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ORIGINAL ARTICLE

TaMYB44-5A reduces drought tolerance by repressing transcription of *TaRD22-3A* in the abscisic acid signaling pathway

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Abstract

Main conclusion **TaMYB44-5A identified as a transcription factor negatively regulates drought tolerance in transgenic *Arabidopsis*.**

Abstract Drought can severely reduce yields throughout the wheat-growing season. Many studies have shown that R2R3-MYB transcription factors are involved in drought stress responses. In this study, the R2R3-MYB transcription factor MYB44-5A was identified in wheat (*Triticum aestivum* L.) and functionally analyzed. Three homologs of *TaMYB44* were isolated, all of which localized to the nucleus. Overexpression of *TaMYB44-5A* reduced drought tolerance in *Arabidopsis thaliana*. Further analysis showed that *TaMYB44-5A* reduced the sensitivity of transgenic *Arabidopsis* to ABA. Genetic and transcriptional regulation analyses demonstrated that the expression levels of drought- and ABA-responsive genes were downregulated by TaMYB44-5A, and TaMYB44-5A directly bound to the MYB-binding site on the promoter to repress the transcription level of *TaRD22-3A*. Our results provide insights into a novel molecular pathway in which the R2R3-MYB transcription factor negatively regulates ABA signaling in response to drought stress.

Keywords ABA signaling pathway · Drought tolerance · Stomatal aperture · *TaMYB44-5A* · *TaRD22-3A* · Wheat

Abbreviations

AbA	Aureobasidin A
CAT	Catalase
EMSA	Electrophoretic mobility shift assay
MBS	MYB-binding site
MDA	Malondialdehyde
POD	Peroxidase
RD22	Responsive to dehydration 22

Introduction

Bread wheat (*Triticum aestivum* L., 2n = AABBDD) is a highly adaptable cereal, providing more than one-fifth of the calories consumed by humans (International Wheat

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Genome Sequencing 2018). Its adaptability is attributed to the distribution of tightly assembled stress-resistance genes in its genome (Itam et al. 2022). Previous reports indicate that wheat production in drought-sensitive regions needs to increase by nearly 30% by 2025 to meet the food demands of the growing global population (Pennisi 2008; Chenu et al. 2017). Therefore, it is essential to identify the functions of drought-related genes for future wheat breeding (Paux et al. 2022). Many key genes responsive to drought stress have been identified in previous studies, including transcription factors, protein kinases, and molecular chaperones (Fujii et al. 2011; Qian et al. 2021; Wang et al. 2021a). For instance, the transcription factor WRKY13 in *Vitis vinifera* negatively regulates drought tolerance by modulating intracellular osmolyte metabolism (Hou et al. 2020).

ABA is a core phytohormone abundantly produced under drought conditions, where it serves a pivotal role in plants' response to drought stress (Nakashima and Yamaguchi-Shinozaki 2013). ABA regulates the expression levels of downstream stress-responsive genes through a precise pathway. Under drought stress conditions, ABA receptors, including the pyrabactin resistance (PYR)/PYR1-like (PYL)/regulatory components of ABA receptor (RCAR), interact with 2C-type protein phosphatases (PP2Cs) to release SNF1-related protein kinase 2s (SnRK2s) from PP2C-dependent repression. Phosphorylated SnRK2s can then phosphorylate downstream ABA-responsive element-binding protein (AREB)/ABRE binding factor (ABF) or other transcription factors. Consequently, the ABA-mediated drought signal is transmitted, allowing downstream genes to directly respond to the signal or influence the upstream system through feedback mechanisms (Nakashima and Yamaguchi-Shinozaki 2013). For instance, upon activation of the ABA pathway, phosphorylated apple (*Malus domestica* Borkh) bZIP44 interacts with MdMYB1 to prevent the degradation of MdMYB1 by the ubiquitin-26S proteasome system, thereby enhancing the response to ABA signaling (An et al. 2018). MdMYB44-like positively regulates drought tolerance via the MdPYL8-MdPP2CA module in apple (Chen et al. 2024). Furthermore, it has been reported that AtMYB44 can interact with AtPYL8 to attenuate the transmission of early signals and suppress the response to drought stress signals (Jaradat et al. 2013). Therefore, MYB transcription factors play crucial roles in the ABA signaling pathway.

MYB transcription factors constitute the largest family of transcription factors in plants (Dubos et al. 2010). Depending on the combination and type of adjacent repeat sequences in the amino acid sequence, MYB transcription factors can be classified into four subfamilies: 1R-MYB, R2R3-MYB, 3R-MYB, and 4R-MYB (Dubos et al. 2010). Members of the MYB transcription factor family are extensively involved in regulating plant photosynthesis, biotic and abiotic stress responses, and the accumulation of plant storage substances, with members of the R2R3-MYB subfamily being the most abundant (Wu et al. 2022). R2R3-MYBs have the capability to bind MYB-binding sites and subsequently regulate the expression of downstream ABA-responsive genes (Wu et al. 2022; Gao et al. 2024). Furthermore, R2R3-MYBs have been identified as crucial components in the response to drought signaling (Jung et al. 2008; Shim et al. 2013; Piao et al. 2019b; Shi et al. 2024). Previous studies have shown that *AtMYB44* overexpression confers weaker drought tolerance and lower ABA sensitivity in *Arabidopsis* (Huang et al. 2007; Jaradat et al. 2013; Li et al. 2014). It has been reported that in the presence of ABA, the expression level of the important ABA signaling marker gene *AtRAB18* is significantly upregulated in the *myb44* (T-DNA insertion mutant) (Huang et al. 2007). In addition, AtMYB44 weakens the drought tolerance in *Arabidopsis* by inhibiting the expression levels of downstream ABA-responsive genes, such as D-1-pyrroline-5-carboxylate synthase 1 (*P5CS1*) and dehydration-responsive gene 22 (*RD22*) (Jaradat et al. 2013). In the ABA sensitivity assays, the germination rate of P35S:*MYBR1*(*AtMYB44*) was higher, while that of *mybr1* was lower, indicating that overexpression of *MYB44* reduces the sensitivity of *Arabidopsis* to ABA (Li et al. 2014). Overexpression of *BnMYBL2* (*Brassica napus* L.) in wheat promotes the accumulation of ABA and anthocyanins, thereby increasing wheat's drought tolerance (Gao et al. 2024). In rice (*Oryza sativa* L.), *OsMYB102* was previously identified as an ABA-dependent regulator of leaf senescence, and *OsMYB102* overexpression reduced the tolerance of drought in transgenic rice (Piao et al. 2019a, 2019b). MsMYBH (*Medicago sativa* L.) can directly bind to the promoters of *MsMCP1*, *MsMCP2*, *MsPRX1A*, and *MsCARCAB* and promote their expression to enhance alfalfa's drought tolerance (Shi et al. 2024). Nonetheless, limited studies have investigated the functions of R2R3-MYBs in the response to drought stress in wheat and the associated mechanism of direct regulation of drought tolerance.

In our study, we identified three homologs of *AtMYB44* in wheat based on their evolutionary relationships. Chinese spring wheat seedlings underwent drought and ABA treatments. Nuclear-localized *TaMYB44*s responded to both drought and ABA stress. Overexpression of *TaMYB44-5A* in *Arabidopsis* led to reduced drought tolerance and decreased ABA sensitivity. Furthermore, the transcriptional regulation analysis revealed that TaMYB44-5A directly downregulates the transcription level of the stomatal aperture regulator *TaRD22-3A*. These findings suggest that TaMYB44-5A may negatively regulate the ABA signaling pathway-mediated drought response.

Materials and methods

Identification of *TaMYB44-5A*

The cDNA sequence of *AtMYB44* (AT5G67300) was obtained from Ensembl Plants (http://plants.ensembl.org/index.html). The coding sequence (CDS) of *AtMYB44* was used in a TBLASTN search against the *Triticum aestivum* IWGSC genome in Ensembl Plants. Based on high sequence similarity scores, TraesCS5A02G159600, TraesCS5B02G157300, and TraesCS5D02G164600 were identified as orthologs of *AtMYB44* in wheat. Specific primers for these genes were designed using Primer Premier 6 (Table S1).

