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Function Analysis of Drought Resistance Related Gene *TaGAPCs* and *TaWRKYs* in Wheat

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Abstract

Backgrounds: Wheat (*Triticum aestivum* L.) is one of the most important food crops in the world. It faces various abiotic stresses during its growth. Drought is one of the main factors limiting the growth and development of wheat. Severe drought stress will Lead to a decline in wheat production. Cytoplasmic glyceraldehyde-3-phosphate dehydrogenase (*GAPC*) is an important member of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) family, which is widely present in plant cytoplasm. Plants play an important role in the process of primary metabolism and stress resistance.

Result: In this study, a comparative transcriptomic analysis of the *TaGAPCs*-RNAi strain of Changwu 134 and the wild-type wheat seedlings of Changwu 134 under natural drought conditions was carried out. A total of 30067 differentially expressed genes were screened in RNAi strains and wild-type strains, of which 19,959 genes were up-regulated in RNAi strains and 10,108 genes were down-regulated in transcription. GO analysis shows that differential genes are mainly enriched in biological regulation, cellular processes, metabolic processes, and responses to stimuli. KEGG analysis showed that the differential genes were mainly concentrated in the biosynthesis of phenylpropane, plant hormone signal transduction and flavonoid biosynthesis pathways. By analyzing the expression levels of differential transcription factors, the significantly down-regulated transcription factor WRKY family member *TaWRKY2 / 22/28/29/33/40/47/52* in wheat was screened out. The *TaWRKY28/33/40/47* gene silencing line was successfully obtained using the barley stripe mosaic virus (BSMV-VIGS) technology. The plants with *TaWRKY28/33/40/47* gene silenced were subjected to natural drought treatment, and physiological and biochemical index tests were carried out. The results showed that the growth status of gene-silenced plants was worse than that of wild-type plants, and the relative water content and chlorophyll content decreased. The content of MDA, H_2O_2 and superoxide anion increases, the activity of antioxidant enzymes (SOD, POD, CAT) decreases, and the content of proline decreases.

Conclusion: The results showed that *TaGAPCs* regulates the expression of some *TaWRKYs* transcription factors, activates antioxidant pathways, enhances tolerance of wheat to drought stress.

Background

Wheat (*Triticum aestivum* L.) is one of the most important gramineous food crops. It faces a variety of abiotic stresses during the growth process, including drought, waterlogging, cold, high temperature, salinity, etc. Drought is the limit one of the main factors of wheat growth and development [1]. The growth and development of wheat include jointing, heading, flowering and filling. Drought stress during the flowering period will cause the flowers to fall off, which will eventually lead to a decline in wheat yield [2]. There are currently about 7.5 billion people in the world, and it is expected that the world population will increase to 9.6 billion by the middle of this century. With the increasing population, the demand for food production and quality is also increasing [3]. In order to meet people the growing demand for food, it is a very difficult task for wheat researchers to increase food production by cultivating drought-resistant wheat varieties [4].

In 1994, S Ishiguro and K Nakamura [5] cloned the first WRKY gene (*SPF1*) from sweet potato, and now people have successfully identified a large number of WRKY genes from a variety of plants, including Ishiguro [5], Corn [6], Arabidopsis [7], Rice [8], Soybean [9], Cotton [10], Wheat [11], Tobacco [12], etc. WRKY transcription factors (WRKY TFs) play an important role in the response of plants to abiotic stress. WRKY can participate in the stress resistance of plants by binding to the specific W-box on the promoter of the target gene to regulate the expression of the target gene. For example, Arabidopsis *WRKY6* specifically binds to the W-box on the *RAV1* promoter, *RAV1* decreased expression level promotes seed germination and early seedling development [13]. Arabidopsis *TTG2* (a WRKY transcription factor) is involved in the regulation of *GL2* transcription during epidermal cell differentiation [14]. After drought and salt stress, *IbWRKY2* in sweet potatoes can up-regulate the expression of *IbVQ4* and improve the abiotic stress tolerance [15]. The interaction between *AtWRKY8* and *AtVQ10* increases the resistance of Arabidopsis plants to gray mold. The interaction between *AtWRKY8* and *AtVQ9* plays an important role in the drought tolerance of Arabidopsis. The interaction between *AtVQ16* and *AtVQ23* and *AtWRKY33* positively regulates the defense of plants against adversity [16–18].

Studies have shown that some members of the wheat transcription factor *TaWRKY1/2/19/33/46/93* are overexpressed in Arabidopsis, significantly increasing the resistance of transgenic lines to abiotic stresses (drought, osmosis, salt and heat) [19–22]. The detached leaves of *TaWRKY40-D* VIGS (virus-induced gene silencing) wheat plants showed a green phenotype, while the Arabidopsis plants overexpressing *TaWRKY40-D* showed premature leaf senescence after JA and ABA treatment. Therefore, *TaWRKY40-D* may positively regulate leaf senescence by changing the biosynthesis and signal transduction of JA and ABA pathway genes [23]. The Arabidopsis strains overexpressing the *TaWRKY142* gene are significantly more resistant to the fungal pathogen Colletotrichum, and this increased resistance is due to the increased expression of the JA signal marker gene *AtPDF1.2* [24]. *TaWRKY51* controls the formation of lateral roots by regulating ethylene biosynthesis [25]. In summary, the WRKY transcription factor in wheat is involved in a variety of biological processes in plants.

