaspp.andOryza.sequencesforphylogeneticanalysis..xlsx](#)
- [TableS3.TheexpressionsoftheWRKYgeneinunderdifferentgrowthanddevelopmentstages..xlsx](#)
- [TableS4.TheexpressionsoftheWRKYgeneresponsestothecoldABAandJA treatment.xlsx](#)
- [TableS5.Allphysiologicalindexesweretreatedat4.xlsx](#)
- [TableS6.AllphysiologicalindexesweretreatedatABA..xlsx](#)
- [TableS7.The relative expression of DoWRKY71 gene in tobacco under different treatments..xlsx](#)