A transcription factor TaMYB5 modulates leaf rolling in wheat

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Leaf rolling is an important agronomic trait in wheat (Triticum aestivum L.). Moderate leaf rolling keeps leaves upright and maintains the relatively normal photosynthesis of plants under drought stress. However, the molecular mechanism of wheat leaf rolling remains unclear. Here, we identified a candidate gene TaMYB5-3A that regulates leaf rolling by using a genome-wide association study (GWAS) in a panel of 323 wheat accessions. Phenotype analysis indicated that the leaves of tamyb5 mutants were flatter than that of the wild type under drought condition. A nucleotide variation in the TaMYB5-3A coding region resulted in a substitution of Thr to Lys, which corresponds to two alleles SNP-3A-1 and SNP-3A-2. The leaf rolling index (LRI) of the SNP-3A-1 genotype was significantly lower than that of the SNP-3A-2 genotype. In addition, TaMYB5-3A alleles were associated with canopy temperature (CT) in multiple environments. The CT of the SNP-3A-1 genotype was lower than that of the SNP-3A-2 genotype. Gene expression analysis showed that TaMYB5-3A was mainly expressed in leaves and down-regulated by PEG and ABA treatment. TaMYB5 induces TaNRL1 gene expression through the direct binding to the AC cis-acting element of the promoter of the target gene, which was validated by EMSA (electrophoretic mobility shift assay). Our results revealed a crucial molecular mechanism in wheat leaf rolling and provided the theoretical basis and a gene resource for crop breeding.

KEYWORDS
GWAS, leaf rolling, TaMYB5, NRL1, canopy temperature, wheat

Introduction

Wheat (Triticum aestivum L.) has been one of the most widely cultivated crops in the world since the Neolithic Age and provides about 20% of calorie intake for humans (Shiferaw et al., 2011). Leaf is the primary organ to process photosynthesis in plants, and the photosynthetic rate is directly affected by its morphological characteristics (Zhang et al., 2015). Leaf shape plays a critical role in photosynthesis and plant development...
by affecting light interception, transpiration, and leaf dehydration (O’Toole and Cruz, 1980). Leaf rolling is a highly complex agronomic trait regulated by genotype and environment (Li et al., 2017). Moderate leaf rolling keeps the leaves upright and improves the population structure, finally enhances light capture and photosynthesis (Zhu et al., 2001; Richards et al., 2002). Under drought stress, moderate leaf rolling maximizes light capture, accelerates dry-matter accumulation, boosts yield, decreases transpiration, changes water uptake capacity, and then affects drought resistance (Lang et al., 2004; Moon and Hake, 2011). In addition, appropriate leaf rolling can decrease the solar radiation on leaves, cumulative transpiration, and water loss, and thus leaf rolling is crucial to improve tolerance of plants to drought stress (Lian et al., 2004; Kadioglu and Terzi, 2007). Therefore, leaf rolling is regarded as a crucial agronomic trait for crop breeding, which is extremely significant for obtaining ideal plant architecture and drought tolerance (Yuan, 1997; Chen et al., 2001).

Due to its agronomic importance, some genes and their pathways involved in controlling leaf rolling have been identified and characterized in crop plants. Most genes could regulate leaf rolling by affecting bulliform cells development or secondary cell wall biosynthesis, for example, srl1 and url1 mutants exhibited adaxial rolling of leaves due to changes in the number and size of bulliform cells (Xiang et al., 2012; Fang et al., 2021). psl1 is a rice photo-sensitive leaf rolling mutant that bulliform cells and cell wall biosynthesis changed significantly, which displayed leaf rolling when exposed to high light intensity (Zhang et al., 2021). NRL1 encodes a cellulose synthase-like protein that is crucial for cell wall formation, and the reduced expression of NRL1 in the mutant displayed adaxially rolled leaves, which was due to the reduction of bulliform cells size in rice (Hu et al., 2010). The loss-of-function mutant of rl14 and cldl displayed leaf rolling via affecting bulliform cells size, and the cell wall formation was affected by changing the content of lignin and cellulose in the cell wall. The loss of CLDL1 function accelerated water loss, reduced leaf water content, and drought resistance of the plant (Fang et al., 2012; Li et al., 2017). The genes of ACL1 and ROC5 have also been reported to regulate leaf rolling by affecting bulliform cell development (Li et al., 2010; Zou et al., 2011). Moreover, the abnormal establishment of adaxial–abaxial polarity also is considered a crucial factor in leaf rolling (Yamaguchi et al., 2012). A maize gene RLD1 encodes an HDZIP III family protein that regulates leaf abaxial establishment (Izuarez et al., 2004). Recent studies have shown that rolled fine striped (RFS) gene causes extreme leaf rolling due to the abnormal development of vascular cells on the adaxial side of leaf in rice (Cho et al., 2018). SLL1 and SRL2 were also found to regulate leaf rolling by influencing the development of abaxial sclerenchymatous cells in rice (Liu et al., 2016).

