Wheat WRKY24-1D, interacts with TaERFL1a, regulates DHAR-mediated ASA-GSH biosynthesis to enhance drought tolerance in wheat

Ge-Zi Li
ligezi@henua.edu.cn

Henan Agricultural University

Jin Liu
Henan Agricultural University

Ying-Ying Wang
Henan Agricultural University

An-Qi Han
Henan Agricultural University

Hai-Tao Liu
Henan Agricultural University

Tian-Cai Guo
Henan Agricultural University

Qiao-Xia Han
Henan Agricultural University

Guozhang Kang
Henan Agricultural University

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Abstract

Drought is a major environmental factor to limit wheat yield and quality. WRKY transcription factors play crucial roles in abiotic stresses, including drought stress. However, the regulatory mechanisms of WRKYs in wheat drought stress tolerance are largely unknown. In this study, we found a WRKY transcription factors TaWRKY24, which identified and screened between drought stress yeast library and drought-induced gene TaERFL1a, and the other yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) found that TaERFL1a was interacted with TaWRKY24-1D, which was more induced than TaWRKY24-1A under drought stress. Moreover, the potential function of TaWRKY24 was examined by using BSMV-VIGS method. Results found that transiently silenced of TaWRKY24 in wheat resulted in enhanced sensitivity to drought stress by increasing the contents of malondialdehyde (MDA) and hydrogen peroxide (H2O2), decreasing the activities of ascorbate peroxidase (APX), superoxide dismutase (SOD), or catalase (CAT), and contents of ascorbate (ASA) and glutathione (GSH), and inhibiting the biomass and relative water content. qPCR showed that the expression levels of ASA-GSH-related genes were also significantly inhibited in TaWRKY24-silenced wheat plants. Among these genes, dehydroascorbate reductase (DHAR) had a lowest expression than other ASA-GSH-related genes, indicating that it expression may be mainly regulate by TaWRKY24. In addition, yeast one-hybrid (Y1H), luciferase complementation imaging (LCI), and luciferase (LUC) assays showed that TaWRKY24 had higher activity to bound the promoter of DHAR than TaERFL1a, suggesting that TaWRKY24 positively regulated the expression levels of DHAR and interacted with TaERFL1a to involve in wheat drought tolerance. Therefore, these results providing a theoretical basis for the molecular regulatory mechanisms of TaWRKY24 in wheat drought resistance, and contributing to the potential candidate genes for breeding the drought resistance wheat varieties.

Introduction

Drought is one of the major environmental stresses, it hinders plant growth and reduces crop productivity, specially in wheat (Triticum aestivum) (Du et al., 2023; IWGSC, 2018). Crops have developed several complex and specific physiological and molecular mechanisms to cope with drought stress (Hu et al., 2014; Giraud et al., 2008). Such as, the lipid peroxidation, which was often employed act as an indicator of oxidative stress, malondialdehyde (MDA), which was used to quantify the degree of plasma-membrane oxidation, and enzymatic (catalase, peroxidase, superoxide dismutase, etc) or nonenzymatic (glutathione, GSH; ascorbate, ASA; etc) were significantly induced or inhibited to scavenge excess reactive oxygen species (ROS) (Rahman et al., 2006; Petrov et al., 2015; Kim et al., 2017; Khurshid et al., 2023). In addition, several regulatory and stress-related genes were induced under drought stress condition, such as ASA-GSH-related stress-induced genes (Hennig, 2012; Kang et al., 2013), transcription factors, or regulatory proteins (Yu et al., 2023; Zhang et al., 2023; Zhang et al., 2021). Thus, to understand the physiological and molecular mechanism, and screen candidate genes, which associated with wheat drought stress tolerance, will provide important insights for improving wheat drought tolerance and breeding wheat varieties.
It has been reported that ASA-GSH biosynthesis enzyme genes, contain *ascorbate peroxidase (APX)*, *dehydroascorbate reductase (DHAR)*, *glutathione-S-transferase (GST)*, *glutathione peroxidase (GPX)*, *glutathione reductase (GR)*, and *monodehydroascorbate reductase (MDHAR)*, played vital role in ROS scavenging under drought stress (Jardim-Messeder et al., 2023; Li et al., 2023). Our previous study showed that these ASA-GSH biosynthesis enzyme genes were up-regulated by drought stress in wheat (Kang et al., 2013), inferring that ASA-GSH biosynthesis enzyme genes could involve plant response to drought stress. And the functions of several ASA-GSH biosynthesis enzyme genes have been extensively studied under drought stress in different plants, such as, silencing of *OsAPX7* enhanced drought stress tolerance (Jardim-Messeder et al., 2023), knocking out of rice *OsGSTU17* gene significantly reduced rice drought tolerance (Li et al., 2023). To our knowledge, however, the regulatory mechanisms of these ASA-GSH biosynthesis enzyme genes in response to drought stress are still poorly understood, especially for DHAR.