The gene silencing induced by barley stripe mosaic virus (BSMV) was determined to be the first system to induce VIGS in monocots [26]. BSMV is a positive RNA virus. its genome consists of three RNA strands: *α*, *β*, and *γ*. The RNA *α* chain encodes the virus's replicase large subunit αa, and the RNA *β* chain encodes the coat protein and the tripartite movement protein TGB1, TGB2 and TGB3, RNA *γ* chain encodes replicase *γ*a and multifunctional protein *γ*b [26, 27]. By constructing multiple cloning sites downstream of the *γ*b gene of the *γ* chain, the foreign gene fragments can be inserted successfully. A BSMV viral vector for silencing the target gene was constructed [28]. BSMV-VIGS technology can be used to study the function of genes in plant growth, development and biotic and abiotic stresses. Zhang and colleague showed that wheat plants infected with BSMV-VIGS/ *TaNAC35* have an effect on wheat rust (Puccinia Triticina Pt) increased resistance to the pathogen *THTT*, indicating that *TaNAC35* can act as a transcriptional activator and negatively regulate wheat Pt resistance [29]. The R2R3 MYB transcription factor *TaMpc1-D4* located on the D chromosome of wheat was used to silence *TaMpc1-D4* in wheat using VIGS technology, the results showed that the relative water content (RWC), proline content, and proline content of the *TaMpc1-D4* gene silenced wheat were compared with the wild type the activity of

antioxidant enzymes is significantly increased, which verifies that *TaMpc1-D4* plays a negative regulatory role in wheat drought stress [30]. Li and colleague used the barley stripe mosaic virus-induced silencing (BSMV-VIGS) method to silence the calcium-dependent protein kinase 34 (*TaCPK34*) gene in the wheat genome, after 14 days of drought stress, compared with the wild type, the *TaCPK34* gene was silenced seedlings have significantly reduced biomass and relative water content, and increased soluble sugar and MDA content [31]. In addition, BSMV-VIGS was used to verify the barley S-adenosylmethionine synthase 3 gene (*HvSAMS3*) actively regulates the tolerance of wild barley (Tibetan wild barley) to drought and salt stress [32]. Using BSMV-VIGS technology to silence *TabZIP74*, *TabZIP74* silenced wheat plants have fewer lateral roots than control wheat seedlings, indicating that *TabZIP74* is involved in the development of wheat roots [33].

The previous research of our research group found that *TaGAPCs*-RNAi strains have reduced drought tolerance, while the transgenic Arabidopsis overexpressed *TaGAPC5* has significantly enhanced drought resistance. In order to further study the biological functions of wheat *GAPC*, the *TaGAPCs*-RNAi mutant strain of Chang Wu 134 and the wild-type strain of Chang Wu 134 were sequenced by transcriptome. The WRKY family member *TaWRKY28/33/40/47*, a member of the WRKY family that significantly down-regulates transcription factors, was screened out in wheat. By studying the function of the drought-resistant transcription factor WRKY, further reveal the function of *TaGAPC*, and provide abundant genetic resources for subsequent transgenic breeding.

Results

GO and KEGG enrichment analysis of DEG in TaGAPCs-RNAi silencing strains and wild-type strains after drought stress

Through the analysis of transcriptome data, a total of 30067 gene expression differences were obtained. Compared with the wild type, RNAi strains significantly up-regulated 19,959 genes and down-regulated 10,108 genes (Fig. 1a).

The GO enrichment analysis analysis of the differentially expressed genes between the gene-silencing line and the wild-type after drought treatment showed that the differentially expressed genes are involved in molecular function, cellular component and biological process. There are 49 sub-class molecular functional classifications are involved under the three major categories. In molecular function, there are 9558 differential genes that are mainly enriched in binding function, and there are 9053 differential genes in catalytic activity function. In terms of cell components, there are 6601 differential genes mainly enriched in the cell composition, and 7133 differential genes in the cell membrane. In the biological process, there are 2210 differential genes in the biological regulation process, 5155 differential genes in the cellular process, and 4529 differential genes in the metabolic process. There are 1973 different genes in response to stimulus (Fig. 1b).

The KEGG analysis of differential genes showed that 12877 differential genes were annotated to 134 metabolic pathways. The differential genes were mainly enriched in drought-related pathways such as

phenylpropane biosynthesis (1450 differential genes) and plant hormone signal transduction (823), flavonoid biosynthesis (329), circadian rhythm (207), α-linolenic acid metabolism (146), carotenoid biosynthesis (127), arachidonic acid metabolism (92), isoflavone biosynthesis (80), Flavonoids and flavonol biosynthesis (78), zeatin biosynthesis (74). (Fig. 1c).

Analysis of differentially expressed transcription factors

After comparing the effective read sequence obtained by sequencing with the reference genome, the gene families encoding transcription factors were classified and counted. A total of 5,631 transcription factors were obtained, which were divided into 34 categories. Among them, the transcription factor families with a large number of genes are MYB, ABI3VP1, AP2 -EREBP, WRKY, etc. Through the analysis of differential transcription factor families, 8 transcription factor families related to drought stress were screened, and the number of differential genes, differential gene expression and the classification of the main KEGG pathways were analyzed. As the research team selected some wheat WRKY transcription factors through yeast one-hybridization in the early stage, this experiment analyzed the *TaWRKY* family of differential transcription factors of *TaGAPC*-RNAi strains, and the results showed that the number of down-regulated genes in the *TaWRKY* transcription factor family is the number of up-regulated genes. After comparing and analyzing the genes with significant differences in down-regulation, the wheat *TaWRKY2/22/28/29/33/40/47/52* genes were screened for significant down-regulation (Table 1). These genes will be important candidates for subsequent experimental verification.