MYB transcription factors (TFs) contain a highly conserved MYB DNA-binding domain (DBD) that generally consists of 1–4 incomplete MYB repeats of approximately 52 amino acids. Based on the number of incomplete repeats in their DBD, the MYB family is divided into four classes, 1R-MYB, 2R3-MYB, 3R-MYB, and 4R-MYB proteins. MYB TFs play an essential role in regulating various developmental processes, primary and secondary metabolism, and a series of stress responses in plants (Dubos et al., 2010; Li et al., 2019a). Interestingly, the MYB TFs are also involved in regulating leaf rolling, such as SLL1 encodes a SHAQKYF class MYB protein belonging to the KAN subfamily and regulates leaf rolling by promoting the development of sclerenchymatous cells. The loss-of-function mutant sll1 displayed defective sclerenchymatous cells due to programmed cell death of the mesophyll cells on the abaxial side (Zhang et al., 2009). In addition, one of the most likely candidate genes in the rolled-leaf mutant (rl10) was also the MYB transcription factor belonging to the KANADI subfamily (Luo et al., 2007). The genes of TaMYB18, OsMYB60, and AtMYB101 have also been reported to regulate leaf rolling (An et al., 2014; Zhang et al., 2016; Liu et al., 2018). Moreover, the MYB TFs regulated leaf rolling by affecting secondary cell wall biosynthesis. Overexpression of OsMYB103L showed that the cellulose content and the expression levels of several cellulose synthase genes (CESAs) were remarkably increased, and resulted in the rolling of leaves in rice (Yang et al., 2014). Recent studies have shown that OsSN2 regulates leaf rolling by promoting the expression of OsMYB61, which affects the biosynthesis of secondary cell walls (Ye et al., 2018).

Several genes that regulate leaf rolling in crops have been reported, but their molecular mechanisms, especially in wheat, remain unclear. In the present study, through genome-wide association study (GWAS), we identified a novel R2R3-MYB gene, TaMYB5, that regulates leaf rolling in wheat. Association analysis showed that TaMYB5 was significantly associated with canopy temperature (CT) in multiple environments, and favorable genotypes of TaMYB5 have been positively selected in different ecological regions of wheat in China. Further studies showed that TaMYB5 directly binds to the promoter through AC cis-acting element to induce the expression of the TaNRL1 gene. The phenotype of tamyb5 mutants under drought stress verified that TaMYB5 was involved in leaf rolling regulation. Our results revealed a crucial molecular mechanism of wheat leaf rolling, and provided the theoretical basis and a gene resource for crop breeding.

Materials and methods

Plant materials and measurement of leaf rolling index

Thirty-two wheat accessions with wide variations (Zhang et al., 2013) were used to detect the nucleotide polymorphism of
the target gene. Three winter wheat populations were employed in this study. Population 323 (P323) was used for GWAS. The accessions include 12 landraces, 36 advanced lines, and 275 modern cultivars, most of whose flowering dates occurred within 1 week, mainly planted in the Yellow and Huai River Valleys Facultative Wheat Zone and the Northern Winter Wheat Zone in China (Li et al., 2019b). Population 157 (P157) (157 landraces) and Population 348 (P348) (348 modern cultivars) collected from all 10 wheat production zones in China were used for haplotype analysis (Wang et al., 2019).