 Mostly regulator factors, such as WRKY, APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF), and NAC (NAM-ATAF-CUC2) families, have been reported to response drought stress tolerance by activating multiple different physiological or metabolic pathways and regulating related target genes expression (Jiang et al., 2017; Mizoi et al., 2012; Cong et al., 2020; Mao et al., 2022; Peng et al., 2022). WRKY transcription factors, which contains many members according to the numbers of WRKY domains and the types of zinc-finger motif, is one of the largest transcription factor families in plant, and they were mostly investigated in plant growth and development, biotic or abiotic stress response, especially in drought stress (Mao et al., 2022; Gong et al., 2020; Li et al., 2020). Such as, rice WRKY5 as a negative regulator in drought stress tolerance by regulating OsMYB2 expression (Lim et al., 2022), ZmWRKY79 enhanced drought stress tolerance by regulating ABA biosynthesis genes AAO3 expression (Gulzar et al., 2021). However, only a few wheat WRKY transcription factors have been identified and their functions were involved in drought tolerance, such as, ectopic overexpression of TaWRKY1, TaWRKY24, TaWRKY33, TaWRKY40, and TaWRKY75-A enhanced drought resistance in *Arabidopsis* (He et al., 2016; Zhang et al., 2019; Ye et al., 2021; Yu et al., 2023), and their regulatory mechanisms are largely not well characterized.

 Here, we employ a yeast two-hybrid (Y2H) assay found that TaWRKY24 interacted with TaERFL1a, which induces and effects drought stress tolerance in wheat in our previous work [29]. Moreover, the transiently silenced of wheat plants were obtained, and then, the phenotype, physiological, and stress related genes expression were measured in transiently silenced of TaWRKY24 wheat plants to explore the molecular regulatory mechanisms of TaWRKY24 under wheat drought stress. It is noting that TaWRKY24 interacts with TaERFL1a to target of ASA-GSH biosynthesis related gene DHAR and activate its expression, and then resulting in enhanced drought tolerance in wheat. These results may be provide new insights into the underlying molecular regulatory basis of TaWRKY 24 in drought resistance and contribute potential target genes for breeding drought-resistance wheat varieties.

**Materials and methods**
Plant materials and growth conditions

Seeds wheat (*Triticum aestivum* L. cv. Bainong 207) were surface-sterilized with the distilled water and subsequently germinated in growth chamber as our previously published literature described (Gao et al., 2018) [30]. Two-weeks-old seedlings, which were well growth, were divided into two different groups for drought (20% PEG-6000) and control (only Hoagland solution) treatment, and the solutions were exchanged every 24 h. All leaves from different plants were separately collected and frozen in liquid nitrogen after drought treatment for five days.

RNA extraction and quantitative RT-PCR analysis

Total RNA from wheat leaves was extracted with the TRIzol RNA Isolation Reagent (Tiangen, China), then, two micrograms of total RNA was prepared for reverse transcribed into cDNA using the First Strand cDNA synthesis kit (Toyobo, Japan). qPCR was performed using the qPCR SYBR Master Mix (Vazyme, China) in QuantStudio 3 (Thermo, USA) system according to the manufacturer's instructions. The relative expression levels of genes were calculated by using the $2^{-\Delta\Delta CT}$ method as in previous study (Livak and Schmittgen, 2001). *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) was used as endogenous control gene. These experiments were conducted with at least three biological replicates with three plant. All the primers used for PCR were listed in Table S1.

