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ORIGINAL ARTICLE

Genome-wide identification of the *MED25 BINDING RING-H2 PROTEIN* gene family in foxtail millet (*Setaria italica* L.) and the role of *SiMBR2* in resistance to abiotic stress in Arabidopsis

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Abstract

Main conclusion **The *SiMBR* genes in foxtail millet were identified and studied. Heterologous expression of *SiMBR2* in Arabidopsis can improve plant tolerance to drought stress by decreasing the level of reactive oxygen species.**

Abstract Foxtail millet (*Setaria italica* L.), a C4 crop recognized for its exceptional resistance to drought stress, presents an opportunity to improve the genetic resilience of other crops by examining its unique stress response genes and understanding the underlying molecular mechanisms of drought tolerance. In our previous study, we identified several genes linked to drought stress by transcriptome analysis, including *SiMBR2* (*Seita.7G226600*), a member of the *MED25 BINDING RING-H2 PROTEIN* (*MBR*) gene family, which is related to protein ubiquitination. Here, we have identified ten *SiMBR* genes in foxtail millet and conducted analyses of their structural characteristics, chromosomal locations, cis-acting regulatory elements within their promoters, and predicted transcription patterns specific to various tissues or developmental stages using bioinformatic approaches. Further investigation of the stress response of *SiMBR2* revealed that its transcription is induced by treatments with salicylic acid and gibberellic acid, as well as by salt and osmotic stresses, while exposure to high or low temperatures led to a decrease in its transcription levels. Heterologous expression of *SiMBR2* in *Arabidopsis thaliana* enhanced the plant's tolerance to water deficit by reducing the accumulation of reactive oxygen species under drought stress. In summary, this study provides support for exploring the molecular mechanisms associated with drought resistance of *SiMBR* genes in foxtail millet and contributing to genetic improvement and molecular breeding in other crops.

Keywords Drought stress · *MBR* gene family; RING-H2 protein · ROS · Transcription pattern · *Setaria italica*

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Abbreviations

CAT	Catalase
MBR	*MED25-BINDING RING-H2 PROTEIN*
MDA	Malondialdehyde
POD	Peroxidase
SA	Salicylic acid

Introduction

Foxtail millet (*Setaria italica* L.) is a crop that originated in China and has been cultivated for over 8000 years (Diao et al. 2014). It serves as an important food and forage in the arid and semi-arid regions of Asia (He et al. 2015). Foxtail millet has become an important model C4 crop for studying stress resistance due to its strong drought tolerance, small gene pool (515 Mb), self-pollination and short life cycle (He

et al. 2015; Peng and Zhang 2020; Fan et al. 2021; Li et al. 2022; Liu et al. 2023; Wen et al. 2024).

The ubiquitin–proteasome system (UPS) is a main way to break down proteins, which is important for changing the activity of proteins that control gene activity and is part of many signalling pathways (Zhou et al. 2024). In plants, the UPS facilitates plant response to internal and external stimuli by rapidly regulating its proteome and plays a crucial role in regulating plant growth, development, and physiology (Chen and Hellmann 2013; Mandal et al. 2018; Xu and Xue 2019). The UPS is characterized by the 26S proteasome and a triad of enzymes responsible for substrate protein ubiquitination: E1 (ubiquitin-activating), E2 (ubiquitin-conjugating), and E3 (ubiquitin ligase) enzymes. E3 ligases mainly choose which proteins to break down, moving ubiquitin from the E2-UPS complex to the chosen proteins (Mandal et al. 2018; Liu et al. 2021). The E3 ligases involve four main types, namely HECT (homologous to the E6-AP carboxyl terminus), RING (really interesting new gene), U-box, and cullin-RING ligases (CRL) (Chen and Hellmann 2013). RING proteins have a region with many cysteine amino acids, about 40–60 in length. In Arabidopsis, the genome encodes over 460 RING proteins, categorized into seven subtypes based on structural motifs: RING-H2, RING-HC, RING-v, RING-C2, RING-D, RING-S/T, and RING-G (Stone et al. 2005; Sun et al. 2019; Han et al. 2022).

Among Arabidopsis RING proteins, Iñigo et al. (2012) reported a RING-H2 group protein that binds to MEDIATOR25 (MED25)/PHYTOCHROME AND FLOWERING TIME1 (PFT1). This led to their naming as MED25-BINDING RING-H2 PROTEIN (MBR). MBR proteins contain the RING-H2 domain close to its C-terminus. Based on protein structure and sequence similarity, four members belong to this small group in Arabidopsis (Iñigo et al. 2012; Zhang et al. 2017b). MBR1 and MBR2 interact with MED25 and promote the degradation of MED25, which delays the flowering time of plants (Iñigo et al. 2012). Furthermore, these MBRs interact with the transcriptional repressor TCP INTERACTOR-CONTAINING EAR MOTIF PROTEIN1 (TIE1), contributing to its degradation and promoting leaf development (Zhang et al. 2017b).

At present, the *SiMBR* gene family in foxtail millet has received limited research attention. In our earlier study, we compared the transcriptomes among three foxtail millet cultivars with varying drought resistance and found 46 genes linked to drought resistance (Guo et al. 2022). One of these genes, *Seita.7G226600* (*SiMBR2*), is part of the MBR gene family related to the protein ubiquitination system in foxtail millet. Under drought stress, its transcription level decreases. To better understand the role of *SiMBR2* in abiotic stress responses, we retrieved and characterized the *SiMBR* gene family from the foxtail millet database, identifying ten members. We examined their phylogenetic relationships, motifs, chromosome locations, gene structures, promoter regions, and predicted expression patterns across different tissues. Additionally, we studied how SiMBR2 responds at the transcriptional level to various abiotic stresses and hormone treatments. In Arabidopsis, over-expression of *SiMBR2* improved plant resistance to drought stress by improving its capacity to neutralize reactive oxygen species (ROS). Our findings support further research into the molecular mechanisms behind *SiMBR* gene-related drought resistance in foxtail millet and could help improve genetics and breeding methods for other crops.

Materials and methods

Identification of genes from the *SiMBR* family in foxtail millet

The complete foxtail millet genome was obtained from the Phytozome database (https://phytozome-next.jgi.doe.gov/, accessed on 30 July 2023; Goodstein et al. 2012). The Arabidopsis *MBR* gene sequences (Iñigo et al. 2012) were submitted to the Pfam database (http://pfam.sanger.ac.uk/, accessed on 30 July 2023) to obtain Pfam ID PF13639 of the MBR Ring finger domain and download the Hidden Markov Model (HMM) profile. Utilizing the TBtools software (version 2.056; Chen et al. 2020), the Simple HMM Search command was employed to scan the complete protein sequences of foxtail millet, with a selection threshold of candidate genes set at an E-value of 1e-4. After removing all redundant sequences, the obtained candidate genes were submitted to the SMART (http://smart.embl-heidelberg.de/, accessed on 30 July 2023) and the National Center for Biotechnology Information Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi, accessed on 30 July 2023) for further confirmation of *MBR* gene family members. The *MBR* genes in other plants (*Brachypodium distachyon, Hordeum vulgare, Sorghum bicolor, Oryza sativa,* and *Zea mays*) were also preliminarily identified using the methods mentioned above.

The SiMBR proteins were further analyzed using ExPASy (https://web.expasy.org/protparam/, accessed on 1 August 2023) to determine their theoretical isoelectric point (pI), molecular weight (MW), and amino acid composition based on protein sequence data (Gasteiger et al. 2003). WoLF PSORT (https://wolfpsort.hgc.jp, accessed on 1 August 2023) was applied to predict the subcellular localization of these proteins (Horton et al. 2007).