Plant material and growth conditions

Chinese Spring wheat (*Triticum aestivum* cv. Chinese Spring) was utilized in our subsequent experiments. The wheat planting method and field management mode were consistent with those described in previous studies (Wang et al. 2021a). Wheat root, shoot, leaves, and spikes were collected at specific growth stages and cryopreserved with liquid nitrogen. *Arabidopsis thaliana* Col-0 type plants were employed in this study. Wild-type (WT) and transgenic *Arabidopsis*, germinated in Murashige and Skoog (MS) medium, were placed in an incubator set at a temperature of 24 °C, a relative humidity of 55%, and a photoperiod of 14-h light/10-h dark.

Stress and hormone treatments

After disinfection with sodium hypochlorite, wheat seeds were sown on MS medium (without hygromycin B) and placed in an incubator at 25 °C with a 9-h light/15-h dark photoperiod. Wheat seedlings were grown for 20 days and then transferred to an aqueous solution containing 30% PEG6000 for drought treatment and 80 μM S-(+)-ABA for ABA treatment. The wheat seedlings were gently wiped to remove any excess root moisture and transferred directly to dry medium for drought treatment. Wheat leaves were collected from all treatments at 0, 1, 3, 6, 12, and 24 h and frozen at − 80 °C. The survival rate was calculated as the percentage of surviving seedlings 4 days after re-watering. For the drought treatment, 4-week-old *Arabidopsis* plants were deprived of water for 14 days, and rosette leaves were collected after 14 days of drought for subsequent experiments.

qRT-PCR analysis

Total RNA was extracted from wheat and *Arabidopsis* subjected to different treatments following the method outlined in a previous study (Wang et al. 2021a). The qRT-PCR reaction procedure and detection instruments were also described in the same study (Wang et al. 2021a). Expression levels were normalized to the expression of the *TaActin1* gene in wheat or the *AtActin2* gene in *Arabidopsis*. Subsequently, the relative expression levels were calculated using the least squares method. All qRT-PCR analyses were conducted using three independent biological replicates. The primers utilized in this study are listed in Table S1.

Subcellular localization

In subcellular localization experiments, *TaMYB44-5A/5B/5D* in the intact open reading frame were cloned into pCAMBIA3301-RFP to generate the fusion vectors TaMYB44-5A/5B/5D:RFP. Fortunately, we borrowed the TaFDL2-1A:GFP fusion vector from Wang et al. (2021a). TaMYB44s:RFP and TaFDL2-1A:GFP were co-transiently transformed into tobacco leaves via *Agrobacterium*-mediated transformation. All injected leaves were collected 48 h after transformation and fluorescent signals were detected using a fluorescent confocal microscope (IX83-FV1200, Olympus Corporation).

Plant transformation

The TaMYB44-5A:RFP fusion vector was used to generate transgenic *Arabidopsis* via *Agrobacterium*-mediated stable transformation, where 1/2 MS medium with 50 μg/mL hygromycin B was used to screen transgenic T1/T2 *Arabidopsis* plants. qRT-PCR was employed to detect the expression levels. 15 transgenic lines were obtained by us.

Drought stress tolerance assays of *TaMYB44-5A* transgenic *Arabidopsis* plants

For the drought tolerance assay, 1-week-old seedlings germinated on half-MS medium were transferred to pots containing soil. Four-week-old plants grown under normal conditions were exposed to drought stress for 14 days. The plants were then re-watered for 4 days and survival was recorded. Detached leaves were air-dried for 7 h and weighed at seven time points (0 h, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h). Water loss was calculated as the weight loss at each time point divided by the initial fresh weight. Three rosette leaves of *Arabidopsis* after 5 weeks of growth were taken to measure their weight and recorded as FW (fresh weight). The leaves were retained in the previous step in ddH_2O to absorb water to

a constant weight, and the measured weight was recorded as TW (turgid weight). The leaves retained in the previous step were put in an oven at 60 °C until they achieved a constant weight, and the measured weight was recorded as DW (dry weight). The relative leaf water content was calculated according to the formula (RWC = (FW–DW)/(TW–DW) × 100%).

Physiological measurements

The physiological parameters were measured using fully expanded leaves obtained from well-watered and drought-stressed plants. The content of malondialdehyde (MDA), proline, soluble protein and the enzymatic activities of peroxidase (POD), and catalase (CAT) were determined as previously described (Luna et al. 2005; Wang et al. 2021b, 2022). Three biological replicates were performed.

Stomatal phenotype and stomatal aperture

The leaves of *Arabidopsis* growing under normal conditions or without water for 6 days were used for the observation of stomatal phenotype. Stomata were photographed using a confocal microscope with an objective lens (UPLSAPO20 × NA: 0.75) under LSM observation mode. Stomatal aperture was calculated as the specific value of width compared with length (Wang et al. 2022).

Transcriptional activity assays and yeast one-hybrid assays

Similar to previous studies, the Matchmaker™ Gold Y2H System (Takara Bio, Beijing, China) was used to detect the transcriptional activity of *TaMYB44-5A* (Wang et al. 2021a; Luo et al. 2022). BD-empty and BD-*TaMYB44-5A* were constructed using the pGBKT7 vector for subsequent transcriptional activity assays. The transcriptional activities of the recombinant vectors (transformed into Y2HGold yeast strain) were tested on SD/-Trp/-His/-Ade medium. The vectors were linearized using fast endonuclease *Bst*BI. *TaMYB44-5A* was fused to the C-terminus of the GAL4 activation domain of pGADT7. All vectors were co-transformed into the Y1H Gold yeast strain as shown in Fig. 7B. All yeast strains were tested on SD/-Ura/-Leu and SD/-Ura/-Leu medium containing 500 ng/mL Aureobasidin A (AbA).

Electrophoretic mobility shift assay (EMSA)

TaMYB44-5A was fused to the pGEX4T vector. Purified GST-TaMYB44-5A and GST proteins were used in subsequent experiments. A LightShift® Chemiluminescent EMSA Kit 20148 (Thermo Fisher Scientific Inc, Waltham, MA, USA) was used to detect the activities of these proteins. All biotin-labeled probes used in the EMSA (modified at the 5') were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The proteins were incubated at 20 °C for 50 min. Each 20 μL reaction system contained 50 fmol of the biotin-labeled probe. The components in the reaction system were separated on a non-denaturing polyacrylamide gel and then transferred to a nylon membrane. Biotin-labeled probe migration was detected using the luminescent system in the kit.

Statistical analysis

SPSS 23 (SPSS. Inc., USA) was used to process all data obtained in this study. The Student's *t*-test (**$P < 0.01$, *$P < 0.05$) was used to determine significant differences between transgenic *Arabidopsis* and WT. Photoshop (CS6, Adobe Systems Incorporated, San Jose, CA, USA) was used to construct all images.

Results

Identification and bioinformatics analysis of *TaMYB44-5A*

To identify homologous genes of *AtMYB44* in wheat, we utilized the coding sequence (CDS) of *AtMYB44* to conduct bioinformatics searches in the Chinese Spring wheat database available in Ensembl Plants. Subsequently, we identified three homologous genes of *AtMYB44* designated as *TaMYB44-5A* (Gene ID: TraesCS5A02G159600), *TaMYB44-5B* (Gene ID: TraesCS5B02G157300), and *TaMYB44-5D* (Gene ID: TraesCS5D02G164600), based on their distinct positions on the chromosomes (Supplementary Fig. S1). *TaMYB44-5A*, *TaMYB44-5B*, and *TaMYB44-5D* encode proteins comprising 336, 349, and 348 amino acids, respectively (Supplementary Fig. S2), with a high sequence similarity of 93.35%. In addition, TaMYB44 shares 42.46% and 75.74% similarity with AtMYB44 and OsMYB102, respectively (Supplementary Fig. S2). Sequence analysis revealed highly conserved N-terminal SANT domains and a C-terminal ethylene-responsive element-binding factor-associated amphiphilic repression (EAR) motif in TaMYB44s, AtMYB44, and OsMYB102 (Supplementary Fig. S2). A previous study suggested that the LXLXL-type EAR motif may be involved in transcriptional regulation or interactions (Kagale and Rozwadowski 2011).