The P323 was planted to investigate agronomic traits at Shunyi (SY) (40°13′N; 116°36′E) and Changping (CP) (40°13′N; 116°13′E) in Beijing, China during the 2016–2017 growing seasons. Two water treatments, rain-fed (drought stress, DS) and well-watered (WW), were applied. The WW plot was irrigated with 750 m³ ha⁻¹ (75 mm) water at pre-overwintering, flowering, and grain-filling stages, respectively, if the rainfall in the corresponding period was insufficient. The total precipitation in the growing seasons was 143 mm. Heat stress (HS) treatment was conducted in a polyethylene-covered greenhouse at the 1-week post-anthesis stage (Li et al., 2019b). Each accession was planted in four rows, with row spacing of 30 cm, length of 2 m, and 40 seeds in each row. Five plants were randomly selected for phenotyping.

For GWAS, P323 was planted in Changping, Beijing, during the 2019–2020 and 2020–2021 growing seasons, and the LRI of 323 accessions was measured. Two water regimes DS and WW were applied in this study. The width of the flag leaf was detected one-third away from the leaf sheath, and the natural leaf width (Ln) and fully unfolded leaf width (Lw) were measured with three replicates, respectively. The LRI data were measured from 8:00 to 10:00 and from 15:00 to 18:00 at the 2-week post-anthesis stage. The LRI of flag leaves was calculated by the formula: 

\[ \text{LRI} = \left( \frac{Lw - Ln}{Lw} \right) \times 100\% \]

Lw means the expanded natural distance of the leaf blade margins, Ln means the natural distance of the leaf blade margins, and the low and high of LRI value represent the slight and severe degree of leaf rolling, respectively (Shi et al., 2007).

**Genome-wide association study of leaf rolling**

GWAS of wheat leaf rolling genes was performed by analyzing the wheat natural variation population P323. The overall LRI used for GWAS was calculated as the best linear unbiased predictions (BLUPs), which were based on a mixed linear model and performed using the lme4 package in R 3.3.0. Genotyping was performed by using the 660K SNP Array as previously described (Li et al., 2019b). After removing nucleotide variations with missing rates \( \geq 0.2 \) and minor allele frequency \(< 0.05\), 395,675 SNPs were used for GWAS in this research. On the whole genome scale, in consideration of the population structure (Q) and familial kinship (K), a mixed linear model (MLM) was applied to analyze the association between molecular markers and traits by using TASSEL 5.0 software (Li et al., 2019c).

**Generation and phenotypic analysis of wheat mutant**

Three wheat mutants (tamyb5) were obtained by exome sequencing (data not published) from a population, which was a wheat variety Aikang58 (AK58) mutant population induced by ethyl methanesulfonate (EMS). These mutants were backcrossed two times with wild-type AK58 to eliminate the noise in the genetic background. To observe the leaf phenotype at the flowering stage, wheat plants were grown in artificial climate chambers under long-day conditions (16 h light, 25°C/8 h dark, 20°C) at 60% humidity. Plants were treated with drought stress at the post-anthesis stage. The leaf phenotyping was performed and the LRI was measured after 18 days of drought treatment.

**Functional marker development and association analysis**

Wheat DNA was extracted from the young leaves by the CTAB method (Stewart and Via, 1993). TaMYB5 was cloned by performing PCR amplification using TransStart FastPfu DNA Polymerase and subsequently ligated into a pEASY-Blunt vector for sequencing (Trans Gen Biotech, Beijing). The PCR conditions were 95°C for 5 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min; followed by a final extension of 72°C for 10 min. Five positive clones were randomly picked for sequencing.

Sequence polymorphism of the TaMYB5 gene was detected in 32 wheat accessions with wide variations. Based on a SNP (C/A) located at 544 bp of the gene coding region, a dCAPS marker was developed to distinguish the C/A genotype by mismatching nucleotide to create the Sal I restriction site. The first-round PCR products were obtained by genome-specific primers and used as the templates for the second round of PCR. Second-round PCR products were digested by Sal I restriction enzyme at 37°C for 2 h and then target fragments were separated by electrophoresis in 4% agarose gel (Wang et al., 2020). Association analyses were conducted by SPSS 16.0 software. \( P < 0.05 \) was considered a significant correlation. The primers are shown in Supplementary Table 1.