Phylogenetic analysis and chromosome location of genes in the *SiMBR* family

Candidate *SiMBR* genes were extracted from the Phytozome database, which provided chromosomal positioning of the genes. The physical map was subsequently generated using

TBtools software (Chen et al. 2020). Phylogenetic trees were constructed using neighbor-joining (NJ) methods and generated after 1000 bootstrap replications using MEGA 7.0 software (Kumar et al. 2016).

Motif composition and gene structure analysis of *SiMBR* family genes

The Multiple EM for Motif Elicitation (MEME) online tool (https://meme-suite.org/meme/tools/meme, accessed on 30 July 2023) was utilized to detect motifs within the candidate SiMBR proteins (Bailey et al. 2009). A MEME search was conducted with the following specifications: the number of repetitions is any and a maximum of 20 motifs, and the motif composition was drawn using TBtools software (Chen et al. 2020). Candidate *SiMBR* gene annotations were extracted from the Phytozome database, from which the genes in exon–intron sequence information were obtained. The exon/intron structure of each *SiMBR* gene was analyzed using the online Gene Structure Display Server 2.0 (https://gsds.gao-lab.org/, accessed on 30 July 2023; Hu et al. 2015).

Cis-acting element analyses and tissue expression patterns of *SiMBR* family genes

Then PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 31 July 2023; Lescot et al. 2002) was used to analyze the 2000 bp upstream cis-acting elements in the *SiMBR* genes. Finally, the results were visualized using TBtools software (Chen et al. 2020). The transcriptomic data for roots, stems, leaves, germinated grains and grains during the filling period S1-S5 (corresponding to the early, early, middle, late, and late filling stages, respectively) were downloaded from the MDSi database (https://foxtail-millet.biocloud.net/home, accessed on 1 August 2023; Li et al. 2023). Utilizing Transcripts Per Kilobase per Million mapped reads data, a heat map representing the expression levels of *SiMBR* genes across various tissues was generated with TBtools software (Chen et al. 2020).

Plant growth conditions and treatment

In the current study, *Setaria italica* "Jingu21" was utilized. Seeds were sourced from Prof. Lizhen Zhang's laboratory at the School of Life Science, Shanxi University, China. The seeds were cultivated in plastic pots filled with a 1:1 mixture of vermiculite and nutrient soil under the following conditions: a temperature range of 23–26 °C, light intensity of 50,000 Lux, relative humidity between 30 and 50%, and a photoperiod 16 h of light/8 h of darkness. At the two-leaf stage, four days post-germination, uniform seedlings were selected and transferred to individual plastic pots, with three plants per pot. For the temperature stress experiments, after a 14-day growth period, "Jingu 21" seedlings were subjected to heat stress (40 °C day /32 °C night) and cold stress (4 °C), for 0 and 24 h in a controlled temperature incubator. To induce long-term drought stress, watering was discontinued for an additional two weeks following the initial 14-day growth period. For other abiotic stresses and phytohormone treatment, the seedlings of "Jingu 21" grown for ten days were transferred to 1/2 MS liquid medium for four days and then treated with salt (200 mmol/l NaCl), PEG6000 (15%), $NaHCO_3$ (150 mmol/l), ABA (100 µmol/l), 6-BA (75 µmol/l), IAA (10 µmol/l), α-naphthaleneacetic acid (NAA; 10 nmol/l), gibberellic acid (GA_3; 1 mmol/l), methyljasmonat (MJ; 100 µmol/l), and salicylic acid (SA; 10 mmol/l) for 0 and 24 h. Each stress treatment had three replicates. Leaf samples were collected and immediately frozen in liquid nitrogen, then stored at –80 °C to facilitate RNA extraction later.

RNA extraction and qRT-PCR

The transcription levels of *SiMBR2* and antioxidant enzyme genes (*AtCAT1*, *AtPOD1* and *AtCSD1*) in plants were detected by qRT-PCR. Total RNA Extraction and qRT-PCR were performed as described before (Zhang et al. 2022). Total RNA was isolated from leaf tissues using the TransZol™ UP Plus RNA Kit (TransGen Biotech, Beijing, China). The extracted RNA was reverse-transcribed into cDNA using the EasyScript® One-step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech). The qRT-PCR reaction mixture had a total volume of 10 µl, comprising 5 µl of TransStart® Tip Green qPCR SuperMix (TransGen Biotech), 1 µl of diluted cDNA template, 0.8 µl of each primer (10 µmol/l), and 3.2 µl of RNase free ddH_2O. The qRT-PCR thermal cycling conditions were set as 94℃ for 30 s, followed by 45 cycles of 94℃ for 15 s and 58℃ for 30 s. All primers (as listed in Suppl. Table S1) were synthesized by Shanghai Sangon Biotech. For foxtail millet, *SiAct2* (*Seita.8G043100*) and *RNA POL II* (*Seita.2G142700*) served as internal controls (Kumar et al. 2013; Zhang et al. 2022). For Arabidopsis, *AtACT2* (*AT3G18780*) and *AtTIP41-like* (*TAP42 239 INTERACTING PROTEIN OF 41 kDa, AT4G34270*) were used as internal reference (Czechowski et al. 2005; Škiljaica et al. 2022). Each experiment was performed with three technical replicates and three biological replicates.

Clone of *SiMBR2*

The open reading frames of *SiMBR2* (*Seita.7G226600*) were amplified from "Jingu21" using gene-specific primers designed with attB1/B2 Gateway attachment sites. The BP reactions in pDONR207 (Invitrogen) produced Entry clones, confirmed by sequencing provided by Sangon Biotech

(Shanghai, China). The destination clones were created using LR Clonase II (Invitrogen) through an LR reaction. For phenotypic analysis in Arabidopsis, the construct was transferred into the pUBN-GFP-Dest vector via LR reaction, as described before (Grefen et al. 2010).

Generation of transgenic Arabidopsis plants and physiology analysis

Stable Columbia-0 Arabidopsis lines expressing *SiMBR2* were obtained through floral dipping (Clough and Bent 1998; Zhang et al. 2015) with Agrobacterium GV3101 carrying pUBN-GFP-SiMBR2, and transformants were selected on a medium containing 50 mg/l Basta (phosphinothricin). The transgene expression in homozygous T3 lines was confirmed by immunoblot analysis, and three independently transformed lines were selected for further experimental use.

Arabidopsis seeds (wild-type and stable transgenic lines) were grown in soil under a 12/12 h (day/night), 23/20 °C (day/night) cycle with 8000 Lux light intensity. Homozygous T3 lines were verified for transgene expression, and three independently transformed lines were chosen for the experiments. For drought stress, watering was stopped for two weeks after four weeks of growth. The water content in the soil was measured by a soil moisture analyzer (TZA-1 K-G, Zhejiang Tuopu Yunnong Technology Co., Ltd). The MDA content (MDA-2-Y), the H_2O_2 content (H_2O_2-2-Y), the glutathione (GSH) content (GSH-2-W), the CAT activity (CAT-2-Y), and the POD activity (POD-2-Y) of the leaf sample were measured using kits purchased from Suzhou Keming Biotechnology co. Ltd (China). To verify protein expression, the immunoblot analysis of plant tissues was performed as described before (Zhang et al. 2017a) using a commercial anti-GFP antibody (Abcam).

Statistical analysis

Statistical analysis of independent experiments is reported as means ± SE as appropriate, with significance determined by Student's t-test or ANOVA.