Surprisingly, upon analyzing the promoter sequences of *TaMYB44*s located 2 kb upstream of the initiation codon, we discovered numerous cis-acting elements associated with the plant drought response and ABA signaling, including ABA-responsive elements (ABRE), dehydration-response elements (DRE), and MYB-binding sites (MBSs; Fig. 1a).

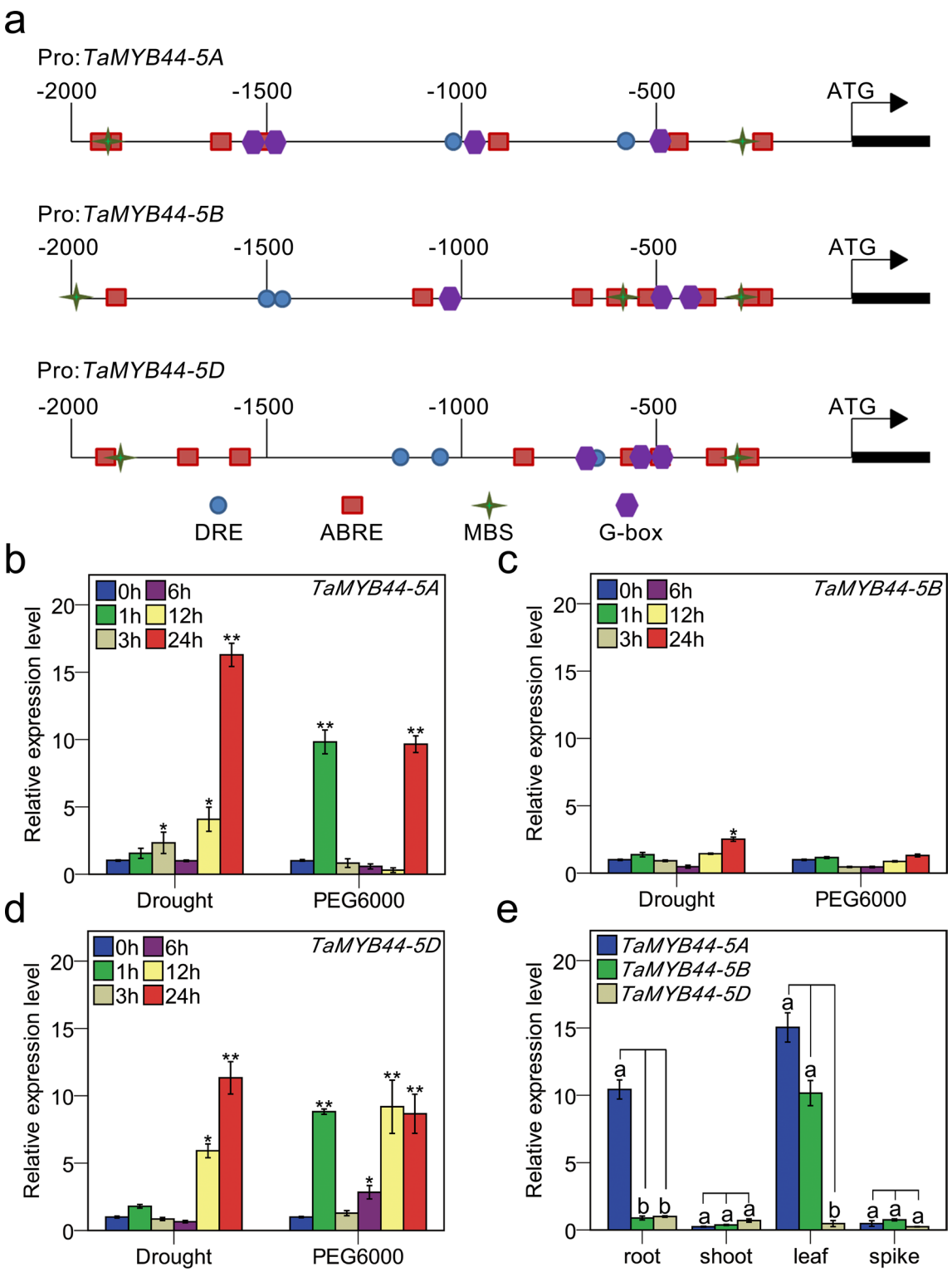


Fig. 1 Expression analyses of *TaMYB44*s. **a** Distribution of major stress-related cis-acting elements in the 2-kb promoter regions of *TaMYB44*s. **b–d** Expression levels of *TaMYB44*s under drought and PEG6000 treatments. The expression levels were normalized against those at 0 h for *TaMYB44*s. The reference for significance analysis is the data at 0 h. **Highly significant difference ($P<0.01$), *significant difference ($P<0.05$). **e** Expression patterns of *TaMYB44*s. Statistical analysis was performed using the LSD method. Data represent mean values ± standard deviations ($n=3$). Values labeled with different letters indicate significant differences, whereas the same letters indicate no significant differences. ($P<0.05$; one-way ANOVA). The reference gene is *TaActin1*. The expression patterns were normalized against the expression level of *TaMYB44-D* at root for *TaMYB44*s