**Gene expression analysis**

Gene expression analysis was performed using wheat variety Hanxuan 10 as the plant material. Tissue-specific gene
expression was analyzed in leaf, root base, and root of seedling at the two-leaf stage, as well as in leaf, spike, penultimate node, root base, root tissues at different depths of jointing stage plants. Wheat seedlings were cultured by hydroponics. Seedlings at two-leaf stage were treated with PEG-6000 (−0.5 MPa) and 50 µM abscisic acid (ABA), and the leaves were collected at 0, 0.5, 1, 2, 3, 6, 12, 24, 48, and 72 h under the treatments (20 h light/4 h dark). Samples at 0 h served as the control. Total RNA was extracted with a TRIzol reagent (TIANMO, Beijing). cDNA was synthesized with a FastQuant RT Kit (TIANGEN, Beijing). Gene expression levels in plant samples were detected using quantitative real-time PCR (qRT-PCR), which was performed with a SYBR Green PCR Master Mix Kit (TAKARA, Beijing). Five plants were harvested for each replicate, and the three biological replicates were set for each treatment.

Subcellular localization

Full-length ORF of TaMYB5 was cloned and inserted into the pCAMBIA1300GFP vector to generate the p35S-TaMYB5-1300GFP construct. The recombinant plasmid was transformed into Agrobacterium tumefaciens strain EHA105 and then transiently expressed into Nicotiana benthamiana leaves (Zhuang et al., 2021). After 48 h of culture, the green fluorescence signal was detected by a confocal microscope (Carl Zeiss, Germany).

Transcriptional activity analysis

To detect the transcription activity, the full-length cDNA of TaMYB5 was fused into p35S-GAL4DB-NOS to generate the p35S-GAL4-TaMYB5-NOS fusion plasmid. The p35S-GAL4-LUC-NOS, p35S-REN-NOS vectors served as the reporter plasmids, and p35S-GAL4DB-NOS served as the effector plasmids (Liu et al., 2020). The TaMYB5-GAL4DB, the firefly luciferase (LUC), and Renilla luciferase (REN) plasmids were co-transformed into tobacco protoplasts to detect the LUC activity ratios (LUC/REN).

Dual-luciferase reporter assay

The p35S-TaMYB5-1-1300GFP and p35S-TaMYB5-2-1300GFP were used as effectors. proTaNRL1 was fused into the pGreenII 0800-LUC vector to generate the reporter constructs. pCAMBIA1300-GFP served as vector control. They were transformed into Agrobacterium tumefaciens strain GV3101 and then transiently expressed into tobacco leaves (Li et al., 2022). The luciferase activities were tested using Dual-Glo Luciferase Assay System (Promega, United States).

Yeast one-hybrid assays

The CDS sequences of TaMYB5-1 and TaMYB5-2 were cloned and fused into the pB42AD as the effector vector. The promoter fragment of TaNRL1 containing the “ACCTAAC” element was cloned and inserted into the pLacZ vector as a reporter. The effector and reporter plasmids were co-transformed into yeast strain EGY48. Empty pB42AD and pLacZ were used as negative controls. Yeast strain was grown on SD/-Trp/-Ura medium containing X-Gal to detect the interaction at 29°C.

Electrophoretic mobility shift assays

The cDNA sequence of TaMYB5 was fused into vector pGEX-4T1 to construct GST-TaMYB5. The empty GST vector pGEX-4T1 and GST-TaMYB5 were transformed into the Escherichia coli expression strain BL21 (Wang et al., 2020). TaMYB5 protein was induced by 0.2 mM IPTG at 16°C for 4 h and then harvested.
10 h and was purified using glutathione-sepharose 4B (GE Healthcare, United States). Biotin-labeled ACC-box and mutant ACC-box, unlabeled ACC-box and mutant ACC-box, and their reverse complementary sequences were synthesized and used as probes. EMSA assay was performed using a LightShift Chemiluminescent EMSA Kit (Thermo, United States).