Results

Identification of *SiMBR* genes in foxtail millet

Based on data of Iñigo et al. (2012), this study got the Arabidopsis MBR gene sequences and used them to receive an HMM profile from the Pfam database. Subsequently, this profile was used to find *MBR* genes in different plants. We found 10 genes in *Setaria italica*, as shown in Table 1. More genes were found in *Oryza sativa* (rice), *Brachypodium distachyon, Zea mays* (maize), *Sorghum bicolor* (sorghum), and *Hordeum vulgare* (barley), as detailed in Suppl. Table S2. The SiMBR proteins in foxtail millet ranged from 347 to 707 amino acids long and had molecular weights between 37.68 and 76.9 kDa. Most of these proteins were predicted to be in the nucleus, except for *SiMBR6* (*Seita.3G158100*), which is in both the nucleus and peroxisomes (Table 1). Like the proteins in Arabidopsis, the SiMBR proteins have a RING domain, either C3H2C3-type or RING-H2 type, that holds

Table 1 Detailed information of predicted *SiMBR* genes in foxtail millet

Gene id	Gene name	Genome position		Chr	Length(aa)	pI	Mw (kDa)	Subcellular localization	Orthologous gene id
		Start	End						
Seita.6G090500	*SiMBR1*	8084538	8089159	Chr6	362	9.24	40.87	Nucleus	*LOC_Os08g14320,LOC_Os04g51400*
Seita.7G226600	*SiMBR2*	29129136	29132418	Chr7	373	8.89	41.12	Nucleus	*LOC_Os04g51400,LOC_Os08g14320*
Seita.1G087400	*SiMBR3*	7774762	7779913	Chr1	576	8.74	64.29	Nucleus	*LOC_Os02g05692*
Seita.4G247100	*SiMBR4*	36977865	36982796	Chr4	580	8.23	64.5	Nucleus	*LOC_Os02g05692,LOC_Os06g48040*
Seita.5G280100	*SiMBR5*	33963948	33969040	Chr5	347	9.39	37.68	Nucleus	*LOC_Os05g47670*
Seita.3G158100	*SiMBR6*	11451163	11454353	Chr3	365	9.65	39.51	Nucleus peroxisomal	*LOC_Os05g47670,LOC_Os01g49770,LOC_Os02g05692,LOC_Os06g48040*
Seita.5G263300	*SiMBR7*	32591418	32599798	Chr5	521	6.22	57.21	Nucleus	*LOC_Os01g47740*
Seita.3G146000	*SiMBR8*	10490525	10493866	Chr3	506	5.86	54.93	Nucleus	*LOC_Os05g48970,LOC_Os01g47740*
Seita.7G032800	*SiMBR9*	10352482	10361641	Chr7	707	6.25	76.9	Nucleus	*LOC_Os04g10680*
Seita.7G262700	*SiMBR10*	31521269	31527177	Chr7	660	6.08	71.97	Nucleus	*LOC_Os04g55510*

two zinc ions with eight metal-binding residues (as illustrated in Fig. S1).

Phylogeny analysis, chromosome location, gene structure, and motif analyses of *SiMBR* genes in foxtail millet

Phylogenetic analysis, using MBR gene protein sequences from various species, showed that MBR proteins are divided into five groups, as shown in Fig. 1. The data also suggested that the *MBR* gene family is conserved across these species, with a similar distribution of genes within each group. The *SiMBR* genes in foxtail millet are closely related to those in maize and sorghum, showing a closer evolutionary connection with these two plants than with the others. This confirms the conservation of the *MBR* gene family and highlights the importance of studying the evolution of plant *MBR* genes to understand their functions in plant biology.

The *SiMBR* genes in foxtail millet are spread across six of its nine chromosomes (Fig. 2). Chromosomes 1, 4, and 6 each have a single gene, while chromosomes 3 and 5 have two genes each, and chromosome 7 harboured three genes. This distribution indicates that the *SiMBR* genes are broadly dispersed throughout various chromosomes in foxtail millet. The analysis of the SiMBR protein motifs found 15 common motifs, illustrated in Fig. 3 (left panel). Each motif shares similar protein sequences (Fig. S2). The motif 1 contains the C3H2C3-type or RING-H2-type Zn binding site. Depending on motifs, these proteins can also be classified into five groups based on their similarities. Similarly, genes in the same group had similar numbers of introns and exons, further supporting the classification from the phylogenetic tree shown in Fig. 3. These results give more information on the structure and evolution of *SiMBR* genes in foxtail millet.

Cis-acting regulatory elements analysis of *SiMBR* genes in foxtail millet

To understand how *SiMBR* genes are controlled, we looked at the 5' upstream region of each gene for cis-acting elements. We found 35 different cis-elements related to resistance mechanisms, as summarized in Fig. 4 and Suppl.

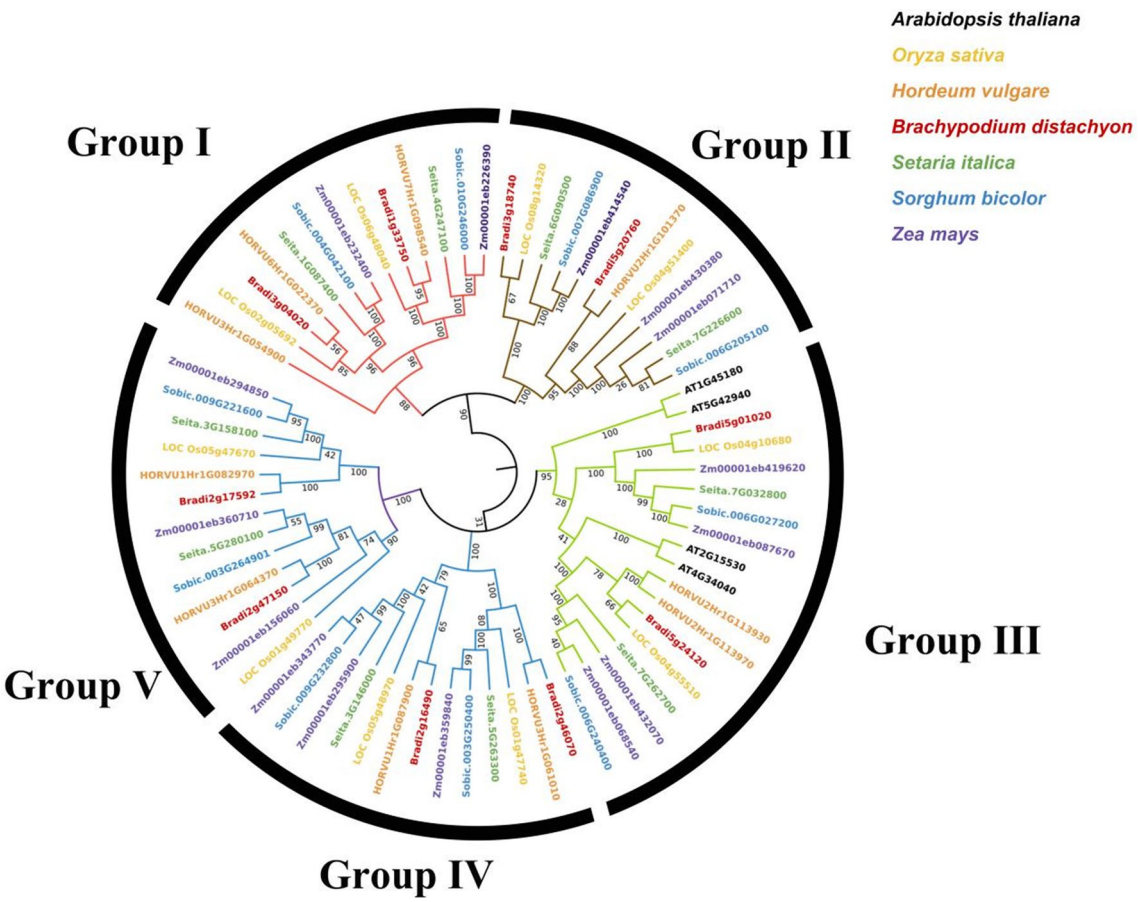

Fig. 1 Phylogenetic analysis and classification of MBR proteins from Arabidopsis (black), *Setaria italica* (green), *Oryza sativa* (yellow), *Hordeum vulgare* (khaki), *Brachpodium distachyon* (red), *Sorghum bicolor* (blue), and *Zea mays* (purple). MBR2 proteins are divided into five distinct groups