Table 1
Expression analysis of *TaWRKY* transcription factors with significant differences in transcriptome sequencing

□□ID Gene ID	□□□□FPKM RNAi FPKM	□□FPKM WT FPKM	log2(RNAi/WT)	□□□□ Gene name
TraesCS5B02G399900	2.71	5.18	-0.89	*TaWRKY2*
TraesCS2A02G433000	76.21	106.44	-0.46	*TaWRKY22*
TraesCS3D02G281900	0.31	11.12	-5.14	*TaWRKY28*
TraesCS4B02G069500	5.32	9.87	-0.88	*TaWRKY29*
TraesCS6D02G136200	21.01	167.67	-2.98	*TaWRKY33*
TraesCS5A02G225600	2.431	29.32	-5.02	*TaWRKY40*
TraesCS3D02G238300	1.623	42.956	-4.69	*TaWRKY47*
TraesCS1A02G410700	1.49	6.94	-2.19	*TaWRKY52*

Quantitative real-time PCR confirms gene expression profiles

In order to verify the accuracy of the RNA-Seq results, 15 potentially important functions were randomly selected for qPCR analysis. The fold change data of RNA-Seq and qRT-PCR after 15 days of drought stress showed that among the 15 tested genes, the trend of drought-induced transcript accumulation changes was same (Fig. 2).

BSMV-mediated TaWRKY28/33/40/47 **gene silencing decreases resistance to drought in wheat**

VIGS vector BSMV:*PDS*andBSMV: *TaWRKYs* were constructed as Fig. 3a ,linearize the recombinant vector. In vitro synthesis of RNA in linear plasmids (Fig. 3b). The bleaching and virus symptoms were observed after the wheat seedlings were inoculated for 10 days. The wheat leaves inoculated with Fes buffer (MOCK) remained green, and the leaves inoculated with BSMV:*PDS* virus appeared bleached symptom, while the leaves inoculated with BSMV:*γ*, BSMV: *TaWRKY28/33/40/47* virus showed slight stripe chlorotic virus symptoms. It indicated that the VIGS system was successful (Fig. 3c). Through qPCR experiment, the gene expression of *TaWRKY28/33/40/47* was lower in the four silenced lines than in the Mock and BSMV:*γ* lines (Fig. 3d). After 15 days of drought treatment, the wheat leaves were severely withered in *TaWRKY28/33/40/47*-silenced lines, while the leaves grew well and upright in the Mock and BSMV:*γ* lines (Supplementary Fig. S1).

TaWRKY28/33/40/47 **reduced drought tolerance by regulating the physiological responses and stress-related genes in gene silencing wheat lines**

Compared with the Mock and BSMV:*γ* lines, the Chlorophyll content and RWC of *TaWRKY28/33/40/47*-silenced lines was significantly decreased under drought stress (Fig.4a-b). Compared with the Mock and BSMV:*γ*, the content of MDA was increased by about 94.3%,155.4%,196.4%and184.2% in genes-silenced lines after drought treatment (Fig.4c). Compared with the Mock and BSMV:*γ*, the content of H_2O_2 was increased by about 21.2%,54.8%,28.1% and 29.7% in genes-silenced lines after drought treatment (Fig.4d). The *TaWRKY28/33/40/47*-silenced lines exhibited more higher O_2^- content after drought treatment as compared to the Mock and BSMV:*γ*(Fig.4e-f). The content of proline and the antioxidant enzyme system (POD, SOD, and CAT enzymes) were significantly decreased in three transgenic lines after drought treatment as compared to the Mock and BSMV:*γ* (Supplementary Fig. S2).

Discussion

Through the enrichment analysis of the differential gene GO, the differentially expressed genes of wheat after drought stress mainly perform molecular functions of binding and catalytic activity. Some differentially expressed genes are involved in the composition of cell membranes, organelles, and cells, and are involved in the process of cell metabolism and response to stimuli. Through the analysis of the enriched GO entries, the results show that drought stress induces the combination and catalysis of certain substances in the plant body, and responds to drought stress by protecting the organelles and cell membranes to maintain the integrity of the cell structure, maintain the normal function of the cell, and reduce The damage to the plant body maintains the normal physiological activity of the plant body by

regulating the cell process and metabolic process to improve the adaptability of wheat to drought stress [34, 35].

KEGG significant enrichment analysis showed that differentially expressed genes are involved in phenylpropane biosynthesis, flavonoid biosynthesis, flavonoid and flavonol biosynthesis, plant hormone signal transduction, carotenoid biosynthesis, brassinosteroid biosynthesis, and zeatin biosynthesis and anthocyanin biosynthesis. Studies have shown that the secondary metabolites of most plants are derived from the phenylpropane metabolic pathway. Phenylpropane compounds play an important role in the growth and development of plants and in response to adversity stress. Studies have shown that 4-Coumarate-CoA ligase (4CL) is a key enzyme in the phenylpropane synthesis pathway. Overexpression of 4CL in Fraxinus mandshurica can enhance the drought stress tolerance of plants [36]. When plants are under drought stress, the ROS content in their bodies increases, and flavonoids can be used as active oxygen scavengers. The production of flavonoids can effectively improve the ability of plants to resist external abiotic stresses. Increased accumulation of flavonols enhances the tolerance of plants to abiotic stresses. Flavonol synthase plays an important role in flavonoid biosynthesis [37]. Dendrobium officinale flavonol synthase is overexpressed in Arabidopsis. *DoFLS1* enhanced flavonol accumulation and abiotic stress tolerance [38]. Transcriptome sequencing results in this experiment showed that 4CL and flavonol synthase were significantly down-regulated in TaGAPCs silenced lines. TaGAPCs silencing in wheat may reduce phenylpropane biosynthesis and flavonoid biosynthesis by regulating 4CL and flavonol synthase, Which reduces the drought resistance of silent strains.