Results

TaMYB5-3A is associated with wheat leaf rolling

In order to characterize genes associated with wheat leaf rolling, we assembled a natural population of P323 consisting of 323 winter wheat accessions from 10 major wheat regions in China, which had been used for GWAS (Li et al., 2019c; Wang et al., 2021). The leaf rolling degree of each accession was analyzed by measuring and calculating the LRI at the flowering stage. There was a large variation of LRI in 323 accessions, ranging from 0.17% to 70.63%, which indicates that the leaf rolling at the flowering stage is suitable for genetic analysis. Two extreme phenotypes of leaf rolling in the population are shown in Supplementary Figure 1. On the whole genome scale, in consideration of the population structure (Q) and familial kinship (K), mixed linear model (MLM) with the correction function was applied to analyze the association between markers and traits. A haplotype block (479,471,884 bp–480,575,035 bp) on chromosome 3A was significantly associated with LRI and contained 12 candidate genes (Figure 1 and Supplementary Table 2). It has been reported that the MYB family genes were involved in regulating leaf rolling in plant; therefore, TraesCS3A02G257800 was selected as a candidate gene for regulating leaf rolling. Protein structure analysis showed that TraesCS3A02G257800 is an MYB protein with two incomplete MYB repeats, belonging to the R2R3-MYB family (Supplementary Figure 2A). Further phylogenetic analysis showed that MYB5 was the closest homologous to TraesCS3A02G257800, hence the gene was named TaMYB5-3A (Supplementary Figure 2B).

Phenotype of tamyb5 mutants

To verify the relationship between TaMYB5 and wheat leaf rolling, we selected three tamyb5 mutants (Supplementary Table 3) and backcrossed the mutants with the wild type (WT, AK58) two times to eliminate background noise. Under drought treatment, the leaves of tamyb5 mutants were flatter than that of WT at the flowering stage (Figure 2A), and the LRI index of WT was more than 60%, while that of mutant plants was less than 10% (Figure 2B). The phenotype of the tamyb5 mutant further confirmed that TaMYB5 was involved in the regulation of wheat leaf rolling.

TaMYB5-3A is associated with leaf rolling and canopy temperature

To further clarify the relationship between TaMYB5 and leaf rolling, we analyzed the polymorphism of TaMYB5
FIGURE 3
Nucleotide polymorphisms and molecular marker development of TaMYB5-3A. (A) Schematic diagram of the TaMYB5-3A structure, including two exons and one intron. One SNP was detected in the coding region of TaMYB5-3A. (B) The TaMYB5-3A-dCAPS marker was developed based on the SNP (C/A) at 544 bp. Red rectangle and dot represent the introduction of the SalI restriction site by base mismatched (A to T), and red letters represent the different bases at 544 bp of the TaMYB5-3A coding region. (C) PCR products were digested by SalI. The 153 bp fragment amplified from accessions with SNP-3A-1 could be digested by SalI into 132 bp and 21 bp, while the 153 bp fragment amplified from accessions with SNP-3A-2 could not be digested. M, 100 bp DNA ladder. SNP-3A-1 (C) was the majority in the wheat population, and this sequence was used in subsequent experiments if not specified.

FIGURE 4
Comparisons of LRI (leaf rolling index), and CT (canopy temperature) in the two TaMYB5-3A genotypes SNP-3A-1 and SNP-3A-2. (A) LRI comparisons of two TaMYB5-3A genotypes in six environments. LRI data were collected in 2020 and 2021. 20, 2020; 21, 2021; DS, drought stress; WW, well-watered; 1 or 2 after 20DS or 20WW refers to two replicates set for each treatment, respectively. (B) CT comparisons of two TaMYB5-3A genotypes in nine environments. CT data were collected in 2017. Plant growth stages: J, jointing stage; G, grain-filling stage. Planting locations: CP, Changping, SY, Shunyi. Treatments: DS, drought stress; WW, well-watered; HS, heat stress. The significance of data differences is tested by Student’s t test. ∗P < 0.05; ∗∗P < 0.01; ∗∗∗P < 0.001. Error bar, ± SE.
FIGURE 5
Frequency and distribution of two TaMYB5-3A genotypes, SNP-3A-1 and SNP-3A-2. Distribution of two TaMYB5-3A genotypes from 157 landraces (A) and 348 modern cultivars (B) in 10 Chinese wheat production zones. I, Northern Winter Wheat Zone; II, Yellow and Huai River Valleys Facultative Wheat Zone; III, Middle and Low Yangtze Valleys Autumn-Sown Spring Wheat Zone; IV, Southwestern Autumn-Sown Spring Wheat Zone; V, Southern Autumn-Sown Spring Wheat Zone; VI, Northeastern Spring Wheat Zone; VII, Northern Spring Wheat Zone; VIII, Northwestern Spring Wheat Zone; IX, Qinghai-Tibetan Plateau Spring-Winter Wheat Zone; X, Xinjiang Winter-Spring Wheat Zone.