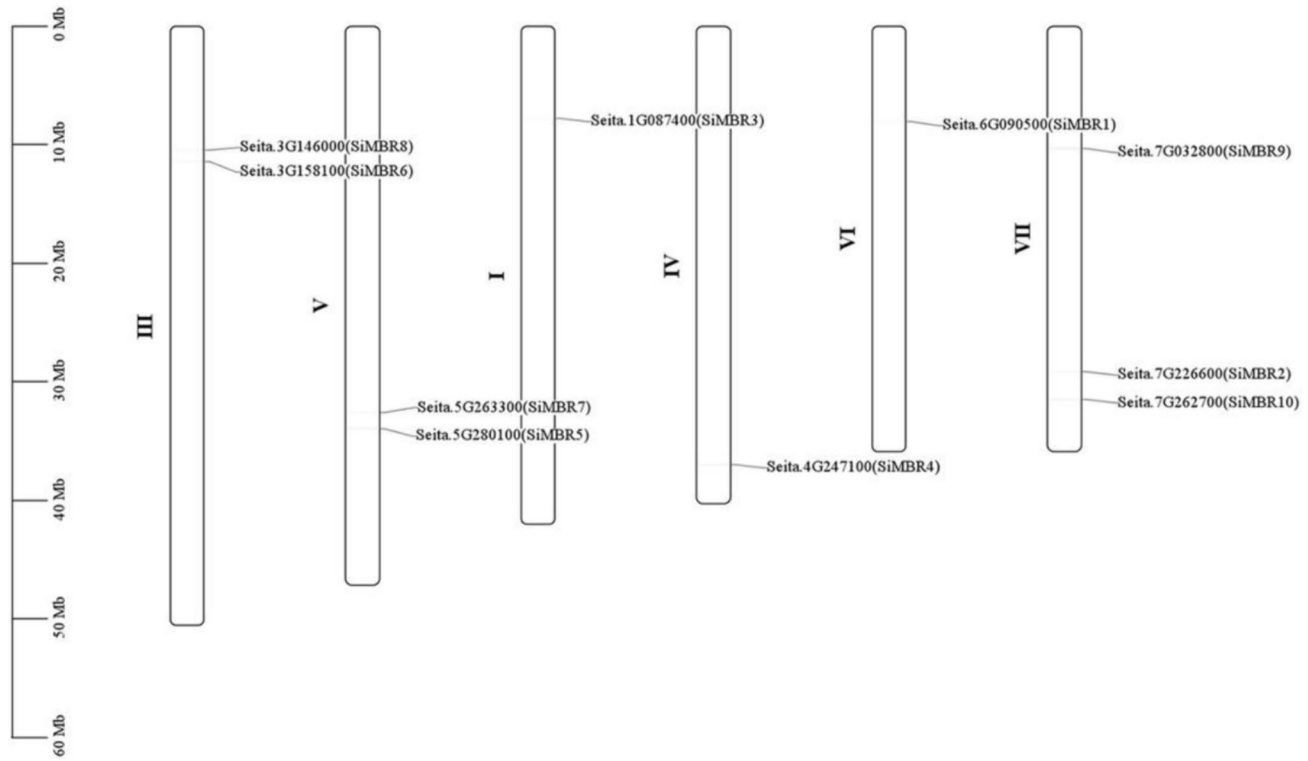

Fig. 2 Chromosomal localization of *SiMBR* genes from foxtail millet. The location information of 10 *SiMBR* genes on the chromosome was obtained from the Phytozome database (*Setaria italica* v2.2)

Table S3. Among these, cis-elements related to plant growth, such as the O2-site, CAT-box, and A-box, were identified in 3, 4, and 8 *SiMBR* genes, respectively. Furthermore, all *SiMBR* genes contained abscisic acid response elements (ABREs). Methyl jasmonate-responsive elements, the CGTCA-motif and TGACG-motif, were in 7 *SiMBR* genes. The auxin response-related TGA-element and the salicylic acid response-related TCA-element were in 4 and 5 *SiMBR* genes, respectively. Many cis-acting elements related to plant stress responses were also present, such as the LTR (low-temperature response), GT1-motif (light response), and MBS (MYB-binding site associated with drought response). This suggests that most *SiMBR* genes are controlled by many factors, including hormones and environmental stresses. For example, *SiMBR2* gene had A-box, ABRE, AE-box, ARE, CGTCA-motif, GT1-motif, LTR, MBS, and other elements, showing it is part of a complex control system.

In silico transcript analysis of *SiMBR* genes in foxtail millet

An examination of publicly accessible RNA-seq data from foxtail millet (multi-omics database for *Setaria italica*; Li et al. 2023) disclosed the expression profiles of *SiMBR* genes across diverse tissues and developmental stages. Figure 5 illustrates that *SiMBR5* (*Seita.5G280100*) is expressed in various tissues, with higher levels in the spikelet and root. The expression of *SiMBR2* was observed in leaves. This suggests that *SiMBR5* and *SiMBR2* are important for foxtail millet's growth and function, probably because they are expressed differently in each tissue.

Expression analysis of *SiMBR2* under different abiotic stresses and phytohormone treatments

In our previous investigation, we found that the transcription of *SiMBR2* decreased in "Jingu21" seedlings under soil drought stress (Guo et al. 2022). We now explore how SiMBR2 affects plant responses to other stresses. The 14-day seedlings of "Jingu21" were subjected to 24 h abiotic stresses (cold, heat, NaCl, PEG, and $NaHCO_3$). The transcription of *SiMBR2* was repressed following 24 h of extreme temperature (both cold and heat), while its expression was induced under salt and osmotic stresses (Fig. 6a). Since we found many hormone-related cis-acting elements within *SiMBR2*, we tested its expression after 24 h of treatment with ABA (100 µmol/l), 6-BA (75 µmol/l), IAA (10 µmol/l), NAA (10 nmol/l), GA_3 (1 mmol/l), MJ (100 µmol/l), and SA (10 mmol/l) (Fig. 6b). Among these, SA and GA_3 increased *SiMBR2* levels, while ABA suppressed it.

