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A genome-wide association study reveals that *DgFH18* and *DgCMO-like* are associated with flowering time in orchardgrass (*Dactylis glomerata*)

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Abstract

Background Flowering is a tightly regulated process influencing yield and promoting plant genetic diversity and conservation. Orchardgrass (*Dactylis glomerata*) exhibits excellent yield traits and stress resistance, making it ideal for animal husbandry and ecological restoration. However, the molecular regulatory factors of the flowering time of orchardgrass are still unknown, limiting its molecular breeding.

Results To speed up molecular breeding to enhance flowering traits in orchardgrass, we conducted a genome-wide association study (GWAS). A diverse panel of 249 orchardgrass accessions was phenotyped for heading stage and flowering time. GWAS analysis identified 359 candidate genes that overlapped or were adjacent to effective single-nucleotide polymorphisms (SNPs), which were considered potential flowering time-related genes. Furthermore, we validated that formin-like protein 18 (*DgFH18*) and choline monooxygenase (*DgCMO-like*) was two important flowering candidate genes by overexpressing them in *Arabidopsis* to unravel their potential functions. Overexpression of *DgFH18* and *DgCMO-like* positively regulated flowering time by inducing the expression of flowering-related genes. Moreover, sucrose treatment could significantly promote the expression of flavonoid pathway genes and enhance the content of total flavonoids and anthocyanins in the *DgCMO-like*-overexpressing lines compared to the wild type.

Conclusion These results provide valuable resources for future orchardgrass breeding programs and broaden the current comprehension of flowering time regulation in perennial grasses.

Keywords *Dactylis glomerata* L., Genome-wide association analysis, Flowering time

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Background

The transition of floral represents a pivotal process in the life cycle of plants, which ensures genetic continuity and variability, controlled by many environmental and endogenous signals [1]. The flowering time traits have a high positive correlation with crop maturity and yield. Plants have been evolvement many complicated regulatory pathways in control flowering time to ensure survival across various environmental conditions, mainly including vernalization, photoperiod, GA and circadian clock [2]. While several genes regulating flowering time have been identified [3, 4], research on flowering time in non-model perennial plants is limited.

Orchardgrass (*Dactylis glomerata* L.), is perennial cool-season grass, originates from northern Africa, western and central Europe, and the temperate and tropical zones of Asia [5]. The genus *Dactylis* is characterized by three ploidy levels, including diploidy, tetraploidy and hexaploidy, with the basic chromosome number of x = 7 [6]. Tetraploids are the most common and widely distributed forms of Dactylis. Germplasm resources of orchardgrass are notably abundant in China, particularly in the southwestern and northwestern regions. These resources predominantly thrive in environments such as forest margins, shrublands, and sub-alpine meadows, typically at altitudes between 1000 and 3600 m [7]. Orchardgrass has been extensively used as pasture due to its excellent forage production, high output, superior quality, good resistance to shade and drought, and strong adaptability to local conditions, significantly contributing to livestock farming and environmental sustainability. Early flowering of orchardgrass is undesirable since livestock avoid eating the flowering stalks, and early flowering also leads to management challenges in spring. Delayed flowering is also not ideal since particular late-flowering varieties have demonstrated diminished net herbage accumulation within rotational grazing systems or compromised survival rate [8, 9]. Thus, proper flowering timing is crucial for the use of orchardgrass in animal husbandry and for its adaptability to varying climatic conditions in different regions and cropping seasons.

Wind pollination, natural selection, and adaptation have led to significant morphological diversity in orchardgrass. Orchardgrass breeding programs have been ongoing for about a century, but most are still focused on mass selection and clonal breeding [10]. Enhancing characteristics in forage grasses necessitates repeated selection across various locations and years of testing, extensive field experiments, and progeny evaluations, all of which can be extremely time-intensive and costly. Therefore, molecular markers and genetics have been employed to hasten the improvement of orchardgrass by quickly creating better varieties [11, 12]. Furthermore, the orchardgrass reference genome and several crucial genomic and transcriptomic resources have been made available, further facilitating the breeding process [13]. The genome map was constructed from orchardgrass accessions through genotyping-by-sequencing (GBS) [14]. These materials are crucial for the rapid advancement of orchardgrass studies.