in wheat tissues at the seedling stage and jointing stage by real-time quantitative PCR. The results showed that TaMYB5 was widely expressed in all tissues, and was mainly expressed in the leaf (Figure 6A), suggesting that TaMYB5 has a function in wheat leaf.

We next performed qRT-PCR to determine whether TaMYB5 is affected by PEG and ABA. The results showed that both ABA and PEG treatments reduced the expression of TaMYB5 in leaves. Under PEG treatment, the expression of TaMYB5 was down-regulated at 0.5 h, and there was no significant change in subsequent expression (Figure 6B). The expression of TaMYB5 decreased significantly after 0.5 h ABA treatment (Figure 6B). Taken together, these results suggest that TaMYB5 may play an important role in plant drought tolerance.

TaMYB5-3A is a nucleus-localized transactivator

To examine the subcellular localization of TaMYB5, we constructed the fusion protein of 35S-TaMYB5-GFP and transiently expressed it in tobacco leaves. The laser scanning confocal microscope images showed that the fluorescence signal of the 35S-TaMYB5-GFP fusion protein was only detected in the nucleus; nevertheless, the fluorescence of the 35S-1300GFP vector was detected across the whole cell (Figure 7A). Moreover, TaMYB5-2 was also localized in the nucleus (Supplementary Figure 4). The result provided that TaMYB5 encoded a nuclear localization protein, which was consistent with its potential localization as a transcription factor.

We further used a dual-luciferase reporter system to detect the transcriptional activity of TaMYB5. The empty GAL4DB vector was used as the negative control, and the fusion vector of VP16 (Herpes Simplex Virus16) was used as the positive control. As shown in Figure 7B, TaMYB5 exhibited stronger transcriptional activity than the empty GAL4DB vector. Together, these data
indicate that TaMYB5 is a transcriptional activator of nuclear localization.

**NRL1 is a putative target gene promoted by MYB5**

To determine the downstream target gene of TaMYB5, TaMYB5-1 and TaMYB5-2 were separately constructed into the pB42AD vector, and the promoters of predicted downstream genes were cloned into the pLacZ vector for yeast one-hybrid assays. We examined whether TaMYB5 binds to the promoter of genes involved in leaf rolling regulation, namely, *ACL1*, *CLD1*, *ROCS*, *CESAs*, *RL14*, *NRL1*, *PSL1*, and *SRL2*. Yeast one-hybrid assay showed that both TaMYB5-1 and TaMYB5-2 could bind to and activate the pro*NRL1* (Figure 8A). A previous study has demonstrated that *NRL1* is involved in leaf rolling regulation in rice, and the loss-of-function mutant *nrl1* displayed leaf rolling by affecting bulliform cell size (Hu et al., 2010). Yeast one-hybrid assay suggested that TaMYB5 could bind to the pro*TaNRL1*, and the A/C nucleotide variation of the *TaMYB5* coding region did not affect the binding to pro*NRL1*.