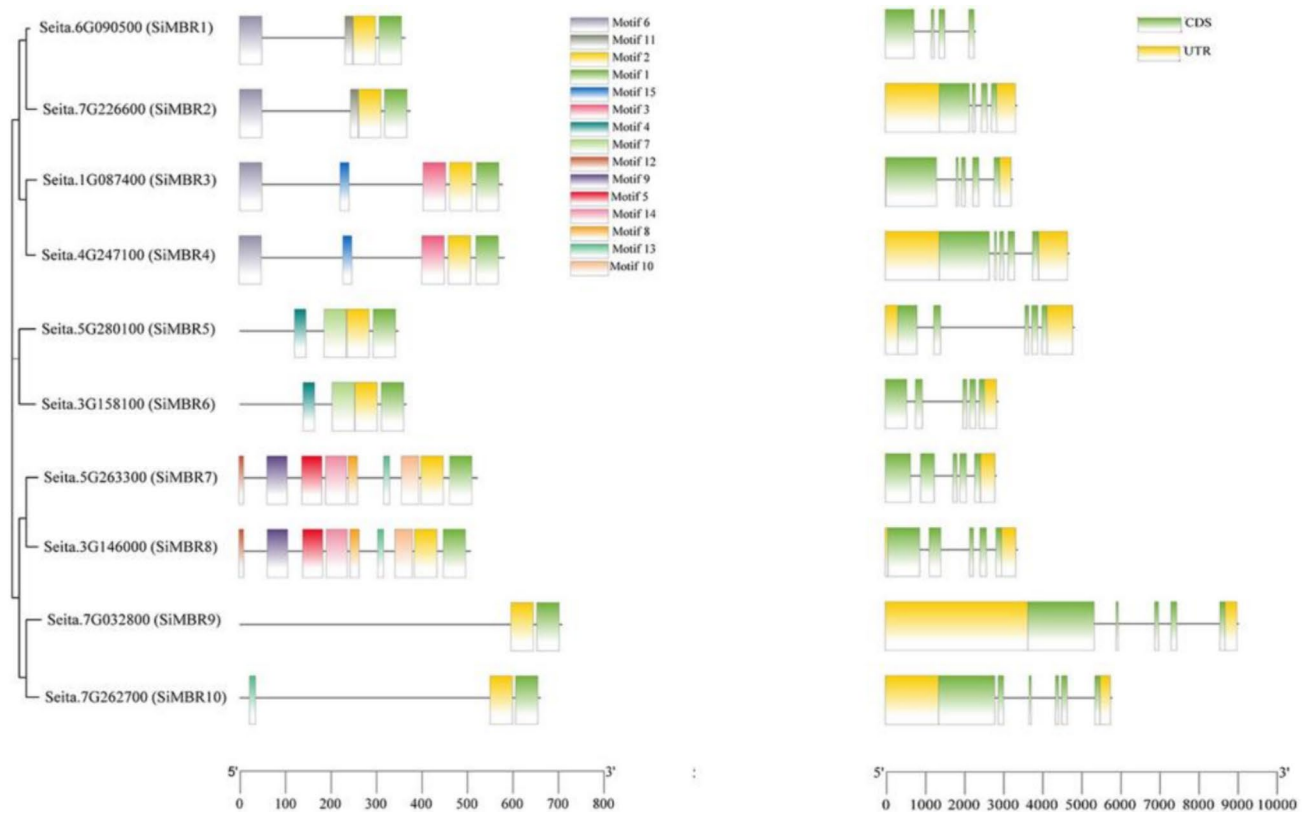

Fig. 3 Protein motifs and gene structures of *SiMBR* from foxtail millet. Left In the SiMBR2 proteins, 15 motifs were identified by the MEME tool, represented by different colors (1–15) and depicted by TBtools. Right The exon–intron structure of these *SiMBR2* genes was predicted by GSDS 2.0. The green boxes represent the coding sequence (CDS), the black lines represent introns, and the yellow boxes represent up/downstream untranslated regions (UTR)

The osmotic stress from PEG mimics drought conditions that plants can tolerate. However, actual drought effects on plants are more complex (Kautz et al. 2015). In this study, we observed that the transcription level of *SiMBR2* increased with PEG treatment. As previously reported, the level of *SiMBR2* decreased after 14 days of drought stress induced by withholding water (Guo et al. 2022). To understand this difference, we monitored changes in the transcriptional levels of the *SiMBR2* gene during a two-week drought process of water deficiency. As shown in Fig. S3, within the 14-day experimental period, *SiMBR2* levels rose, then fell in both control and stress groups, peaking on day 1 and day 3 (the day when treatment group's soil water content below 10% was considered as day 0). After 14 days of water-deficit drought, the stress group had lower *SiMBR2* levels than control, matching our earlier findings (Guo et al. 2022). This suggests that PEG-induced drought and water-deficit drought affect *SiMBR2* gene expression differently.

Over-expression of *SiMBR2* in *Arabidopsis* seedlings contributes to abiotic stresses tolerance

To elucidate the physiological function of the *SiMBR2* gene in plant stress responses, we introduced this gene into Arabidopsis for constitutive expression. After two weeks of soil water deprivation, plants over-expressing *SiMBR2* were more tolerant to drought (Fig. 7a). We checked the levels of H_2O_2 and MDA, and the activity of antioxidant enzymes in leaf samples on day 10, when the leaves of plants were still green. Under drought stress, control plants exhibited elevated levels of H_2O_2 in their leaves, leading to an increase in MDA content and increased activities of antioxidant enzymes such as CAT, POD, and superoxide dismutase (SOD; Fig. 7b–f). In contrast, plants overexpressing *SiMBR2* showed higher activities of these antioxidant enzymes, effectively reducing the rise in ROS and