The correct flowering time is crucial for both reproductive success and the yield of crops. Inappropriate flowering time restricts yield potential, disrupts harvest operations and hinders breeding progress. Previous studies on the flowering time of orchardgrass mainly focused on a certain variety, and the molecular mechanism of flowering time in population genetics is still limited. Current genome-wide association studies (GWAS) have pinpointed genomic areas linked to flowering time traits in various species, including tree peony [15], switchgrass [16], and wheat [4]. Consequently, investigating the molecular mechanisms underlying the flowering time through GWAS is significant interest within the field of population genetics of orchardgrass. In this study, we gathered data on the heading stage and flowering time for 249 orchardgrass accessions in four different environments and conducted GWAS analysis to identify key candidate genes of flowering. The GWAS analysis showed that *DgFH18* and *DgCMO-like* may be significant contributors to the regulation of flowering time in orchardgrass. Identifying single nucleotide polymorphisms (SNPs) associated with flowering time provides a deeper understanding of molecular breeding in orchardgrass and other perennial plants and is a valuable resource to improve the breeding process.

Materials and methods

Plant materials

This study used 249 orchardgrass accessions obtained from the Chinese Grass Germplasm Resource Library and the National Plant Germplasm System of the United States. The information on the 249 orchardgrass accessions is shown in Table S1. These accessions were evaluated for heading stage and flowering time in two regions in 2021 and 2022: (a) the Ya'An in Sichuan province, China (YA region, 103.01 E, 29.98 N, 2084.9 mm average annual rainfall, 610 m above sea level) (b) and the Da'Yi in Sichuan province, China (DY region, 103.52 E, 30.58 N, 918.3 mm average annual rainfall, 1100 m above sea level). Two independent biological replicates were conducted in a completely random design for each field experiment.

Heading stage and flowering time measurements

Each experimental field was subjected to appropriate management practices, such as weed removal, disease prevention, and fertilization tailored to the local environment. We utilized a previously described method for

phenological period assessment with slight modifications [17]. The heading stage represented the full emergence of the first inflorescence from the flag leaf while flowering time was defined as 50% of florets blossoming from the first inflorescence. Heading stage and flowering time traits were recorded two times a week.

GWAS analysis

The genomes of all accessions were sequenced using GBS at a sequencing depth of 12X. The FastQC-0.12.0 was used to quality control of raw data and then aligned to the reference genomes of orchardgrass with the accession number PRJNA471014 [11, 18]. The SNPs of all orchardgrass geneotypes used in the GWAS analysis are publicly available under the accession number PRJCA018363 [14]. The obtained SNP markers and the heading stage and flowering time phenotypic data collected from the 249 accessions across the two years were used for GWAS analysis. The GWAS analysis of heading stage and flowering time was performed utilizing a diploid analysis model and the ridge regression best linear unbiased prediction (RR-BLUP) model available in the rrBLUP package within the R software [19, 20]. For the RR-BLUP model, individual genotypes were randomly split into training and validation sets with 80 and 20% ratio, respectively, and repeated for 500 iterations [21]. A General Linear Model (GLM) analysis was employed to filter out the effective SNPs based on the modified Bonferroni correction was adopted to determine the significant associations, utilizing a significance threshold of $p > 10^{-4}$ [22, 23]. Manhattan and Quantile-quantile (Q-Q) plots were preformed using the ggplot2 package within the R software [24]. The candidate genes of heading stage and flowering time were screened in the 50 kb on either side of effective SNPs [25].

Subcellular localization and plant transformation

The ORFs of *DgFH18* and *DgCMO-like* were inserted into the pAN580-35 S-GFP vector (Table S2). The empty plasmid served as a negative control. The pAN580-*DgFH18* recombination vector and the empty vector were infiltrated into the lower epidermises of tobacco leaves for one month, and the plants were kept under dim light conditions for two days. The methodologies for transferring the fusion vector of pAN580-*DgCMO-like* into rice protoplasts were referred to previous reports. Nikon C2-ER laser confocal microscope system was used to assess the location of *DgFH18* and *DgCMO-like* proteins, respectively.