The AC box (ACCTAAC) is a specific cis-acting element regulated by the R2R3-MYB transcription factor (Prouse and Campbell, 2012). Notably, the *TaNRL1* promoter region contains the AC element (Figure 8A). After the induction and purification of the TaMYB5 protein (Supplementary Figure 5), electrophoretic mobility shift assay (EMSA) was performed to examine whether the TaMYB5 protein binds to the AC element in the *TaNRL1* promoter (Figure 8B). The migration rate of the biotin-labeled AC probe was significantly reduced with the addition of TaMYB5-GST protein, whereas the addition of GST protein alone did not affect the migration rate of the AC probe. In the competition
TaMYB5 binds to the wheat TaNRL1 promoter region. (A) TaMYB5 activated expression in yeast of the LacZ reporter gene driven by the AC box of TaNRL1 promoter. proNRL1 are base pairs from 1,150 bp to 2,000 bp in the promoter of TaNRL1. LacZ and pB42AD were used as negative controls. TaMYB5-1 and TaMYB5-2 correspond to SNP-3A-1 and SNP-3A-2 amino acid sequences, respectively. (B) EMSA (electrophoretic mobility shift assay) of the TaMYB5-GST protein and the AC box probe. The lower bands show the free probes and the upper bands show that the TaMYB5-GST protein binds to the biotin-labeled AC box probe. (C) Dual-luciferase assay of transformed tobacco leaves to detect the interaction between TaMYB5 and the proNRL1. Schematic diagrams of the effector and reporter constructs are shown. Data are means (± SE) of three biological replicates.

In the experiment, the unlabeled AC probe could compete with the biotin-labeled AC probe, while the mutated probe did not affect the binding of the biotin-labeled probe to TaMYB5 protein, suggesting that TaMYB5 could specifically bind to the AC cis-acting elements.

We also performed a dual-luciferase reporter assay to further verify that TaMYB5 binds to the TaNRL1 promoter and regulates its transcription. The p35S-TaMYB5-1-1300GFP and p35S-TaMYB5-2-1300GFP were constructed as effectors, and the TaNRL1 promoter was inserted into the LUC vector as a reporter. Compared with the negative control, the LUC activities were higher when the effector and reporter were co-expressed in tobacco leaves (Figure 8C). However, the LUC activity between the two alleles of TaMYB5 was not a significant difference. Taken together, the results suggest that TaMYB5 directly binds to its promoter to promote TaNRL1 expression through AC cis-acting element, while the A/C variation of the TaMYB5 coding region does not affect the binding to the TaNRL1 promoter.

Discussion

Leaf is the main organ of photosynthesis in wheat, and its morphology directly affects the photosynthetic rate. Moderate leaf rolling can keep the leaves upright, improve the population structure, and finally enhance the light use efficiency and yield of wheat (Richards et al., 2002; Zhang et al., 2015). Therefore, it is important to reveal the molecular mechanism of wheat leaf rolling. Here, we identified a novel gene TaMYB5 in wheat involved in leaf rolling by using GWAS. A SNP identified in the TaMYB5 coding region was significantly associated with leaf rolling, and the degree of leaf rolling of SNP-3A-2 was significantly higher than that of SNP-3A-1.
MYB proteins in the plant constitute one of the largest TF families involved in diverse processes, namely, plant growth, development, metabolic and stress responses. Previous studies reported that MYB proteins are involved in leaf rolling regulation. Ectopic expression of *GhMYB7* in *Arabidopsis thaliana* led to leaf rolling by activating a suite of genes involved in secondary cell wall formation, resulting in increased *thaliana* led to leaf rolling by activating a suite of genes involved in diverse processes, namely, plant growth, metabolic and stress responses. Previous studies reported that MYB proteins are involved in leaf rolling regulation. Ectopic expression of *GhMYB7* in *Arabidopsis thaliana* led to leaf rolling by activating a suite of genes involved in secondary cell wall formation, resulting in increased cellulose and lignin content (Huang et al., 2016). A MYB gene NbPHAN controlled leaf development by suppressing the *NTH20* expression gene and regulating plant tolerance to drought stress. NbPHAN-silenced plants exhibited severe downward rolling and an increased rate of water loss (Huang et al., 2013). Here, the leaves of the *tamyb5* mutant did not exhibit a severe curling response to drought stress compared with WT (Figure 2). The results confirmed that *TaMYB5* was involved in leaf rolling regulation of wheat. Moreover, we found that *NRL1* was a downstream target gene of *TaMYB5* (Figure 8). *NRL1* plays a critical role in leaf rolling by regulating bubble cell development (Hu et al., 2010). Mature leaves usually showed adaxially leaf rolling under water stress, which may be related to the bulliform cells (Fujino et al., 2008; Li et al., 2010). Under drought stress, dehydration reduces the turgor pressure of bulliform cells, resulting in leaf fold up on both sides and eventually leaf rolling. Once the water is restored, these cells absorb the water and expand again, and the leaves unfold again (Hsiao et al., 1984; Price et al., 1997). Therefore, it is speculated that the regulation of bulliform cells may be an intermediate step of *TaMYB5* in regulating wheat leaf rolling under drought stress.