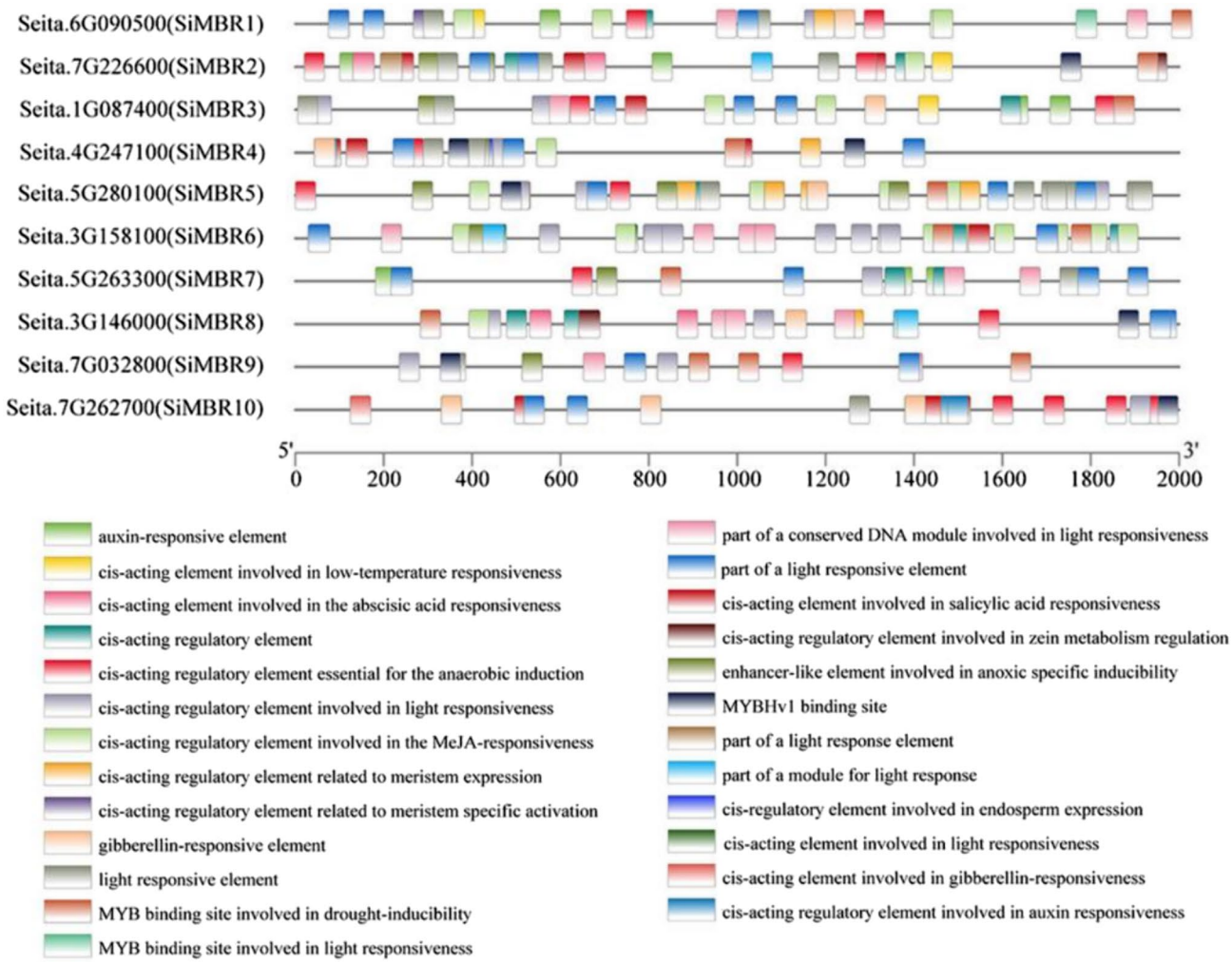

Fig. 4 Cis-acting element analysis of the promoter regions of *SiMBR* genes from foxtail millet. Based on the functional annotations, the cis-acting elements were shown in boxes with different colors

MDA levels. Additionally, we investigated the expression of representative antioxidant enzyme genes, including the superoxide dismutase isoform *AtCSD1*, peroxidase *AtPOD1*, and catalase *AtCAT1* (Amoah and Seo 2021; Lan et al. 2022; Wang et al. 2023), in transgenic plants (Fig. 8). The data show that *AtPOD1* and *AtCAT1* were up-regulated in plants with extra *SiMBR2* under drought. The transcription level of *AtCSD1* remained consistently high in plants overexpressing *SiMBR2*, regardless of drought conditions (Fig. 8). This suggests that over-expression of the *SiMBR2* gene in Arabidopsis improves their drought resistance, which is linked to increased gene activity and better ROS detoxification.

Discussion

Foxtail millet, a C4 plant, exhibits remarkable resistance to various external stresses, and identifying the genes linked to its stress tolerance could help improve crop genetic engineering (Peng and Zhang 2020; Panchal et al. 2022; Zhang et al. 2023). With the full genome sequencing of foxtail millet now accessible (Bennetzen et al. 2012; Zhang et al. 2012), performing systematic analyses of millet genes using bioinformatics coupled with experimental methods has become feasible. The *SiMBR* genes belong to the RING proteins, which are part of the E3 ubiquitin

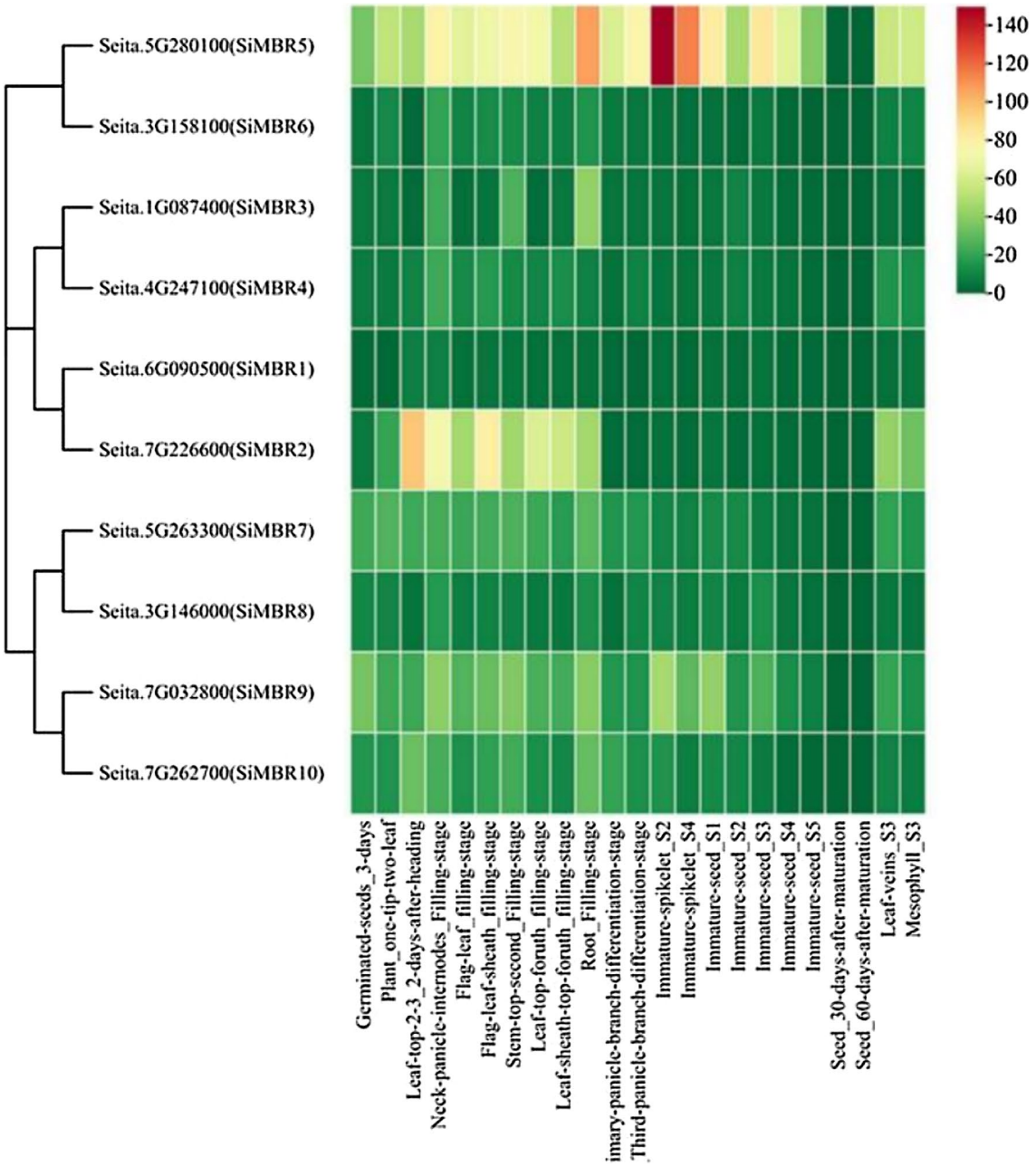

Fig. 5 Expression profile of *SiMBR* genes in different tissues and developmental stages of foxtail millet. Gene expression data are downloaded from the MDSi database

ligase family. The functions of several RING proteins have been investigated, revealing their involvement in various plant processes such as responses to abiotic and biotic stresses, hormone signalling, leaf development, flowering, and more (Dong et al. 2006; Stone et al. 2006; Lazaro et al. 2012, 2015; Lee and Seo 2015; Zhang et al. 2017b; Sun et al. 2019). However, the functions of RING proteins are not yet fully elucidated, and the majority of research on these proteins' function has been focused on model organisms like Arabidopsis, with limited investigation in crop species. Arabidopsis contains over 460 RING proteins (Stone et al. 2005; Sun et al. 2019), rice has 425 (Lim et al. 2010), and wheat has 1255 (Parveen et al. 2021). For the RING-H2 type alone, Arabidopsis has 291 and rice has 281. RING-type proteins play a role in plant growth, development, and responses to various abiotic stresses, including drought, salinity, temperature extremes, and toxic metals (Sun et al. 2019; Han et al. 2022). According to our initial transcriptome screening results (Guo et al. 2022), one *SiMBR* gene, *Seita.7G226600*, reacts to