Furthermore, the ORFs of *DgFH18* and *DgCMO-like* were inserted into pHG-35 S-GFP fusion vectors and subsequently introduced into *Arabidopsis* using the floral dip method [26] (Table S2). Overexpressing plants the transformed genes were selected on 1/2 MS medium enriched with 25 $\mu g.ml^{-1}$ hygromycin. The seeds of WT and homozygous T3 *DgFH18* and *DgCMO-like* overexpressing *Arabidopsis* were sown on soil in plant growth chamber (12 h light / 12 h dark, 23 °C), with 200 μmol m^{-2} s^{-1} light intensity and 80% humidity. Flowering time and the number of rosette leaves were counted once the bolt was 0.5 cm tall [27].

Physiological index determination of *DgCMO-like* Transgenic plants

To explore the potential function of *DgCMO-like* in responding to sucrose stress, the seeds of WT and homozygous T3 *DgCMO-like*-overexpressing *Arabidopsis* were sown in soil. We measured physiological indexes after a week of treatment with 1% sucrose, such as the concentrations of malondialdehyde (MDA), total flavonoids, and anthocyanins. These physiological indexes were measured with specialized assay kits provided by Grace Biotechnology Co., Ltd., Suzhou, China.

qRT-PCR analysis

The extraction of RNA and synthesis of cDNA refer to previous descriptions [28]. The $2^{-\Delta\Delta Ct}$ technique was employed to assess the result of qRT-PCR [29]. *Atactin* and *Atactin2* were employed as reference genes in this study. Primer sequences were designed on NCBI and are presented in Table S3.

Data analysis

Hierarchical clustering was performed using the factoextra package in R. Venn diagram was shown using VennDiagram package in R (http://www.r-project.org/). Gene Ontology (GO) analysis was performed using the ggplot2 package in R. The transcriptome data of expression levels in different tissues were obtained from previous research [11]. Heatmaps of expression patterns were performed in R. Homologous protein sequences of DgFH18 and DgCMO-like were identified through the BLASTP tool on NCBI. The evolutionary tree was constructed using the maximum likelihood (ML) method in MEGA 7.0 software, with 1,000 bootstrap replicates [30]. Protein domains were identified using the Conserved Domain Search tool available on NCBI (https//www.ncbi.nlm.nih.gov) and visualized with the TBtools application. The data of histograms were analyzed by Microsoft Excel 2017 (Microsoft, Redmond, USA) and were shown as mean ± SD using GraphPad Prism 9.2.0 (GraphPad, Boston, USA). The statistical difference was compared and obtained by ANOVA (least significance difference, LSD test) analysis using SPSS version 27.0 (IBM, Armonk, USA).

Results

The phenotypic variation of heading stage and flowering time in the GWAS population

The natural variation among 249 orchardgrass accessions in heading stage and flowering time were evaluated across four different environments. A considerable variation was observed in the heading stage and flowering time among the accessions, with the duration of the heading stage ranging from 58 to 179 days, and the flowering time spanning from 73 days to over 190 days (Table S4). In addition, we found that the orchardgrass population in the YA region continuously formed heads and flowered earlier than that in DY, potentially due to the varying climatic conditions of the DY region. A notable positive relationship was reported previously between the heading stage and flowering time in several species of the grass family [31]. To determine the degree of correlation between the heading stage and flowering time in orchardgrass, we conducted a correlation analysis based on their phenotypic data (Fig. S1). A positive relationship was observed between the flowering time and the heading stage in orchardgrass ($p > 0.8$).