Transcription factor is the main regulator of transcription reprogramming. The expression of many TF genes is affected by drought, including auxin-responsive transcription factor (ARF), zinc finger protein transcription factor (C_2H_2), GRAS, LOB, MYB Transcription factors such as, NAC, TCP, WRKY, and bZIP, which participate in plant response to drought stress by regulating downstream genes. In this experiment, transcription factors that respond to drought stress have up-regulated expression in *TaGAPCs*-RNAi strains. There are also down-regulated transcription factors. Through the analysis of transcriptome data, the number of down-regulated WRKY transcription factor family is 2.67 times the up-regulated number. Some significantly different WRKY transcription factors have been screened out. Studies have shown that the overexpression of *SlWRKY8* in tomato is enhanced. In view of its tolerance to drought stress [39], *WRKY46*, *WRKY54* and *WRKY70* in Arabidopsis play a negative regulatory role in drought response [40]. The WRKY transcription factor was studied to further reveal the molecular mechanism of *TaGAPCs* in response to drought.

In this experiment, we successfully constructed the BSMV:TaWRKY28/33/40/47 silencing vector, and inoculated the recombinant vector in vitro transcription product into the two-leaf and one-heart stage Chinese spring wheat seedlings. The phenotype of the plants after the inoculation was analyzed and verified by real-time fluorescent quantitative PCR. Indicating that the *TaWRKY28/33/40/47* gene silencing line was successfully obtained.

After drought stress, it will affect the growth and photosynthesis of plants. The water status of plant tissues has a certain relationship with their physiological functions. The relative water content (RWC) index can reflect the characteristics of changes in plant water status. The reduction of chlorophyll content under drought stress is also considered a typical symptom of oxidative stress, which may be the result of pigment photooxidation and chlorophyll degradation. The loss of chlorophyll seriously affects the photosynthetic rate of plants [41]. In this experiment, the relative water content and chlorophyll content of the *TaWRKY28/33/40/47* gene-silencing line were lower than the control MOCK after drought stress. The results showed that the water retention capacity of the wheat leaves of the *TaWRKY28/33/40/47* gene-silencing line was weak and Photosynthesis is also affected. MDA is the final breakdown product of lipid peroxidation, which can reflect damage to plant membrane system and plant resistance [42]. The results of this experiment showed that the VIGS gene silencing line accumulated more MDA than the control MOCK line under drought treatment, indicating that *TaWRKY28/33/40/47* gene silencing promoted plant lipid oxidation. Drought may lead to the accumulation of reactive oxygen species (ROS) and the damage related to ROS. This experiment detects that the *TaWRKY28/33/40/47* gene-silencing line after drought stress accumulates more superoxide anions and hydrogen peroxide than MOCK Excessive superoxide anion will affect DNA replication and protein synthesis, and also damage the membrane system that maintains cell morphology. Excessive production of H_2O_2 can cause leaf cell death. In order to combat reactive oxygen damage, antioxidant enzymes are widely present in plants Systems, such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) are ROS scavenging enzymes, which can further convert H_2O_2 into H_2O, etc., and play an important role in regulating the content of H_2O_2 in cells Role [43, 44]. In addition, proline is one of the main osmotic adjustment substances in plants. When plants are under drought stress, it can remove excess active oxygen accumulated in plants due to drought, and has a protective effect on the integrity of cell membranes [45]. This experimental study shows that when subjected to drought stress, the antioxidant enzyme activity in plants will have an upward process. When subjected to drought stress, the *TaWRKY28/33/40/47* gene-silencing line showed lower CAT SOD, POD activity than MOCK plants. The antioxidant enzyme activity of *TaWRKY28/33/40/47* gene-silencing lines is lower than MOCK, which may reflect the stronger oxidative damage of gene-silencing lines under drought stress, but the weaker ability of scavenging reactive oxygen species. These results indicate that silencing *TaWRKY28/33/40/47* affects the antioxidant system, and *TaWRKY28/33/40/47* may be involved in the ROS-mediated wheat response to drought signaling pathway.

Methods

plant materials

The wild-type strain of Chang Wu 134 wheat is a resistant to drought and Chang Wu 134 *TaGAPCs*-RNAi mutant strain is a susceptible variety to drought, which is a 333-bp conserved *TaGAPC* fragment was amplified with specific primers to construct the wheat *GAPC* RNA interference (RNAi) vector. The amplified PCR product was purified and recombined in antisense and sense orientations to flank the 500-bp rice Adh gene intron of PTCK303, forming the *TaGAPCs*-RNAi vector. Using particle bombardment

technology to genetically transform the *TaGAPCs*-RNAi vector into Changwu 134 wheat and obtain a stable genetic transgenic line. The wheat Changwu 134 *TaGAPCs*-RNAi strain and Changwu 134 wild-type seeds were planted, and the wheat seedlings were grown to the two-leaf and one-heart stage and subjected to natural drought stress. On the 15th day, wheat leaf tissues were taken as experimental samples. The Changwu 134 *TaGAPCs*-RNAi strain and the Changwu 134 wheat RNAi group and WT group were each set up with 3 biological replicates, and the collected fresh samples were quick-frozen with liquid nitrogen and placed in a refrigerator at -80°C for later use.