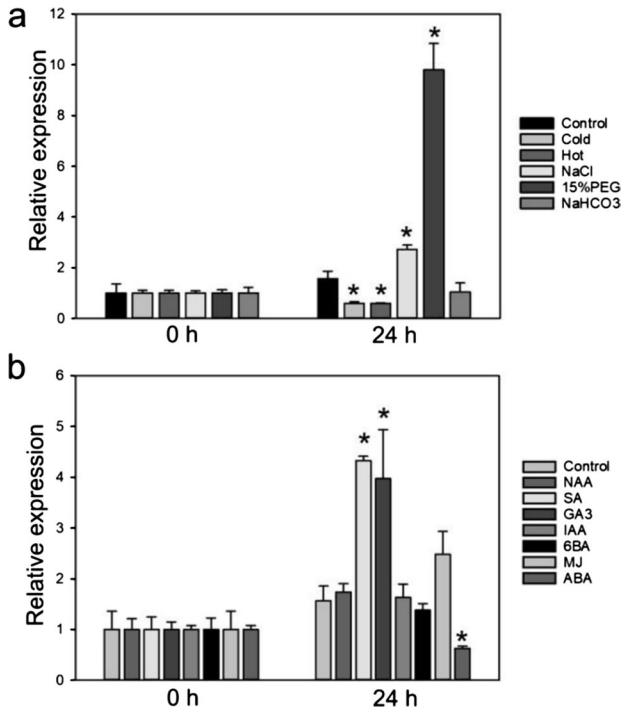

Fig. 6 The relative transcript levels of *SiMBR2* gene in foxtail millet under different abiotic stresses (**a**) and phytohormone treatments (**b**). The qRT-PCR analyses were used to access transcript levels of *SiMBR2* gene from two-week-old foxtail millet seedlings with or without 12 and 24 h cold (4 ℃), hot (40 ℃ day/32 ℃ night), salt (200 mmol/l NaCl), osmotic stress (15% PEG), abscisic acid (ABA, 100 µmol/l), 6-benzylaminopurine (6-BA, 75 µmol/l), auxin (IAA, 10 µmol/l), 1-naphthaleneacetic acid (NAA, 10 nmol/l), gibberellic acid (GA_3, 1 mmol/l), methyljasmonat (MJ, 100 µmol/l), and salicylic acid (SA, 10 mmol/l) treatments. Each bar represents the mean ± SE normalized to *SiAct2* (*Seita. 8G043100*) and *RNA POL II* (*Seita. 2G142700*). All samples were run in three biological and three technical replicates. Asterisk indicates that the gene expression under stress has a significant difference compared with the control (*$P < 0.05$)

drought stress. Considering many members of the RING gene family and their complex roles, we chose to do a detailed analysis on the *MBR* family instead of the whole *RING* gene family.

The gene *Seita.7G226600*, which we named *SiMBR2*, exhibits similarity to the *MBR* genes found in Arabidopsis. Using the four *MBR* genes from Arabidopsis as a reference, we found that foxtail millet, as well as rice, barley, *Brachypodium distachyon*, and sorghum, has ten members of this gene family, and maize has 15 (Fig. 1 and Table S2). Phylogenetic analysis showed these MBR genes from different species fall into five distinct groups. Each group within foxtail millet shares comparable motifs and gene structures (Fig. 3). The *SiMBR* genes from foxtail millet show greater similarity to their orthologous counterparts in maize and sorghum than to those in Arabidopsis, indicating that the diversification of the *MBR* gene family predates the evolutionary

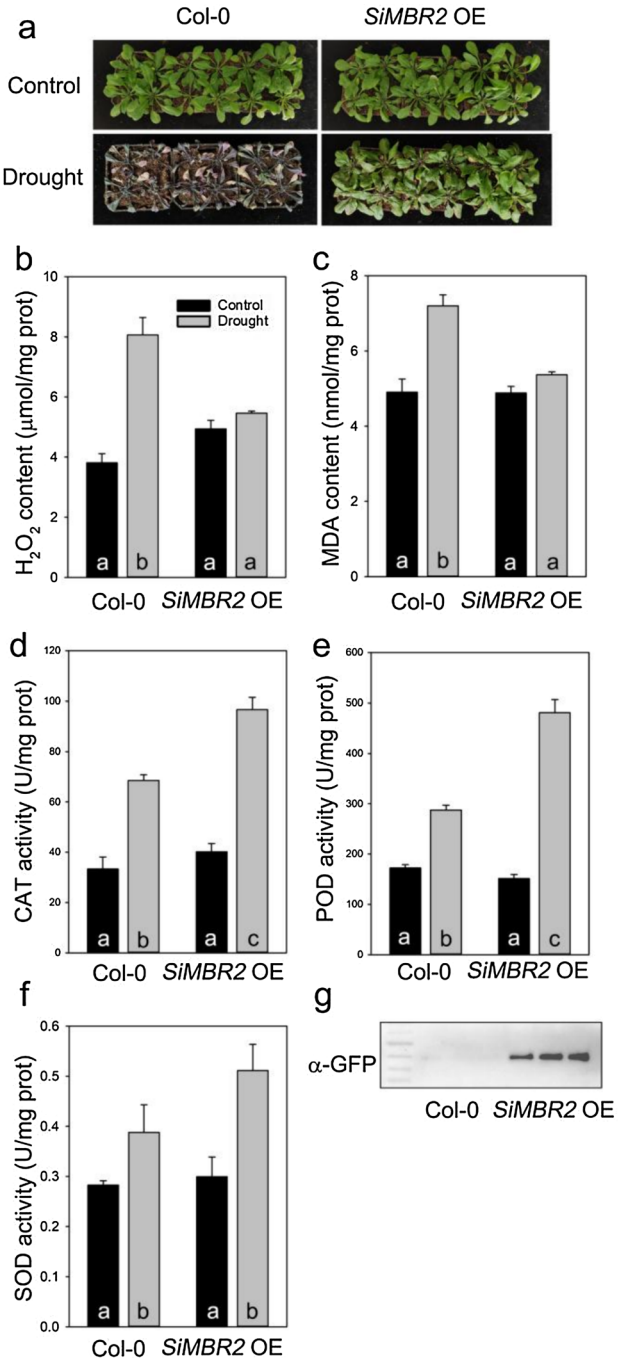

Fig. 7 Comparison of drought tolerance of wild-type and transgenic Arabidopsis conferred by *SiMBR2*. **a** Growth phenotypes of 4-week-old soil-grown plants of wild-type and transgenic lines treated with natural drought for 2 weeks. Control plant kept water during the whole experiment. **b** H_2O_2 level. **c** MDA level. **d** CAT activity. **e** POD activity. **f** SOD activity. **g** The expression of *SiMBR2* in Arabidopsis was confirmed by Western blot using anti-GFP (abcam). The leaf samples were harvested after 10 days of stop watering. The results were shown as means ± SE from three independent experiments. For **b**–**f**, different letters indicate significant differences ($P < 0.05$)