Yeast two-hybrid

TaERFL1a was used to screen the cDNA library, which treated with drought of wheat seedlings, as our previously published literature described (Gao et al., 2018). And then, CDS of TaERFL1a and TaWRKY24 were separately ligated into the pGBKT7 and pGADT7 vectors. For yeast two-hybrid (Y2H) assays, the recombinant plasmids of TaERFL1a-BD and TaWRKY24-AD were co-transformed into the yeast strain Y2H Gold, and then they were grown on SD-Trp/Leu and SD-Trp/Leu/His/Ade for protein interaction analyses.

Bimolecular fluorescence complementation assays

For bimolecular fluorescence complementation (BiFC) assays, the full-length TaERFL1a or TaWRKY24 was fused into the N-terminal yellow fluorescent protein and C-terminal yellow fluorescent protein (cYFP) to form YN-TaERFL1a and YC-TaWRKY24, respectively. Then, these recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101, and were co-infiltrated into leaves of *N. benthamiana* as our previously studied (Gao et al., 2018). And the yellow fluorescent protein (YFP) signals were observed by a Leica confocal microscope (FV3000; Olympus).

BSMV-VIGS-mediated gene silencing

Knockdown the expression of TaWRKY24 by using BSMV-VIGS method as our previously published literature described (Gao et al., 2018). One 153 bp fragment (from 175 ~ 249 bp) of TaWRKY24 was cloned and amplified into the BSMV-γ vector to form a silencing vector of TaWRKY24 (BSMV-TaWRKY24), and BSMV-GFP was used as a control as our previously described (Dong et al., 2019). After
inoculation for 8 ~ 10 days, the inoculated wheat seedlings were observed obvious phenotype, and its leaves were collected for RNA extraction to measure the gene expression levels. And then, the wheat seedlings, which were successfully inoculated BSMV-TaWRKY24 or BSMV-GFP, were separately selected and transferred to drought (Hoagland solution supplemented with 20% PEG6000) and Control (full-length Hoagland solution) treatments for 3 ~ 5 days. The plants of leaves or roots were collected for measuring the stress parameters.

**Determination of the antioxidant system in leaves of BSMV-TaWRKY24 wheat seedlings**

The accumulation of hydrogen peroxide (H$_2$O$_2$) and O$_2^-$ in wheat seedlings were detected by DAB (3,3-diaminobenzidine) staining and NBT (nitro blue tetrazolium) staining by the methods of previously studies (Daudi and O’Brien, 2012; Huang et al., 2019), respectively. After staining, the leaves were soaked in 95% ethanol to remove the chlorophyll, and then these stained leaves were washed and observed by using stereoscopic microscope (Nikon, Japan).

Moreover, the H$_2$O$_2$ content was tested using Hydrogen Peroxide assay kit (Nanjing Jiancheng Bioengineering Institute, China). The activities of antioxidant enzymes including APX, SOD, POD, and CAT were determined using Assay Kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer’s instructions. Fresh samples (0.1 g) were ground in liquid nitrogen and phosphate buffer (10 mM, pH 7.8), which containing 0.1 mM EDTA and 1% PVP. And then, the homogenate was centrifuged at 8,000g at 4°C for 10 min, and the supernatant was separated and stored in a new tube in ice for measuring the activities of APX, SOD, POD, and CAT and the H$_2$O$_2$ content.

Meanwhile, the malonaldehyde (MDA) content was measured by using thiobarbituric acid (TBA) according to the method of Hodges and his colleagues (Hodges et al., 1999). Ascorbate (ASA) content were determined recorded at the absorbance of 525 nm according to the method of Kampfenkel and his colleagues (Kampfenkel et al., 1995) by using the standard curves. Reduced glutathione (GSH) were analyzed by using DTNB, which recorded at the absorbance of 412 nm, according to the method of our previously described (Li et al., 2013).