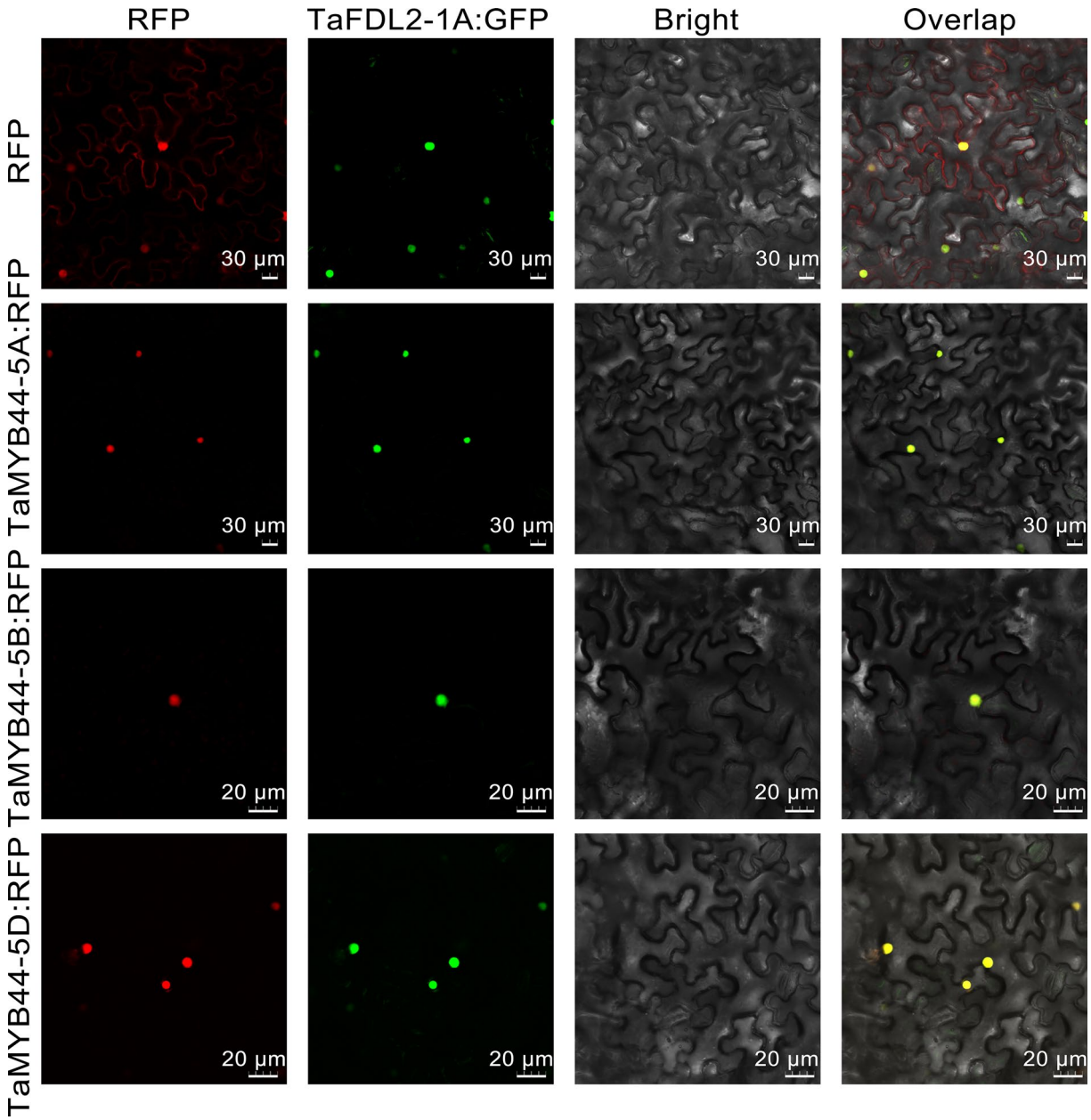


Fig. 2 Subcellular localizations of TaMYB44s. TaMYB44-5A:RFP, TaMYB44-5B:RFP, and TaMYB44-5D:RFP were co-transformed with TaFDL2-1A:GFP into *Nicotiana benthamiana* leaves. RFP was transformed into tobacco leaves alone as a blank control. Scale bars are shown in the lower right corner of images

Therefore, our findings strongly suggest that *TaMYB44*s may play a significant role in drought tolerance in wheat.

Expression patterns and subcellular localization of *TaMYB44*s

To verify whether *TaMYB44*s participates in the drought response, we used real-time quantitative PCR (qRT-PCR) to detect the changes in the expression levels of *TaMYB44*s in wheat under drought and dehydration stress (using PEG6000) (Fig. 1b–d). The results indicate that *TaMYB44*s are induced to higher expression levels at 1 h by drought, followed by a slight decrease in expression at 3 h (*TaMYB44-5A* showing a decrease at 6 h). With increasing treatment time, *TaMYB44*s are ultimately induced to the highest level at 24 h in a similar pattern. Under PEG6000 treatment, both *TaMYB44-5A* and *TaMYB44-5D* exhibit higher expression levels at 1 h and 24 h, while the expression level of *TaMYB44-5B* shows a slight increase. All these findings suggest that these three homologous genes may have

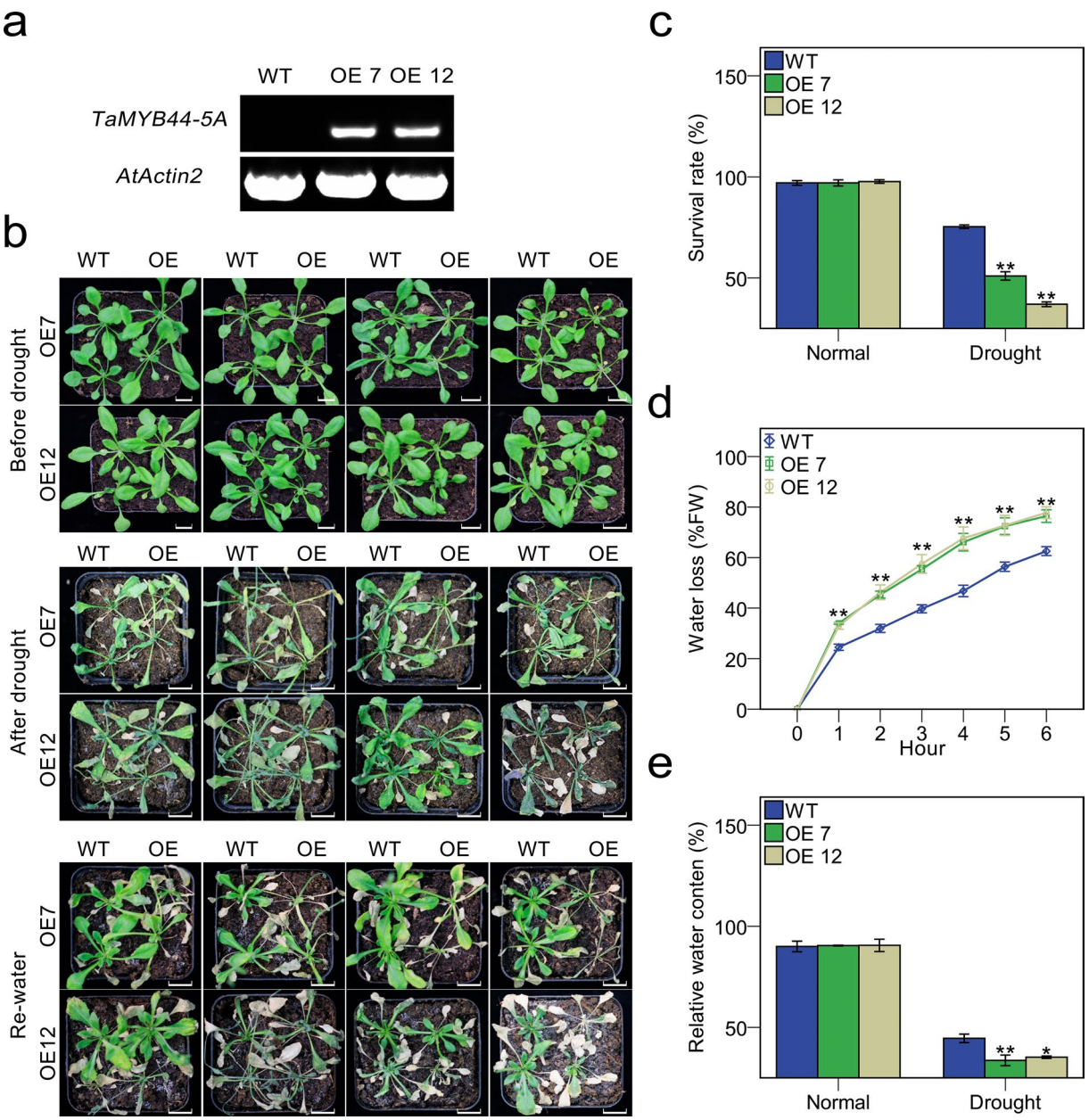
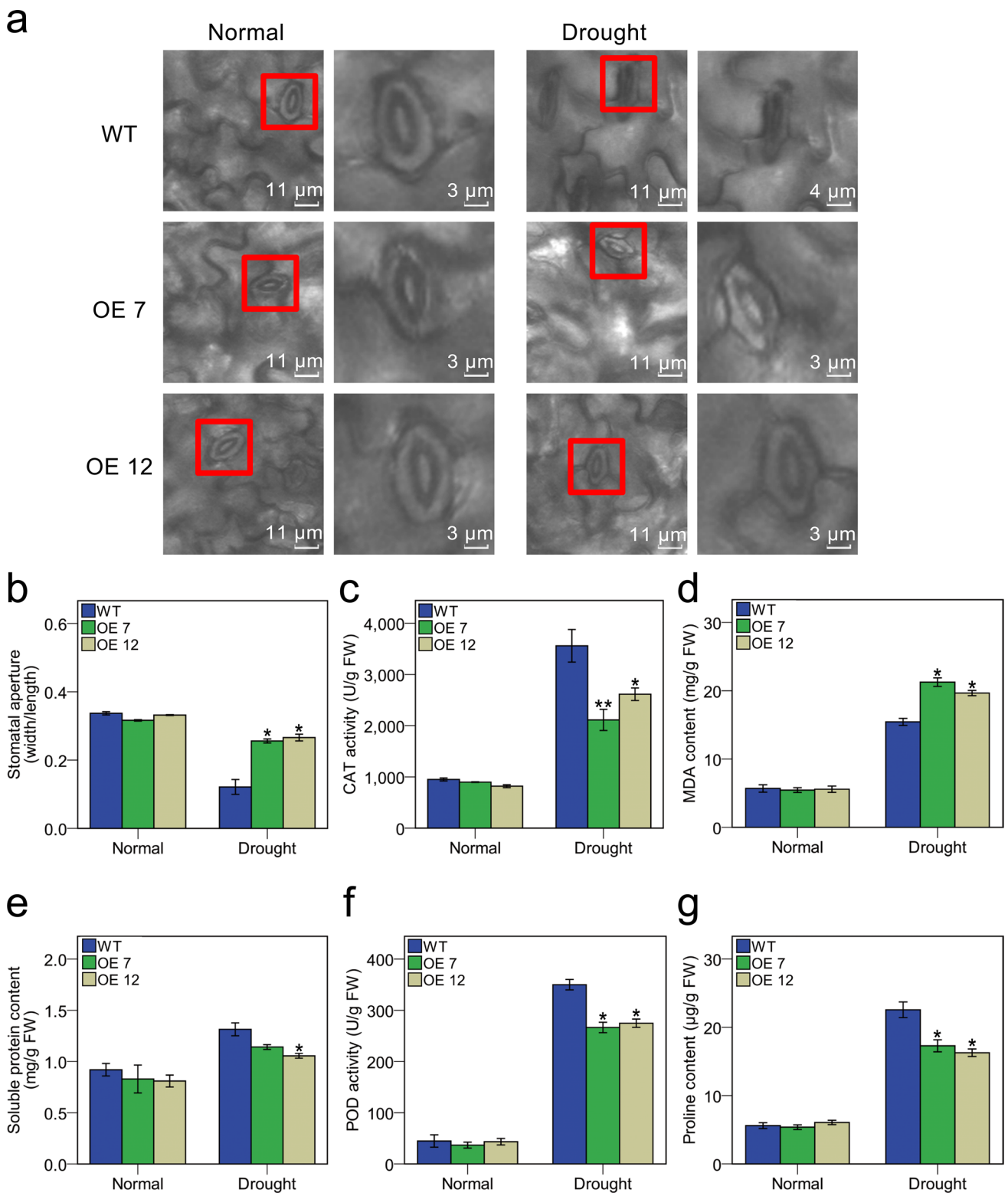