Leaf rolling limited transpiration and increased canopy temperature under drought stress (Lian et al., 2004; Yan et al., 2009). Canopy temperature is negatively associated with yield under water stress (Melandri et al., 2020). These two traits are important considerations for plant development and drought tolerance. However, few studies addressed functional markers for these traits. Based on the SNP locus of 544 bp in the *TaMYB5* coding region, we developed a functional marker related to leaf rolling and canopy temperature (Figures 3, 4), which could be used for marker-assisted selection for leaf morphology and drought resistance. These results indicate that *TaMYB5* may have multiple effects on plant development and drought tolerance. In addition, we identified two alleles of *TaMYB5*, SNP-3A-1, and SNP-3A-2, based on the SNP locus of 544 bp. Based on the genotyping analysis of landraces and modern varieties in China, we found that the SNP-3A-1 genotype with low CT was positively selected in arid areas, while the SNP-3A-2 genotype with high leaf rolling was positively selected in humid areas (Figure 5). This may be due to the different breeding objectives of each wheat zone. The SNP-3A-1 genotype in arid areas is associated with increased drought tolerance, while the SNP-3A-2 genotype in humid areas is associated with enhanced photosynthesis. It is reported that the genotypes with a high degree of leaf rolling generally have less water uptake in the field under drought stress (Cal et al., 2019). The SNP-3A-2 genotype with a high degree of leaf rolling may not be selected in arid areas because it absorbs less water.

We tried to find out the reasons for the differences between the two *TaMYB5* genotypes by yeast one-hybrid and LUC assays, but no difference was found between them. Meta-analysis of large-scale phosphoproteomic data showed protein phosphorylation mainly through forming an ester bond with Ser (80–85%), Thr (10–15%), or Tyr (0–5%) residues and then modifying the proteins in plants (Champion et al., 2004; Wijk et al., 2014). We observed a difference of Thr and Lys in the amino acids of the two proteins of *TaMYB5* and then predicted their phosphorylation. Surprisingly, the differences in phosphorylation sites between the two proteins were identified (Supplementary Figure 6). Then, we selected eight accessions for each of the two extreme leaf rolling phenotypes and identified 13 variations in their promoter sequences. We then detected the expression level, but there was no significant difference in their expression levels (Supplementary Figure 7). So we inferred that the difference between the two haplotypes was caused by variations in the coding region rather than that in the promoter. Therefore, we speculated that a certain kinase might affect the phosphorylation of *TaMYB5*, resulting in different functions of the two proteins, which requires further experimental verification.
Overall, our research reveals a possible regulatory network, as shown in Figure 9: TaMYB5 modulates leaf rolling by promoting the expression of the TaNRL1 gene in wheat. Under well-watered condition, TaMYB5 did not affect the expression of TaNRL1, which inhibited leaf rolling and showed flat leaf. Under drought stress, the expression of TaMYB5 was reduced, and the expression of TaNRL1 decreased along with the decrease of TaMYB5, which weakened the inhibitory effect of NRL1 on rolling leaves (Hu et al., 2010), thus leaves showed a rolling state.

Collectively, we identified a novel MYB gene TaMYB5, which is a transcriptional activator and regulates leaf rolling by promoting the expression of TaNRL1. In addition, TaMYB5 is also associated with canopy temperature. The functional marker TaMYB5-3A-dCAPS will be helpful for future wheat breeding.

Data availability statement

The original contributions presented in this study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

RJ, JgW, CL, and JC conceived the idea. ZZ, JgW, CL, XM, GH, and JpW performed the experiments. ZZ, JgW, and LL analyzed the data. ZZ and JgW wrote the manuscript. RJ, JC, and JgW revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.897623/full#supplementary-material

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