RNA extraction and preparation of the cDNA library

Total RNA was extracted using TRIzol from wheat seedlings and converted to cDNA using the PrimeScript™ RT reagent kit (TaKaRa, Japan). The preparation of the cDNA library was completed by Huada Biological Company using the BGISEQ-500 platform and machine sequencing with the HiSeq 4000 sequencing system.

RNA-seq analysis of wheat leaves after drought

The raw reads obtained from Trimmomatic were filtered after obtaining clean reads. Clean data (clean reads) were obtained by removing reads containing adapters, reads containing poly-N and low-quality reads from the raw data. Thereafter, clean sequence reads were mapped to the available wheat genome. We used HISAT (Hierarchical Indexing for Spliced Alignment of Transcripts) to align the clean reads to the reference genome sequence.

Gene ontology (GO) and KEGG analysis of DEGs

As described by Anders and Huber, the DESeq R (1.18.0) package was used to analyse the differential expression between the treated and control transcripts. DEGs were defined as genes that had absolute values of $\log_2$-fold change (Log_2FC) ≥ 2 or $\text{Log}_2\text{FC} \leq -2$ and a false discovery rate (FDR) ≤ 0.05. According to the GO and KEGG annotation results and the official classification, we functionally classified the DEGs and used the phyper function in R software for enrichment analysis. FDR correction was then performed on the p values. The functions with Q value ≤ 0.05 were considered significantly enriched.

Validation of RNA-Seq results by qRT-PCR

To verify the accuracy of the RNA-Seq results, 15 DEGs with potentially important functions were randomly selected from drought for 15d for qPCR and RNA-SEq. cDNA was synthesized by reverse transcription. QPCR primers were designed using Primer5 software, and primer specificity was evaluated by blasting primer sequences against the NCBI database (Supplementary Table S1). Three technical replicates for each of three biological replicates were performed.

BSMV-mediated TaWRKY28/33/40/47 gene silencing in wheat

Plasmids (*α*, *β*, *γ*, and *γ*-PDS) used for VIGS system were constructed according to [46]. Four segments (196 bp,187bp,187 bp and 186 bp) of *TaWRKY28/33/40/47* were cloned using the primers with the restriction enzymes *Pac*I and *Not*I (Supplementary Table S2). The wheat phytoene desaturase (*PDS*) gene was replaced with four specific *TaWRKY28/33/40/47* sequences. The RNA was synthesized in vitro from linearized plasmids following the instructions of RiboMAX™ Large Scale RNA Production System and Ribo m7G Cap Analog Kits (Promega, Madison, Wisconsin, USA).

The BSMV inoculum was combined 10 μL of *α*, *β* and four modified *γ* transcripts (BSMV:*γ*, BSMV:*PDS*, BSMV: *TaWRKY28*, BSMV: *TaWRKY33*, BSMV: *TaWRKY40* and BSMV: *TaWRKY47*, respectively) with 70 μL of Fes buffer (viral inoculation buffer). The viral inoculation solution was inoculated on the second leaves of 10-day-old wheat seedlings by the sliding friction with gloved fingers according to the method of [47]. Control inoculations (MOCK) were performed using Fes buffer. BSMV:*γ* and BSMV:*PDS* were used as negative and positive controls for BSMV infection, respectively. After inoculation, the wheat seedlings were cultured in a growth chamber set at 25℃ for 24 h in the dark, and then shifted to 16 h/8 h light/dark cycle at 25℃. About 10 days after virus inoculation, the symptoms were examined.

QRT-PCR analysis to assay silencing efficiency

For assays of silencing efficiency, total RNA was extracted from the leaves of silenced plants on the 14th day using Trizol reagent (TIANGEN), and cDNA synthesis was conducted using an M-MLV reverse transcriptase kit (Takara) according to the manufacturer's instructions. Real-time PCR reactions were performed in a total volume of 20 μL containing cDNA (100 ng μL^{-1}) 1.0 μL, 0.5 μL of each primer (10 mM), and 10 μL of BeyoFast™ SYBR Green qPCR Mix (2X). At the end of the reaction, the melting curve was observed to ensure that the product was specifically amplified. The relative expression level of the target gene was presented as fold change compared with the BSMV-*γ* using the $2^{-\Delta\Delta CT}$ method, three biological and three technical replicates were conducted in the transcript profiles of genes. Three independent experiments were conducted.

Physiological and biochemical tests of gene silencing plants under drought stress

The *TaWRKY28/33/40/47* gene silencing wheat plants were withheld water for 15 days. Drought symptoms of wheat were photographed after drought treatment. The fourth leaves of seedlings were sampled to do further experiments after drought treatment. The leaves were harvested to determine the contents of chlorophyll (Chl), MDA, H_2O_2, proline, and relative water content (RCW),. The activities of POD (EC 1.11.1.7), SOD (EC 1.15.1.1), CAT (EC 1.11.1.6) enzymes were also measured. The Chl content of the leaves was extracted in 96 % ethanol for 24 h until the green of the wheat leaves fades to colorless as described by Jia [48]. The content of MDA was assessed using the thiobarbituric acid method [49]. The H_2O_2 content was measured as described by Negi [50].The proline content was measured by the ninhydrin reaction method [51].To determine the O_2^- content according to the method of wang [52].The RCW detection method as described by Nauš [53].The activity of POD was estimated following the method of Huyskens-Keil [54]. The activity of SOD was examined by monitoring the inhibition of

photochemical reduction of NBT [55]. The activity of CAT was determined according to Sousa [56]. Three biological replicates were analyzed and three independent experiments were conducted.