Fig. 3 Overexpression of *TaMYB44-5A* reduced drought tolerance in *Arabidopsis*. **a** Identification of *TaMYB44-5A* overexpression lines by PCR. *AtActin2* was used as a background control. **b** Phenotypic changes in WT and transgenic lines before and after drought stress, and after re-watering. The white scale bars represent a length of 1 cm. **c** Survival rates of WT and transgenic lines after drought stress. **d** Under normal growth conditions, the rosette leaves from WT and transgenic lines were used to measure the water loss rate. **e** Relative water contents of rosette leaves from WT and transgenic lines before and after drought stress. Data represent mean values ± standard deviations based on three independent replicates. **Highly significant difference ($P < 0.01$), *significant difference ($P < 0.05$)

similar functions in response to drought. Subsequently, we analyzed the expression of *TaMYB44-5A*, *TaMYB44-5B*, and *TaMYB44-5D* in different tissues, and observed that *TaMYB44-5A* exhibits high-level expression in both root and leaf (Fig. 1e).

To detect the possible regulatory functions of *TaMYB44*s, we determined their subcellular localization. The three proteins were fused to the N-terminal of red fluorescent protein (RFP) and transformed into *Nicotiana benthamiana* leaves. The red fluorescent signals of TaMYB44-5A:RFP, TaMYB44-5B:RFP, and TaMYB44-5D:RFP were detected in the nucleus (Fig. 2). Their co-localization with green fluorescent protein (GFP) and nuclear protein TaFDL2-1A (TaFDL2-1A:GFP) confirmed that TaMYB44-5A,



TaMYB44-5B, and TaMYB44-5D may function in the nucleus. Therefore, based on the expression patterns and subcellular localizations of *TaMYB44*s, we selected *TaMYB44-5A* for further study.

Overexpression of *TaMYB44-5A* reduced the tolerance of drought in transgenic *Arabidopsis*

TaMYB44-5A overexpression lines were created in *Arabidopsis*, and then we selected *TaMYB44-5A* overexpression

◂**Fig. 4** *TaMYB44-5A* can alter the stomatal aperture and physiological indicators related to plant drought tolerance in transgenic *Arabidopsis* under drought stress. **a**, **b** Rosette leaves of WT and transgenic lines before and after drought stress, and stomata photographed at different resolutions. Stomatal aperture was calculated as the specific value of width compared with length. Data are presented as the mean ± SE based on three independent biological replicates, and 10 stomata were observed per experiment. The corresponding scale bars are shown in the figures. **c** CAT activities in WT and transgenic lines under normal and drought conditions. **d** MDA contents of WT and transgenic lines under normal and drought conditions. **e** Soluble protein contents in WT and transgenic lines under normal and drought conditions. **f** POD activities in WT and transgenic lines under normal and drought conditions. **g** Proline content in WT and transgenic lines under normal and drought conditions. Data represent mean values ± standard deviations based on three independent replicates. **Highly significant difference ($P < 0.01$), *significant difference ($P < 0.05$)

line 7 (OE7) and OE12 for the highest *TaMYB44-5A* expression as follow-up research objects (Fig. 3a and Supplementary Fig. S3). After drought treatment, the rosette leaves of wild type (WT), OE7, and OE12 were severely damaged, with leaf curling or yellowing (Fig. 3b). Compared with WT, the OE7 and OE12 lines exhibited greater mortality (Fig. 3c). Subsequent analysis showed that the water loss rate by the overexpression lines (OE7 and OE12) was significantly higher than that of WT (Fig. 3d). The contents of proline and soluble protein, CAT enzyme activity, and peroxidase (POD) enzyme activity were lower in the OE lines than WT, and the malondialdehyde (MDA) contents was higher than those in WT (Fig. 4c–g).

Leaves are the main transpiration organs of plants, so we then examined the physiological status of the rosette leaves. After drought treatment, the leaf water content was significantly higher in WT than in overexpression lines (Fig. 3e), and the stomatal aperture was maintained at a high level in the overexpression lines (Fig. 4a, b). These results indicate that the overexpression of *TaMYB44-5A* reduced the tolerance of drought in *Arabidopsis*.

ABA-induced *TaMYB44-5A* might decrease ABA sensitivity in *Arabidopsis*

The ABA signaling pathway regulates the stomatal aperture under drought conditions (Aalto et al. 2012). To determine whether *TaMYB44-5A* is involved in the ABA signaling pathway, we conducted qRT-PCR and ABA sensitivity assays. The expression of *TaMYB44-5A* in Chinese Spring wheat treated with exogenous ABA increased gradually over time (Fig. 5c), indicating that *TaMYB44-5A* responds to exogenous ABA treatment. Subsequently, we assessed the percentage of green cotyledons under both ABA treatment and control conditions. The number of seedlings with green cotyledons was significantly higher in the OE7 and OE12 lines compared to WT under ABA treatment, while there was no significant difference between OE7, OE12, and WT under control conditions (Fig. 5a, b). These results suggest that ABA-induced overexpression of *TaMYB44-5A* might reduce the sensitivity of transgenic *Arabidopsis* to ABA.