**Yeast-one hybrid assays**

For yeast-one hybrid assays, the promoter of DHAR were amplified and inserted into the pHIS vector, and then transformed into Y187 to create the reporter yeast strains (pHIS-DHAR-pro). These reporter yeast strains were transformed with pGAD-TaERFL1a and pGAD424-TaERKY24, respectively. The interaction between TaERFL1a or TaWRKY24 and the DHAR promoter was analyzed by using different concentrations of 3-amino-1,2,4-triazole (3-AT), which may be suppress the background of yeast. Primers used for these vector construction are listed in Table S1.

**Luciferase complementation imaging and reporter assays**
Luciferase complementation imaging (LCI) assay was carried out as described previously (Du et al., 2023). The DHAR promoter fragment was cloned into luciferase (LUC) reporter vector pGreenII-0800, and the full-length coding sequences of TaERFL1a or TaWRKY24 were cloned into the pCAMBIA1300 vectors, respectively. And then, these two recombinant constructs were transformed into Agrobacterium tumefaciens strain GV3101, and co-infiltrated into leaves of Nicotiana benthamiana. After infection for 48h, the LUC signals were visualized by using the ChemiDoc™ Imaging System (Bio-Rad, USA), and the activity of LUC/REN reporter were assayed by using Dual-Luciferase reporter assay system (Promega, USA) as our previously described (Li et al., 2018).

**Statistical analysis**

SPSS 17.0 software was used for statistically significant differences analyses via Duncan's test with a significance threshold of $P < 0.05$. And means and standard deviation (SD) from each treatment were calculated at least three biological replicates.

**Results**

**TaWRKY24 interacts with TaERFL1a**

Our previous study found that the TaERFL1a played important and positive roles in wheat responses drought stress (Gao et al., 2018). In order to elucidate the molecular regulator mechanism of TaERFL1a-mediated drought tolerance, Y2H was used to screen its interaction factors by using yeast cDNA library (Fig. S1), which derived from leaves of drought-stressed wheat. WRKY24 (LOC109755167), a WRKY family transcription factor, was screened from TaERFL1a-interaction proteins (Table S2). Then, CDS of WRKY24 was screened in wheat IWGSC database (http://www.wheatgenome.org/), and found that WRKY24 is located on the chromosome 1 and contains TraseCS1A02G121500.1 (TaWRKY24-1A) and TraesCS1D02G122400.1 (TaWRKY-1D) two homologous copies (Fig. S2). The qPCR measured their transcription levels found that TaWRKY24-1D had more highly expressed than TaWRKY24-1A under drought stress (Fig. 1), suggesting that TaWRKY24-1D could play important roles under drought stress. Thus, TaWRKY24-1D was used to explore the function of TaWRKY24 under drought stress in wheat.

TaWRKY24-1D, contains one exon and encodes a putative protein with 194-amino acids (possesses WRKYGQK sequence and a C2HC zinc finger motif) (Fig. S3A and S3B), were cloned and inserted into pGADT7 yeast vector to confirm the interaction between TaWRKY24-1D and TaERFL1a. Results showed that the combination of BD-TaERFL1a + AD-TaWRKY24-1D was grown well under SD mediums of -Trp/-Leu/-His/-Ade, while the combinations of BD-TaERFL1a + AD, BD + AD-TaWRKY24-1D, and BD + AD were not grown (Fig. 2A), suggested that TaWRKY24-1D and TaERFL1a was interacted in yeast cells. And their interaction was further validated by using the BiFC, results found that co-expression of YN-TaERFL1a and YC-TaWRKY24-1D was co-localized in the nuclear of tobacco leaves (Fig. 2B). These results demonstrated that TaWRKY24-1D interacts with TaERFL1a.
Silencing of TaWRKY24 reduces drought tolerance in wheat seedlings