Statistical analysis

The data were first to analyze using the Microsoft Office Excel 2019. The error bars represented standard error (SE). The analysis of the significance level was performed according to Duncan's method at $*P < 0.05$, and $**P < 0.01$, through the SPSS Statistics 20.0 software. The figures were generated using the Origin 9.0 and Adobe Photoshop software.

Declarations

Availability of data and materials

The dataset supporting the conclusions of this article is included within the article and its additional files. the plant material complies with relevant institutional, national, and international guidelines and legislation.

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Contributions

Q.Z.W. and L.Z.designed the experiments. Q.Z.W. performed the simulations and analyzed the corresponding results. Q.Z.W. and L.Z wrote the paper. S.S.Y. supervised this whole process and reviewed this paper.

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Additional information

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Supplemental Figure

Supplementary Figure S2 is not available with this version.

Figures

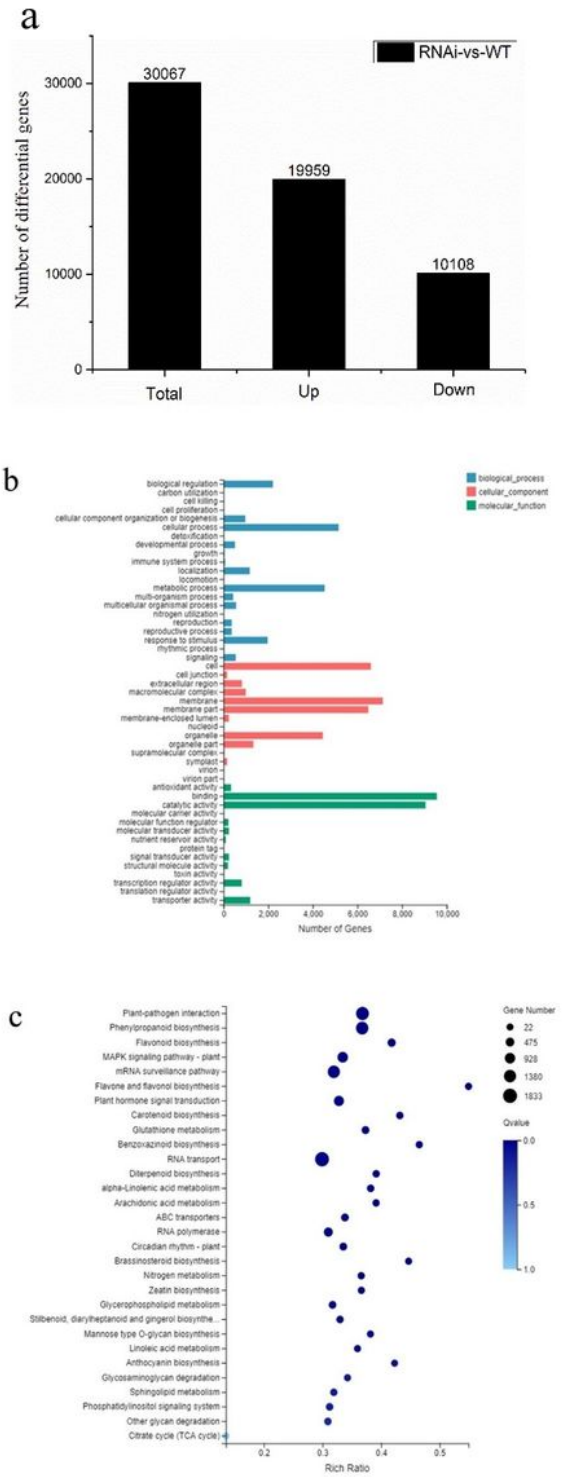

Figure 1

GO and KEGG enrichment analysis of DEG. a. differentially expressed gene statistics map. b. differential expression gene GO enrichment analysis. c. differentially expressed genes KEGG pathway enrichment analysis.