To identify the function of *TaMYB44-5A* in the ABA signaling pathway under drought conditions, we conducted qRT-PCR to determine the expression levels of several drought- and ABA-responsive genes comprising *AtP5CS1*, *AtRD22*, *AtRAB18*, ABA-responsive element-binding protein 3 (*AtAREB3*), ABA insensitive 5 (*AtABI5*), and senescence-associated gene 29 (*AtSAG29*). The expression levels of *AtP5CS1* and *AtRD22* were all significantly lower in transgenic *Arabidopsis* compared with WT under drought treatment (Fig. 6b, c). However, except for the significant downregulation of *AtSAG29* in OE7, there were no differences in the expression levels of the other genes (Fig. 6a, d, e, f). We performed a dual-luciferase assay to confirm whether TaMYB44-5A could directly regulate the transcriptional levels of *AtP5CS1*, *AtRD22*, and *AtSAG29* (Supplementary Fig. S4a). However, only the transcriptional level of *AtRD22* was significantly repressed by TaMYB44-5A (Supplementary Fig. S4b). These findings also indicate that TaMYB44-5A may negatively regulate drought tolerance by repressing the expression of drought- and ABA-responsive genes.

Transcriptional regulation assay of TaMYB44-5A

A previous study demonstrated that OsMYB102 is an R2R3-MYB transcription factor that can directly bind to the MBS element in the promoter to regulate the expression of rice (*Oryza sativa* L.) cyclophylin A-like protein 707-6 (*OsCYP707A6*) (Piao et al. 2019a). Thus, we examined the autoactivation activity of TaMYB44-5A using the yeast system. Similar to BD-empty, BD-*TaMYB44-5A* could grow on SD/-Trp medium but could not survive on SD/-Trp/-His/-Ade medium, indicating that BD-*TaMYB44-5A* could not independently activate the synthesis of His or Ade. Therefore, TaMYB44-5A does not possess autoactivation activity (Supplementary Fig. S5a and b).

Next, we performed a dual-luciferase assay to confirm that TaMYB44-5A can mediate changes in the transcription level of *TaRD22-3A* (the homologous gene of *AtRD22* in wheat) (Liu et al. 2019). TaMYB44-5A significantly reduced the relative luciferase activity in the Reporter compared with the empty effector (Fig. 7a). Furthermore, we found two MBS motifs in the promoter within 1000 bp of *TaRD22-3A* (Fig. 7c). Therefore, we speculated whether TaMYB44-5A regulates the transcription level of *TaRD22-3A* by directly binding to the MBS motif.

Therefore, we performed yeast one-hybrid assays (Fig. 7b). In SD/-Ura/-Leu/ + AbA medium, empty pGADT7 was significantly nonviable whereas the

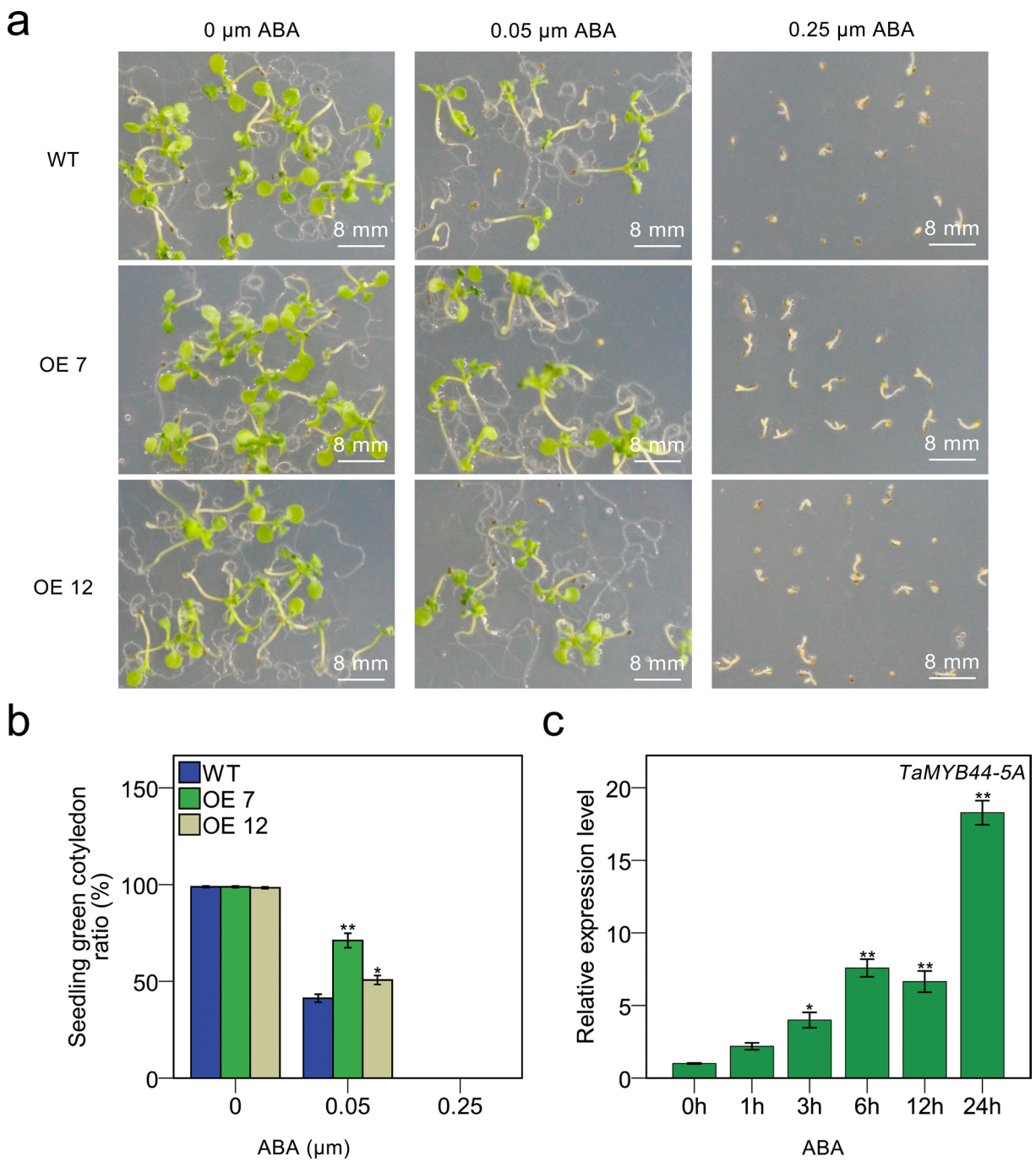


Fig. 5 *TaMYB44-5A* attenuated the sensitivity of transgenic lines to ABA. **a** Seedling establishment for *TaMYB44-5A* overexpression and WT plants in normal, 0.25 μM ABA, and 0.75 μM ABA conditions. **b** Seedling greening ratios are shown for the plants reported in **a**. **c** Expression levels of *TaMYB44-5A* under ABA treatments. Data represent mean values ± standard deviations based on three independent replicates. The reference for significance analysis is the data at 0 h. **Highly significant difference ($P < 0.01$), *significant difference ($P < 0.05$)

other pAbAi-MBS strains grew very well, although the AACAG-type strain was slightly less viable (Fig. 7d).

Subsequent analysis by EMSA confirmed our speculation. The migration band exhibited a decrease trend in specificity only in group P1 as the unlabeled probe concentration increased (Fig. 7c, e). Our results indicate that TaMYB44-5A can directly mediate the transcription level of *TaRD22-3A*, and the changes in its transcription level were consistent with the expression level of homologous *AtRD22* in transgenic *Arabidopsis* with reduced drought tolerance.