To verify the function of TaWRKY24 under drought stress, the silencing of TaWRKY24 two copies in wheat genome was performed by using BSMV-VIGS. To simultaneously silence of TaWRKY24 gene, a high similarity cDNA fragment of TaWRKY24 two copies (Fig. S2) was selected and constructed into BSMV-VIGS-TaWRKY24 vectors. And then, two-weeks-old wheat plants were inoculated with the BSMV-VIGS-TaWRKY24 or BSMV-VIGS-GFP (control) vectors, the wheat plants showed exhibited growth or photobleaching phenotype after inoculation for 8 days (Fig. S4A). Compare with the control, the expression levels of TaWRKY24 were remarkably decreased by 51.6% in BSMV-VIGS-TaWRKY24-inoculated wheat plants (Fig. S4B), suggested that TaWRKY24 was successfully silenced in wheat. Moreover, the expression levels of TaERFL1a were also significantly decreased by 67.6% in above silenced of TaWRKY24 wheat plants (Fig. S4C), suggested that TaWRKY24 regulated the expression of TaERFL1a.

Subsequently, the successfully silenced TaWRKY24 wheat plants were treated with or without 20%PEG in Hoagland solutions. After 3d drought stress, BSMV-VIGS-TaWRKY24-inoculated wheat plants exhibited more curled and wilted leaves than the control (Fig. 3A and 3B), and the relative water content was significantly decreased by 22.7% compared with control (Fig. 3C). Besides these symptoms, the biomass of fresh or wry weight was significantly reduced by 39.1% or 21.6% in BSMV-VIGS-TaWRKY24-inoculated wheat seedlings compared with the BSMV-GFP-inoculated seedlings, respectively (Fig. 3D and 3E). Moreover, the expression levels of TaWRKY24 and TaERFL1a were also significantly decreased by 89.3% and 76.9% in BSMV-VIGS-TaWRKY24-inoculated wheat seedlings (Fig. 3F and 3G), respectively.

Silencing of TaWRKY24 increases the ROS accumulation and decreases the antioxidant activity of wheat seedlings under drought stress

After 5 days drought stress, the ROS accumulation was determined by using the DAB and NBT staining, which represent the content of O$_2$ and H$_2$O$_2$. Under drought stress, the leaves of BSMV-VIGS-TaWRKY24-inoculated wheat seedlings were deeper ROS staining than those in BSMV-GFP-inoculated seedlings (Fig. 4A and 4B). Moreover, contents of MDA and H$_2$O$_2$, which acted as the stress marker, were significantly increased by 62.65% and 24.45% in leaves of BSMV-VIGS-TaWRKY24-inoculated wheat seedlings (Fig. 4C and 4D).

Furthermore, the activities of APX, SOD, POD, and CAT were measured in these BSMV-VIGS-TaWRKY24-inoculated wheat seedlings. compared to control, the activities of APX, SOD, and CAT were decreased by 50.2%, 16.6%, and 9.9% in leaves of BSMV-VIGS-TaWRKY24-inoculated wheat seedlings after drought stress, excepted to POD (Fig. 5A ~ 5D). Similar patterns were found in the ASA and GSH content, which decreased by 36.2% and 20.2% in leaves of the above BSMV-VIGS-TaWRKY24-inoculated wheat seedlings (Fig. 5E and 5F), respectively.
Silencing of TaWRKY24 changes the expression of stress response genes in wheat seedlings under drought stress

To investigate the molecular role of TaWRKY24 gene under drought tolerance, some stress response genes, which had been studied and associated with the drought tolerance (Kang et al., 2013), were selected and measured by using qPCR. Compare with the control, the expression levels of ASA-GSH system genes were significantly decreased in leaves of BSMV-VIGS-TaWRKY74-inoculated wheat plants under drought stress, respectively. Such as, the expression levels of APX, DHAR, MDHAR, GR, GSH, GST, GPX1, and GPX2 were separately decreased by 44.7%, 99.9%, 65.9%, 36.0%, 80.2%, 44.7%, 67.6%, and 16.2% in leaves of the BSMV-VIGS-TaWRKY24-inoculated wheat plants (Fig. 6), respectively. These results suggested that TaWRKY24 regulated ASA-GSH system gene expression under wheat drought stress, especially of DHAR gene.