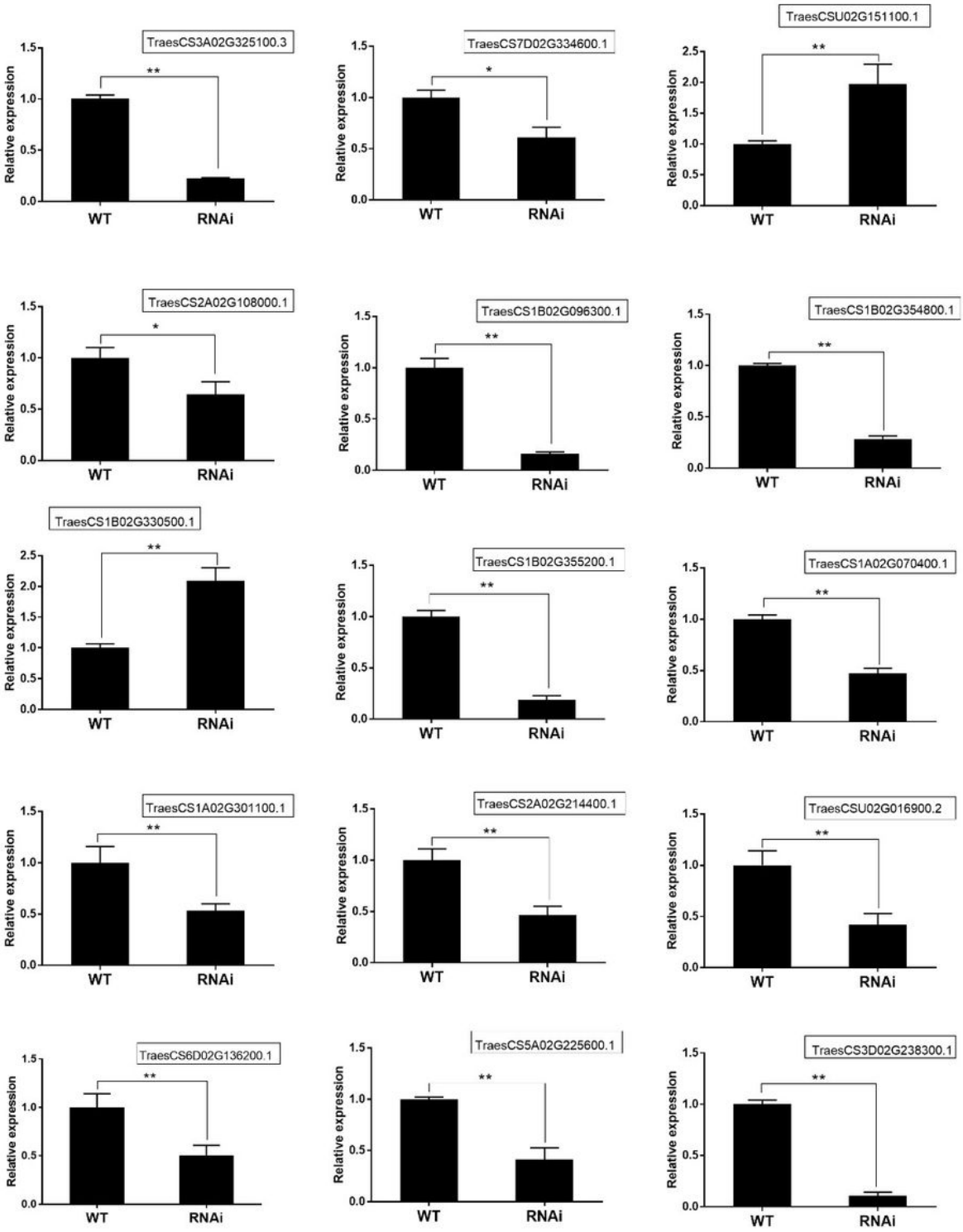

Figure 2

RT-qPCR verification of differential gene expression results of transcriptome sequencing.