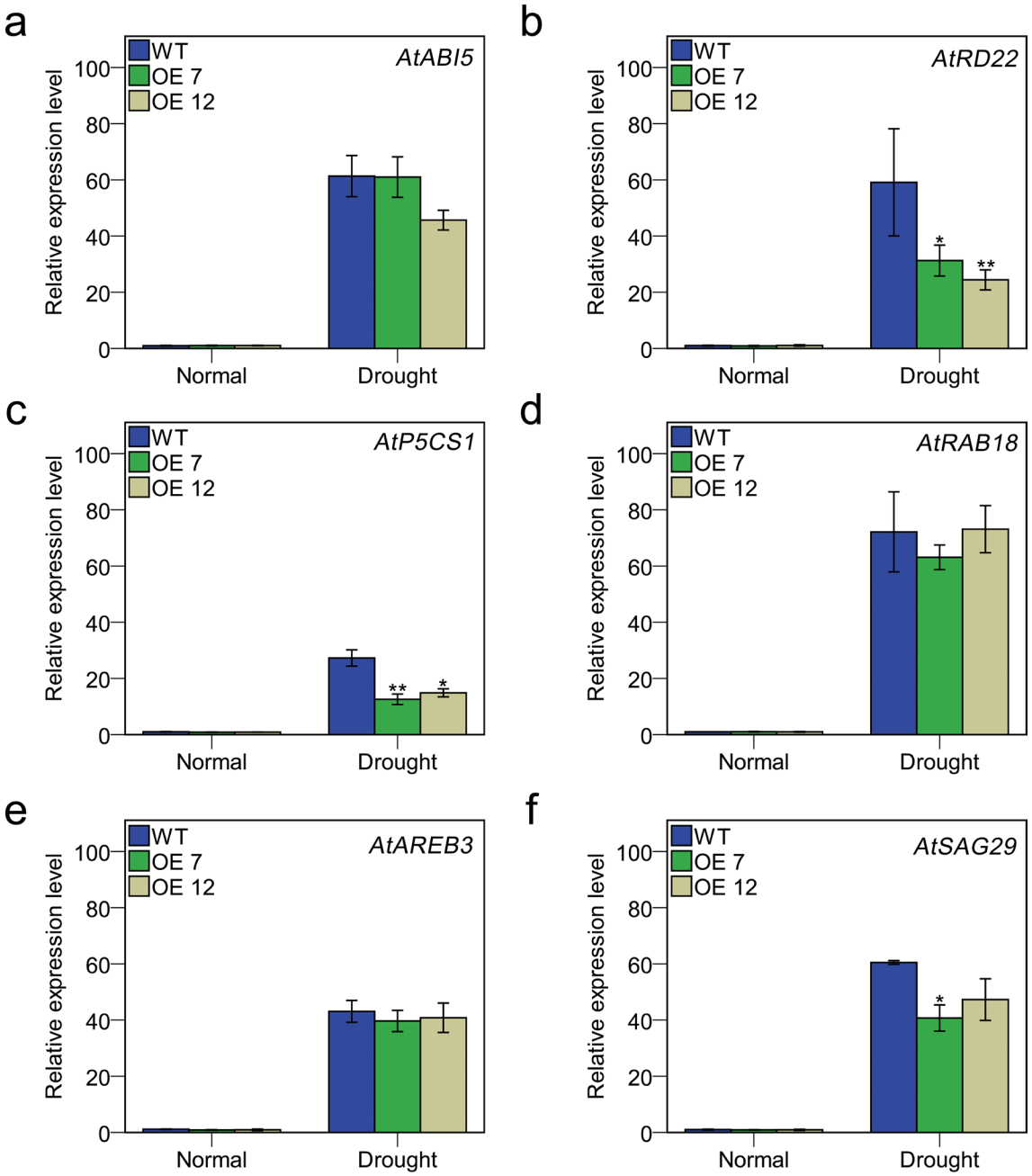


Fig. 6 *TaMYB44-5A* reduced the expression of ABA-responsive genes. **a–f** Expression levels of *AtABI5*, *AtRD22*, *AtP5CS1*, *AtRAB18*, *AtAREB3*, and *AtSAG29* in WT and transgenic lines under normal and drought conditions. Data represent mean values ± standard deviations based on three independent replicates. **Highly significant difference ($P < 0.01$), *significant difference ($P < 0.05$)

Discussion

Drought can severely damage sessile plants but plants have also evolved molecular mechanisms to cope with drought stress (Zhu 2016). These molecular mechanisms typically involve transcriptional activators or repressors induced by drought. Therefore, identifying drought-associated transcription factors is crucial for modern molecular breeding (Paux et al. 2022; Han et al. 2023). For example, wheat (*Triticum aestivum* L.) N-acetylcysteine 071-A (*TaNAC071-A*) promotes the expression of genes related to water use efficiency to improve the drought tolerance of transgenic wheat (Mao et al. 2022). Similarly, the expression level of *OsMYB2* was significantly increased in *OsWRKY5*-knockout transgenic rice to enhance tolerance of drought (Lim et al. 2022). The R2R3-MYB transcription factor in sessile plants is