TaWRKY24 and TaERFL1a binds to the promoter of DHAR and actives its expression under drought stress

To confirm the regulatory relationship between TaWRKY24 and ASA-GSH system genes, the Y1H and dual-luciferase assays were separately performed in this study. Interestingly that there was three WRKY core motifs in DHAR promoter, and others ASA-GSH system genes only had one or two WRKY core motifs(Fig. S5). Thus, the binding between TaWRKY24 and DHAR promoter was used for next experiment. The promoter of DHAR was cloned into yeast pHIS2.0 vector, and the TaWRKY24 or TaERFL1a was inserted into pGADT7. And then, the DHAR promoter and TaWRKY24 or TaERFL1a were cotransformed in Y187. Results showed that the yeast cells of cotransformed with TaWRKY24-AD and pHIS2.0 vector were inhibited growth on SD/-Trp/-Leu/-His media by 50 mM 3-aminotriazole (3-AT) (Fig. 7A), which a inhibitor of His synthase, while those growth of TaWRKY24 and DHAR promoter were grown well, suggested that TaWRKY24 could be bind the promoter of DHAR. Moreover, the cotransformed of TaERFL1a-AD and pHIS2.0 vector was not completely inhibited by 50 mM 3-AT, but they were less growth than that in TaERFL1a-AD and DHAR promoter, suggested that TaERFL1a could also be bind the promoter of DHAR.

Moreover, the DHAR promoter and TaWRKY24 and TaERFL1a were co-transformed in tobacco leaves by using LCI and LUC assays. Results shown that the LUC fluorescence signals of TaWRKY24 + TaERFL1a + Pro-DHAR were higher than only TaWRKY24 + Pro-DHAR or TaERFL1a + Pro-DHAR (Fig. 7B). Moreover, the LUC fluorescence signals of TaWRKY24 + Pro-TaDHAR were also higher than TaERFL1a + Pro-DHAR (Fig. 7B). Consistently, the ratios of LUC/REN was highest in TaWRKY24 + TaERFL1a + Pro-TaDHAR, which was higher 2.42-folds and 1.84-folds than TaWRKY24 + Pro-TaDHAR and TaERFL1a + Pro-DHAR (Fig. 7C), respectively. These results suggested that TaWRKY24 was more regulated DHAR expression than TaERFL1a by binding its promoter under drought stress.

Discussion
WRKY transcription factors, which were exclusively found in the plant kingdom, involve in modulating numerous processes, such as plant senescence, seed development, and drought stress responses in various plant species, especially in *Arabidopsis* and rice (Banerjee et al., 2015; Li et al., 2020; Khoso et al., 2022). However, since wheat (*Triticum aestivum* L.), one of major food crops, had complex and huge genome size wheat genomes (highly repetitive sequences, > 85%), thus, the knowledge about the role of WRKY transcription factors in wheat stress responses was limited, especially in drought stress.

To improve the knowledge of WRKY transcription factors in wheat, Okay and his colleagues identified and characterized 160 wheat WRKY transcription factors response to drought stress in wheat by using sequence similarity, motif type, and phylogenetic relationships (Okay et al., 2014). In this study, we revealed the function of TaWRKY24-1D, which screen from Y2H and identified from IWGSC, in wheat response drought stress tolerance. Our study found that TaWRKY24-1D, which expression levels were similar with Yu and his colleagues found that it were induced by drought stress (Yu et al., 2023a), is more higher induced than TaWRKY24-1A under drought stress. Moreover, Y2H and BiFC found that TaWRKY24-1D interacted with TaERFL1a, suggesting that TaWRKY24 may play an important role under wheat drought stress. Moreover, base on the results, we found that silencing of TaWRKY24 decreased drought resistance by inhibiting the plant biomass and enhancing ROS accumulation, and decreasing antioxidant activity and ASA-GSH content (Figs. 3 ~ 5). Similar results have been found in different plants, such as rice WRKY50 enhances drought tolerance through scavenging ROS accumulation (Yang et al., 2020); SbWRKY30 enhances drought tolerance through increasing proline contents and SOD, POD, and CAT activities (Ma et al., 2019).