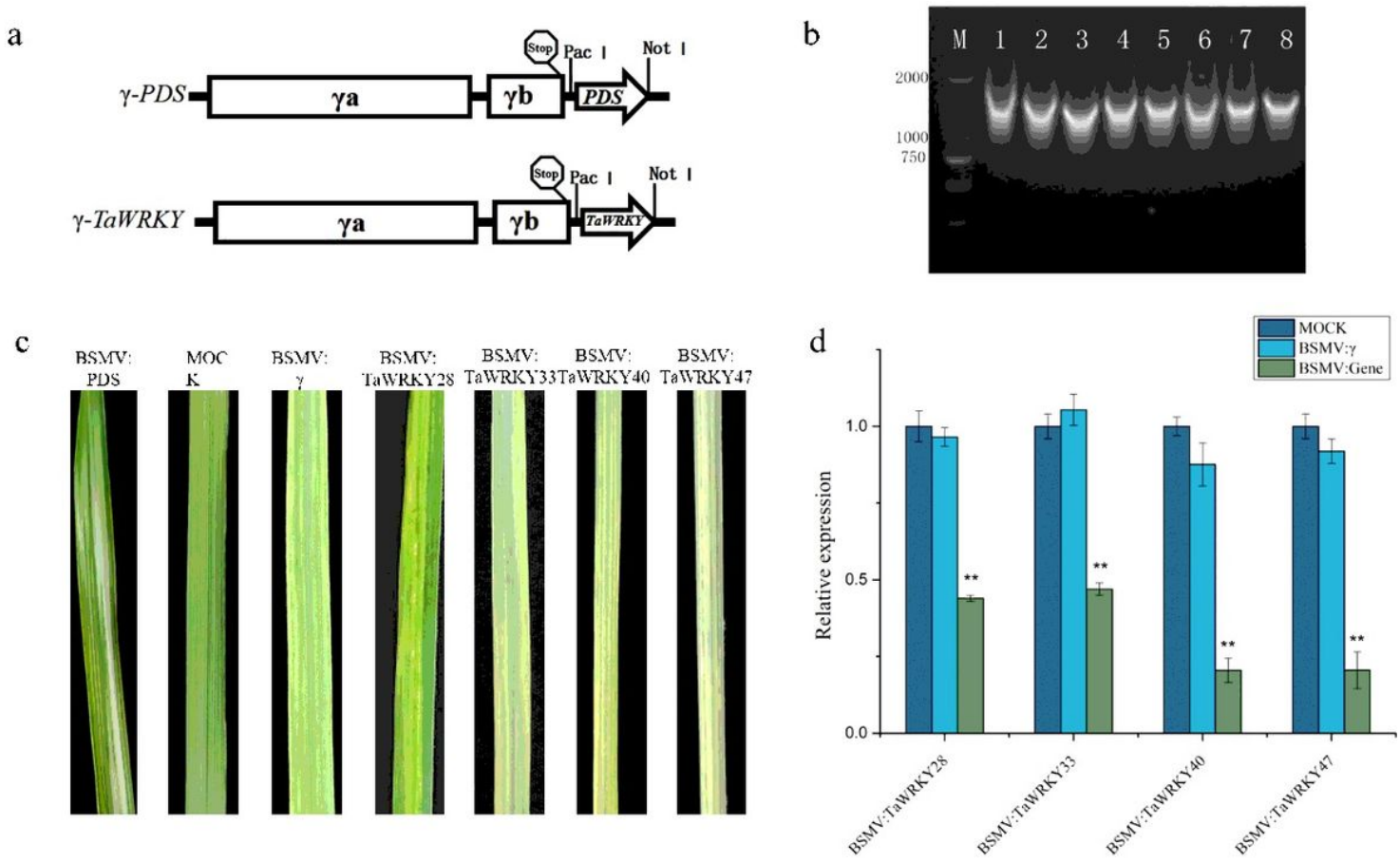

Figure 3

a Vector construction mode diagram. b in vitro transcription results of linearized viral plasmids. c phenotype of wheat leaves inoculated with BSMV virus. d silencing efficiency of wheat BSMV:TaWRKY28/33/40/47 gene silencing plants

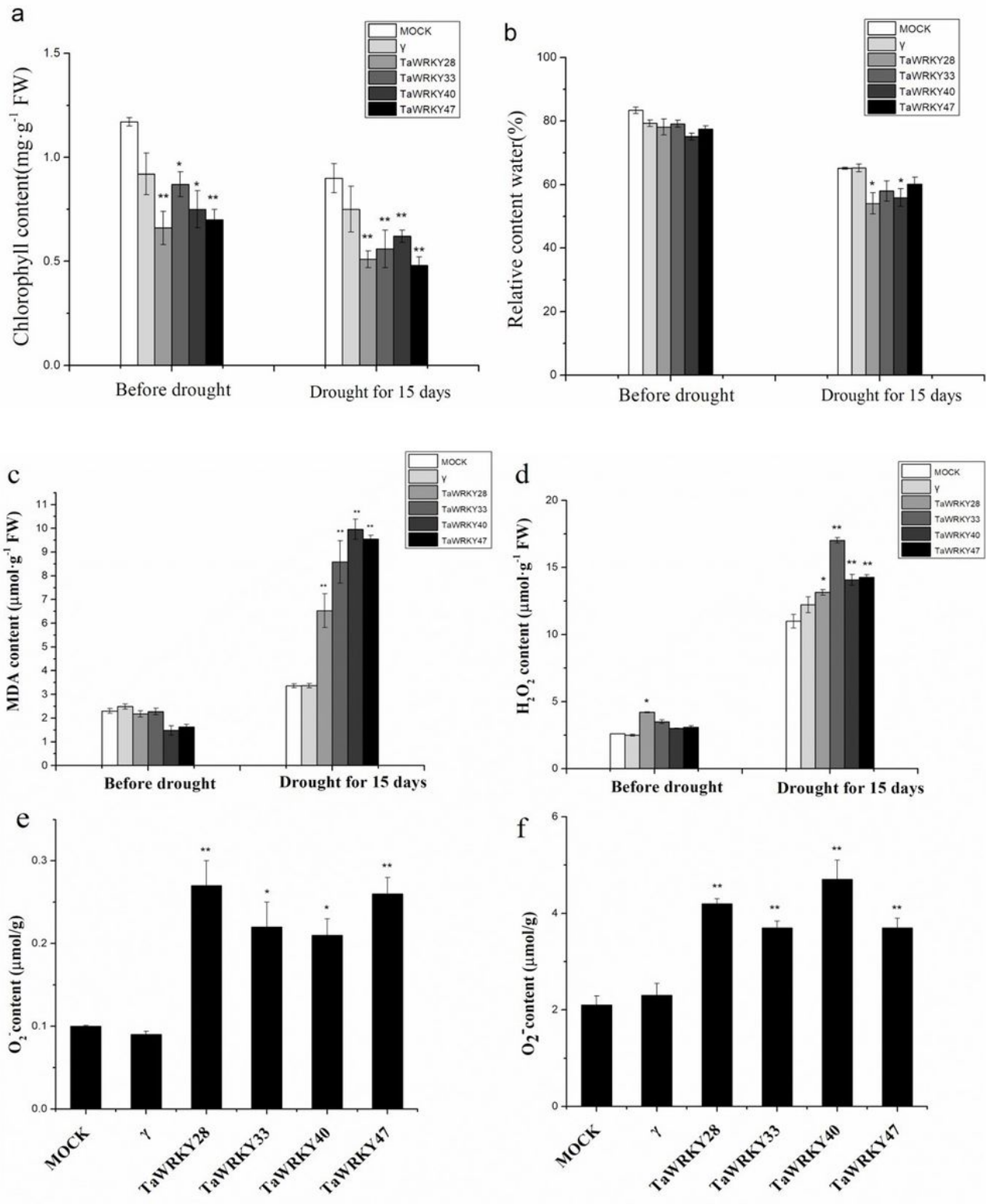

Figure 4

chlorophyll content, RWC, MDA, H2O2 and superoxide anion content and of wheat TaWRKY28/33/40/47 gene-silencing plants before and after drought treatment.

Supplementary Files

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