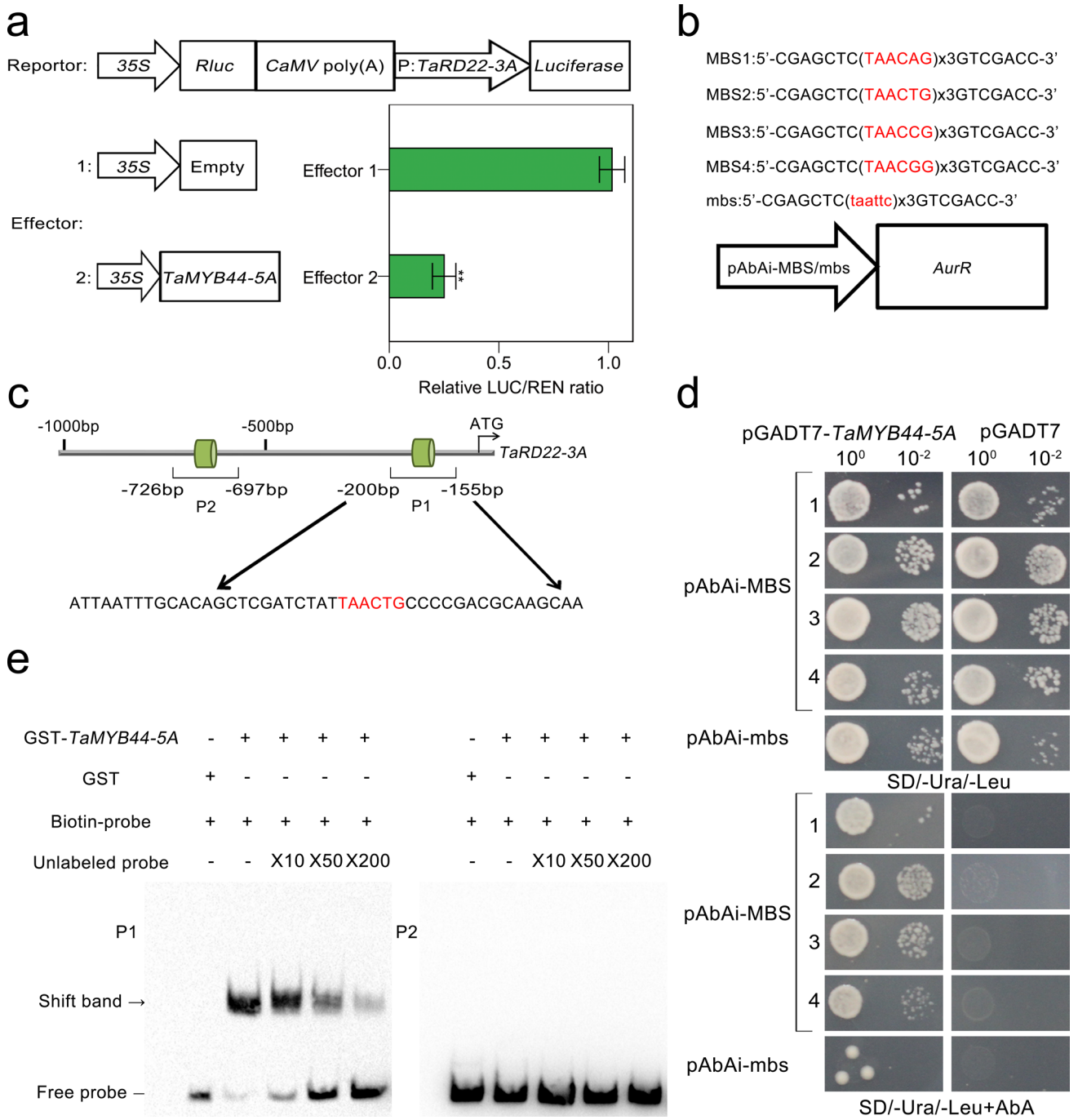


Fig. 7 Transcriptional regulation assay for *TaMYB44-5A*. **a** The promoter of *TaRD22-3A* was fused upstream of the luciferase gene in the pGreenII 0800-LUC vector. *TaMYB44-5A* was fused into the pGreenII 62-SK vector and empty pGreenII 62-SK was used as a control. **b** Three consecutive copies of AACNG (MBS) and mutant taattc (mbs) were fused to the pAbAi vector. *TaMYB44-5A* was fused to the pGADT7 vector and the unmodified pGADT7 was used as a control. The corresponding vectors were co-transformed into the Y1H Gold strain. **c** Distribution of MBS within 1 kb upstream of *TaRD22-3A* promoter. **d** 1–4 correspond to MBS1-4. The yeast suspension was dropped on SD/-Ura/-Leu and SD/-Ura/-Leu + AbA (500 ng/mL) medium at two concentrations. **e** The probes used in EMSA are indicated by green columns. Purified GST-TaMYB44-5A protein and partial fragment promoter of *TaRD22-3A*. "+" and "–" indicate that the components were added or not added; "×10," "×50," and "×200" denote tenfold, 50-fold, and 200-fold molar excesses of unlabeled probe relative to biotin probe, respectively. Data represent mean values ± standard deviations based on three independent replicates. **Highly significant difference ($P < 0.01$)

considered a good candidate gene for improving the drought tolerance of plants (Dubos et al. 2010). In the present study, we found that *TaMYB44-5A* (R2R3-MYB) overexpression reduced drought tolerance by repressing the genes associated with the ABA response in transgenic *Arabidopsis* (Figs. 3b and 6).

Physiological indexes related to drought combined with the identification test of drought tolerance can more comprehensively study the specific biological functions of target genes (Luna et al. 2005; Chenu et al. 2017; Langridge and Reynolds 2021; Li et al. 2022; Yao et al. 2022). Wheat accumulates a large amount of hydrogen peroxide under drought conditions, and CAT and POD can remove the accumulated hydrogen peroxide in wheat (Luna et al. 2005). Overexpression of *CAT1/2* can reduce the accumulation rate of hydrogen peroxide in wheat under severe drought condition, to improve the drought tolerance of wheat (Luna et al. 2005). *StNAC053* in potatoes can enhance drought tolerance and salt tolerance of transgenic *Arabidopsis* by increasing POD enzyme activity (Wang et al. 2021b). The MDA content represents the stability of the cell membrane after the plant is subjected to drought stress (Wang et al. 2022). Overexpression of *TaFDL2-1A* can attenuate the degree of drought stress damage to the wheat cell membrane system (Wang et al. 2022). Osmotic adjustment substances can help plants maintain intracellular osmotic pressure under drought stress. *TaNAC071-A* can increase the proline content of wheat to help wheat maintain a higher level of photosynthesis under water deficit (Mao et al. 2022). In this study, lower CAT enzyme activity, POD enzyme activity, and osmolyte content demonstrated that *TaMYB44-5A* negatively regulated the drought tolerance of transgenic *Arabidopsis* (Fig. 4c, e, f, g). Simultaneously, the higher MDA content proved that the overexpression of *TaMYB44-5A* reduced the stability of the transgenic *Arabidopsis* cell membrane system under drought stress (Fig. 4d). Therefore, the drought tolerance of *Arabidopsis* overexpressing *TaMYB44-5A* was weaker.

The ABA signaling pathway is one of the most critical pathways that allow plants to respond to drought stress (Jaradat et al. 2013; Zhu 2016; Boija et al. 2018). In this pathway, many transcription factors are induced to upregulate the expression levels of genes and then activate the downstream drought tolerance mechanism (Nakashima and Yamaguchi-Shinozaki 2013). ABA-induced soybean (*Glycine max* L.) WRKY16 can promote the expression of *RD29A* to enhance drought tolerance in transgenic plants (Ma et al. 2018). Differently, *AtMYB44* can be induced to express at high levels by exogenous ABA treatment, and it inhibits the expression of downstream responsive genes in the ABA signaling pathway. Therefore, researchers believe that *AtMYB44* can sustain the growth of *Arabidopsis* under stress conditions (Jaradat et al. 2013). In this study, we have shown that *TaMYB44-5A* also can be induced by ABA treatment and inhibited the ABA further response (Fig. 5). Previous studies demonstrated that *RD22* is a downstream component induced by ABA in response to drought stress and it enhances drought tolerance in plants (Goh et al. 2003; Wang et al. 2012). In the present study, the transcriptional level of *AtRD22* was significantly reduced (Fig. 6b). The subsequent LUC and binding assays also confirmed the role of *TaMYB44-5A* in the direct inhibition of *TaRD22-3A* (Fig. 7a–d). Thus, the mechanism that allows *TaMYB44-5A* to negatively regulate drought tolerance is mediated through the ABA signaling pathway.

Stomata on the leaves are important organs for controlling water loss by transpiration under drought stress. Plants have evolved many mechanisms to regulate stomatal aperture and prevent the excessive loss of water under drought conditions, such as stomatal closure controlled by the ABA signaling pathway (Goh et al. 2003; Aalto et al. 2012). *TaFDL2-1A* overexpression in wheat increases the sensitivity of transgenic wheat to ABA and leads to ABA-dependent stomatal closure (Wang et al. 2022). In the present study, compared with WT, the sensitivity to ABA was significantly lower in transgenic *Arabidopsis* (Fig. 5a, b), and drought treatment resulted in significantly increased stomatal aperture (Fig. 4a, b). In a previous study, *RD22* was shown to regulate stomatal movement in *Arabidopsis* under drought conditions in an ABA-dependent manner (Goh et al. 2003; Wang et al. 2012). Therefore, we speculate that *TaMYB44-5A* may be involved in ABA-dependent stomatal movement.

Transcription factors recognize and bind to specific motifs in the promoters of downstream genes to regulate stress signal transduction and stress-response networks. Many R2R3-MYB transcription factors recognize and bind to AAC and TTG core motifs, including TAACNG (MBSI) and A/GTTGA/TT (MBSII), before then regulating the expression of downstream genes (Dubos et al. 2010; Shim and Choi 2013; Piao et al. 2019a; Fu et al. 2020). Previous studies based on protein-binding microarray analysis found that *AtMYB44* can specifically bind to the AACNG motif. In addition, analysis using a random promoter fragment showed that sequences with migrating bands in EMSA experiments all contained AACNG (Jung et al. 2012). Therefore, we directly tested all types of TAACNG using the yeast one-hybrid system (Fig. 7b, d) and, as expected, the results showed that TaMYB44-5A could recognize and bind to the AACNG-specific motif. Furthermore, subsequent EMSA assays demonstrated that TaMYB44-5A could specifically bind to the MBS element in the *TaRD22-3A* promoter (Fig. 7e). The AACNG motif is widely distributed in promoter sequences in the wheat genome (International Wheat Genome Sequencing 2018), so TaMYB44-5A may reduce the expression of drought- and ABA-induced genes in wheat by directly binding to this motif.

In summary, we found that *TaMYB44-5A* encoding an R2R3-MYB could reduce the tolerance of drought and sensitivity to ABA in transgenic *Arabidopsis*, and it regulated the expression levels of downstream genes related to drought tolerance. Furthermore, TaMYB44-5A bound specifically to the MBS element in the promoter of *TaRD22-3A* to respond to ABA-mediated drought stress. Therefore, TaMYB44-5A may act as a negative regulator in response to drought stress via the ABA signaling pathway in wheat.

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Author contribution DP and LL conducted the study, analyzed data, and wrote the manuscript. AW, ML and YL collected the samples. LZ and BW contributed to the development of materials. XL and YX conceived and designed the study, and revised the manuscript. All the authors read and approved the final manuscript.

Data availability Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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