In wheat, it is increasing evidence indicated that WRKYs play an important role in drought stress response. For example, TaWRKY44 may act as a positive regulator in drought, salt and osmotic stress responses in transgenic *Arabidopsis* (Wang et al., 2015); TaWRKY33 was involved in drought, high- and low-temperature and ABA responses in transgenic *Arabidopsis* (He et al., 2016); TaWRKY75-A was ectopic expressed in *Arabidopsis* enhanced drought tolerance (Ye et al., 2021); TaWRKY24 was involved in drought tolerance (Yu et al., 2023); Moreover, the molecular regulatory mechanisms of WRKYs in wheat also had been studied under drought stress. For example, Lv and his Colleagues found that TaWRKY133 participated drought tolerance by regulating stress-related genes of POD, CAT and P5CS expression in plants (Lv et al., 2022); TaWRKY1-2D interacts with TaDHN3 and regulates the expression levels of TaPOD, TaCAT, and TaSOD (Fe) under drought stress (Yu et al., 2023b); TaWRKY31 regulates the expression levels of TaSOD (Fe), TaPOD, TaCAT, TaDREB1, and TaPYL5 to enhances drought resistance. To our knowledge, however, the molecular regulatory mechanisms of TaWRKY24 in wheat drought stress are unclear. In this study, thus, we found that silencing of TaWRKY24 inhibiting the expression levels of TaERFL1a and ASA-GSH biosynthesis related genes (Figs. 3 and 6), suggesting that TaWRKY24 regulates their expression under drought stress. Among these genes, the expression levels of ASA-GSH biosynthesis related gene, DHAR, were mostly significantly decreased than other genes in silencing of TaWRKY24 wheat plants (Fig. 6), suggesting that TaWRKY24 may be more regulated the expression of DHAR than others under wheat drought stress. Like other transcription factors, Y1H, LIC, and LUC found that TaWRKY24 could directly bond DHAR promoter and activated its expression (Fig. 7). Furthermore,
the LUC activities assays found that both TaWRKY24 and TaERFL1a plus DHAR promoter were higher than the only one of TaWRKY24 or TaERFL1a (Fig. 7B and C), and the LUC activities of TaWRKY24 + DHAR promoter were also higher than TaERFL1a + DHAR promoter (Fig. 7C), suggesting that TaWRKY24 interacts with TaERFL1a, and more regulates and actives the expression of DHAR under wheat drought stress. These results were similar with Yang and his Colleagues found that JrERF2-2 interacts with JrWRKY7 control the expression of GSTs by binding the promoters of GSTs in Juglans regia (Yang et al., 2021).

Conclusions

In summary, TaWRKY24-1D, which interacts with TaERFL1a, was identified by using Y2H, and its expression was induced by drought stress. Moreover, the function of TaWRKY24 was further investigated via BSMV-VIGS method. Silencing of TaWRKY24 in wheat results in decreasing drought resistance and increasing the ROS accumulation, and further experiment found that TaWRKY24 was more regulated DHAR expression, which involves ASA-GSH biosynthesis-related genes, than TaERFL1a. Taken together, our results found that regulation pathway of TaWRKY24-TaERFL1a-DHAR in response to wheat drought tolerance (Fig. 8), and these results not only improve the understanding of molecular regulatory basis in wheat drought stress resistance, and also lay theoretical foundation contribute for breeding drought-resistance wheat varieties in future.

Abbreviations

APX, ascorbate peroxidase; ASA, ascorbate; AP2/ERF, APETALA2/ETHYLENE RESPONSIVE FACTOR; BSMV-VIGS, barley stripe mosaic virus-induced gene-silencing; BiFC, bimolecular fluorescence complementation; CAT, catalase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GPX, glutathione peroxidase; GSH, glutathione; GST, glutathione-S-transferase; H2O2, hydrogen peroxide; LUC, luciferase; LCI, luciferase complementation imaging; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; ROS, reactive oxygen species; SOD, superoxide dismutase; Y1H, yeast one-hybrid; Y2H, yeast two-hybrid; YFP, yellow fluorescent protein.