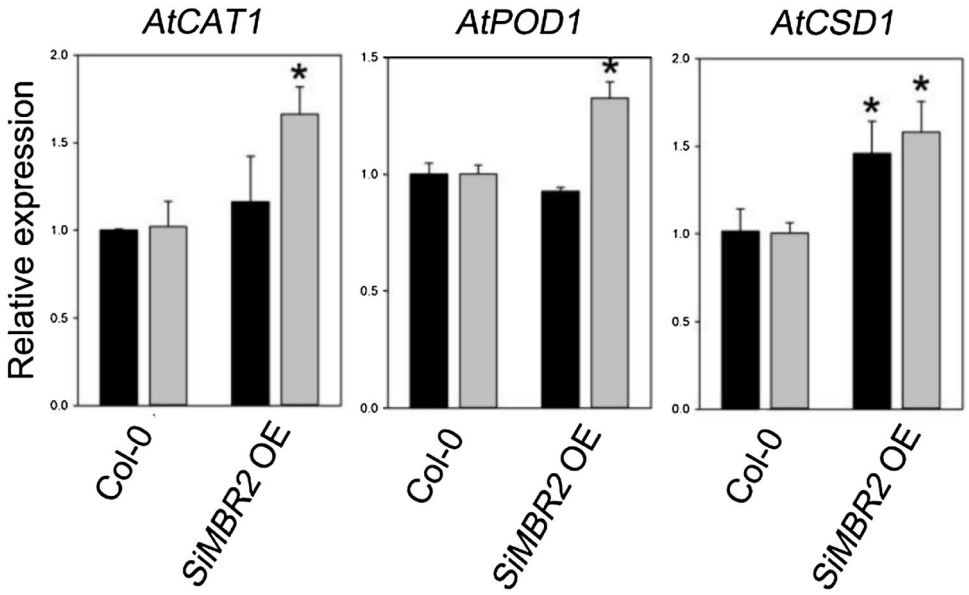

Fig. 8 The relative transcript levels of *AtCSD1*, *AtPOD1*, and *AtCAT1* in *SiMBR2* overexpressed in Arabidopsis. The qRT-PCR analyses were used to access transcript levels of *AtCSD1*, *AtPOD1*, and *AtCAT1* genes from two-week-old *SiMBR2* overexpressing Arabidopsis with stopping watering treatment. *AtACT2* (*AT3G18780*) and *TIP41-like* (*TAP42 INTERACTING PROTEIN OF 41 kDa*, *AT4G34270*) were used as the internal reference. Each bar represents the mean ± SE. All samples were run in three biological and three technical replicates. Asterisk indicates that the gene expression under stress has a significant difference compared with the transcript level at control plant (*$P < 0.05$)

split between monocots and dicots. Analysis of cis-acting elements (Fig. 4) indicates a regulatory network controlling *SiMBR* gene expression, involving multiple factors.

Using previously obtained RNA-seq data, we further analyzed the expression profiles of the *SiMBR* genes (Fig. 5). *SiMBR5* was highly expressed in various tissues, with significantly elevated levels in the spikelet and root during the grain-filling stage. In contrast, *SiMBR2* expression was highest in leaf tissue. Since SiMBR2 expression decreased under drought stress (Guo et al. 2022), we then focused on this gene to study its transcriptional regulation in leaves under different phytohormone treatments and abiotic stresses (Fig. 6). The findings indicated that *SiMBR2* expression was up-regulated by PEG and salt stress, as well as by treatment with SA and GA_3, while it was down-regulated under cold or heat stress, corroborating the presence of multiple cis-regulatory elements in the gene.

In two-week-old foxtail millet seedlings, a 24-h treatment with PEG6000 caused up-regulation of the *SiMBR2* gene (Fig. 6). However, our earlier research showed that withdrawing water for 14 days led to down-regulation of the gene (Guo et al. 2022). To clarify this discrepancy, we further investigated the expression levels of *SiMBR2* at various time points after stopping watering in two-week-old millet seedlings. We found that the gene up-regulated from day 0 to day 3, regardless of irrigation, matching RNA-seq data showing increased expression during leaf development (Fig. 5). After day 5, expression levels decreased in both the control and drought-stressed groups, with significantly lower expression in the drought-stressed group after 14 days, confirming our previous transcriptome analysis. Our research findings demonstrate that there are differences between PEG-simulated drought stress and water-deficit drought stress in regulating *SiMBR2* expression. This distinction may be due to the osmotic stress induced by PEG-simulated drought is rapid, while plants experience a slower and more complex response to water-deficit stress. It also suggests that PEG-simulated drought and actual water-deficit drought are different and cannot be simply substituted in molecular mechanism research.

To elucidate the role of *SiMBR2* in plant responses to drought stress, we introduced the gene into Arabidopsis. As shown in Fig. 7, expressing *SiMBR2* in Arabidopsis conferred tolerance to drought conditions, indicated by improved antioxidant enzyme activity, lower ROS levels, and reduced MDA damage. Subsequent experiments involved quantifying the transcript levels of key antioxidant enzyme genes *AtCSD1*, *AtPOD1*, and *AtCAT1* in Arabidopsis (Amoah and Seo 2021; Lan et al. 2022; Wang et al. 2023). Under drought stress, we found increased transcription levels of *AtPOD1* and *AtCAT1* in plants expressing *SiMBR2*. In contrast, *AtCSD1* levels remained consistently elevated in these plants, irrespective of stress conditions (Fig. 8). These results suggest that expressing *SiMBR2* in

Arabidopsis enhances antioxidant enzyme gene expression, improving ROS scavenging and plant tolerance to drought.

In Arabidopsis, MBR binds to PFT1 and promotes its degradation, ultimately promoting flowering (Iñigo et al. 2012). PFT1 is a subunit of the plant Mediator complex, which links the transcription factor to the RNA Polymerase II and regulates gene expression (Chen et al. 2022). Zhang et al. (2017b) reported that MBR could also interact with the transcription factor repressor TIE1, promote its degradation and control leaf defects. *AtCSD1*'s consistent up-regulation in *SiMBR2* overexpressing plants, even without drought stress, suggests a complex regulation of gene expression by *SiMBR2*, as *AtPOD1* and *AtCAT1* levels increased only under stress. Whether this involves SiMBR2-mediated degradation of the Mediator complex or transcription regulators requires further investigation.

It is also important to note that our current method involves rapidly screening and evaluating gene functions through heterologous expression in Arabidopsis. However, given the substantial differences between Arabidopsis and foxtail millet, further functional characterization of the gene must be performed within the millet system.

Conclusion

We identified 10 *SiMBR* genes in the foxtail millet genome. We analyzed their phylogenetic relationships, conserved motifs, chromosomal locations, gene structures, promoter regions, and predicted expression profiles in different tissues and developmental stages, as well as responses to plant hormones and abiotic stresses. We found that *SiMBR2* is regulated by various stress factors. Its heterologous expression in Arabidopsis improved drought tolerance by reducing ROS. This research opens avenues for further exploration into genetically enhancing foxtail millet and other crops for better resistance to environmental stressors.

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Author contributions Experiments were designed by BZ, PY, and LZ. Bioinformatics analysis was performed by YF, YG, ZL, and BZ. Plant physiology experiments were performed by YF, RH, HZ, PY, and BZ. Plant molecular experiments were performed by YF, RH, and PY. BZ, ZL, and LZ analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Availability of data and materials All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

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

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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