Declarations

Authorship contributions

Ge-Zi Li and Guo-Zhang Kang: Funding acquisition, Methodology, Visualization, Writing-original draft. Jin Liu: Data curation, Formal analysis, Resources, Software. Ying-Ying Wang: Data curation, Investigation, Validation. An-Qi Han: Methodology, Project administration. Hai-Tao Liu: Project administration, Validation, Visualization. Qiao-Xia Han, Qiang Ge, and Tian-Cai Guo: Formal analysis, Project administration.

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Data availability

All data generated or analyzed during this study are included in this manuscript. And raw data will be made available on request.

Declarations

All authors declare that they have no financial or personal relationships that could have appeared to influence the work reported in this manuscript.

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Figures
Fig. 1 Expression levels of *TaWRKY24-1A* and *TaWRKY24-1D* in different tissues of wheat plants under drought stress. Two-week-old wheat seedlings were used for 20% PEG6000 treatment, and the *GAPDH* gene has been used for the internal control gene. Data present means ± SD (n = 3). Different letters represent significant difference at $P < 0.05$.

**Figure 1**

See image above for figure legend.
**Figure 2**

See image above for figure legend.
Fig. 3 Phenotype and growth characteristics of BSMV-GFP- or BSMV-TaWRKY24-inoculated wheat plants after drought stress. A, Phenotype of BSMV-GFP- or BSMV-TaWRKY24-inoculated wheat plants after 20% PEG6000 stress for 5 days. B and C, Fresh weights and dry weights of BSMV-GFP- or BSMV-TaWRKY74-inoculated wheat plants at 5 days after drought stress. D and E, The relative water content and absolute water content of BSMV-GFP- or BSMV-TaWRKY74-inoculated wheat plants at 5 days after drought stress. F and G, The relative expression levels of TaWRKY24 and TaERF11a in leaves of BSMV-GFP- or BSMV-TaWRKY74-inoculated wheat plants at 5 days after drought stress. Data are given as means ± SD of three biological replicates. Different letters represent significant difference at $P < 0.05$.

Figure 3

See image above for figure legend.
Fig. 4 The effect of ROS accumulation in leaves of BSMV-GFP- or BSMV-TaWRKY24-inoculated wheat plants after drought stress. A, DAB staining in leaves of wheat plants under drought stress. B, NBT staining in leaves of wheat plants under drought stress. C and D, the contents of MDA and H2O2 in leaves of wheat plants under drought stress. Data present means ± SE (n = 3). The different letters represent significant difference at $P < 0.05$.

Figure 4

See image above for figure legend.
Fig. 5 The activities and contents of antioxidant enzymes in leaves of BSMV-GFP- or BSMV-TaWRKY24-inoculated wheat plants under drought stress. A, APX activity. B, SOD activity. C, POD activity. D, CAT activity. E and F, Contents of ASA and GSH. Data present means ± SE (n = 3). The different letters represent significant difference at P < 0.05.

**Figure 5**

See image above for figure legend.
Fig. 6 Relative expression levels of genes in ASA-GSH cycles after drought stress. A, APX expression. B, MDHAR expression. C, DHAR expression. D, GSH expression. E, GR expression. F and G, GST expression. H and I, GPX1 and GPX2 expression. Data present means ± SE (n = 3). The different letters represent significant difference at $P < 0.05$.

Figure 6

See image above for figure legend.
**Fig. 7 Bind relationship between DHAR promoter and TaERFL1a or TaWRKY24-1D.** A, Y1H assay the bind relationship between TaWRKY24-1D or TaERFL1a and DHAR promoter. B, Dual-luciferase assay the bind relationship between TaWRKY24-1D or TaERFL1a and DHAR promoter. C, The ratio of LUC/REN between TaWRKY24-1D or TaERFL1a and DHAR promoter. Data present means ± SE (n = 3). The different letters represent significant difference at P < 0.05.

**Figure 7**

See image above for figure legend.
Fig. 8 The proposed model of TaERFL1a interacts TaWRKY24 mediated drought stress tolerance in wheat.

**Figure 8**

See image above for figure legend.

**Supplementary Files**

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- SupplementalFigures.pdf
- SupplementalTables.pdf