Cluster analysis categorized the 249 accessions were divided into six different classes (I-VI) (Fig. 1). Class VI exhibited the largest number of accessions numbers (78), followed by class IV (67 accessions), class V (42 accessions), class II (33 accessions), class III (27 accessions), and class I (2 accessions) (Table S5). The accessions in class II, such as PI325294, PI315424, Jinniu, PI598423, and PI231474, showed obvious late flowering phenotype (Fig. 2), while those in class III, including PI235124, PI230117, PI308542, PI269885, and PI287818, showed an obvious early flowering phenotype.

GWAS analysis of the candidate genes linked to heading and flowering characteristics of orchardgrass

The Q-Q plots indicated that the GLM model was suitable for the identification of the SNPs significantly associated with heading stage (HS) and flowering time (FT) traits (Fig. 3). The GWAS analysis detected 150 effective SNPs, and the 50 kb regions associated with these SNPs contained 359 candidate genes, which could be related to the HS and FT traits (Fig. S2a; Table S6). In 2021, the candidate genes linked to the HS and FT traits were mainly significantly enriched in "pattern binding," "polysaccharide binding," and "coenzyme binding" molecular functions of the GO enrichment analysis (Fig. S2b). In 2022, the linked genes were predominantly associated with "peroxidase activity," "oxidoreductase activity" and "antioxidant activity" molecular functions and the "photosynthesis, light reaction" biological processes (Fig. S2c).

In addition, functional annotation yielded 12 candidate genes related to orchardgrass flowering (Table 1). Ten of these genes have homologs in other species and

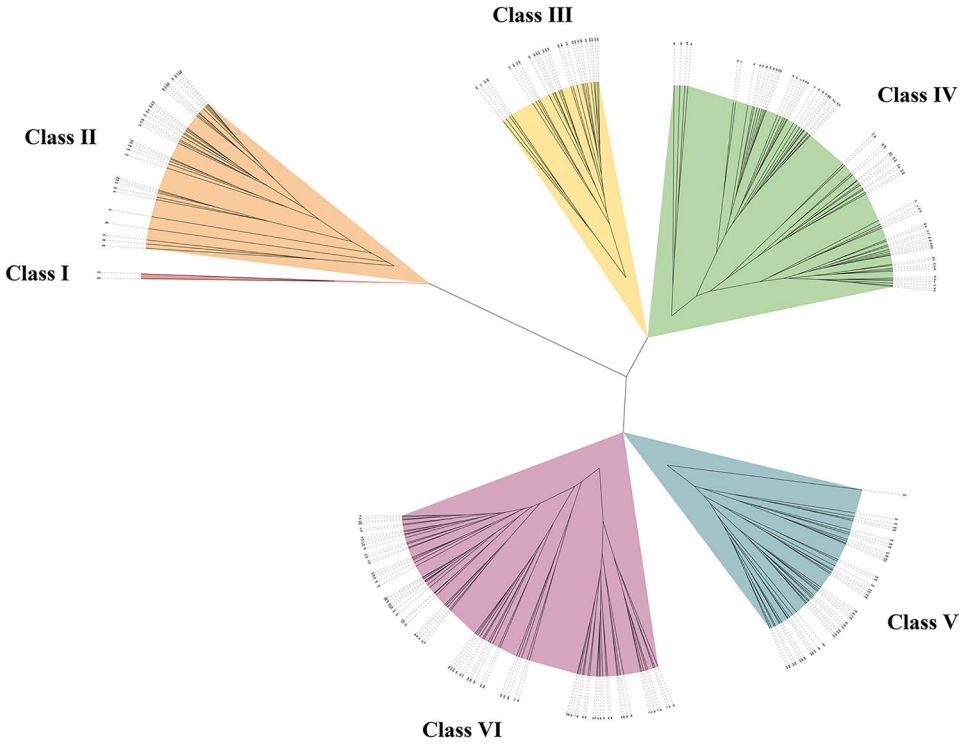

Fig. 1 Hierarchical cluster analysis of the flowering time of the 249 orchardgrass accessions. Each cluster is color-coded as follows: Class I (Light Pink), Class II (Orange), Class III (Yellow), Class IV (Green), Class V (Blue), and Class VI (Purple). The branching patterns indicate the degree of similarity between the accessions, with closer branches representing higher genetic similarity

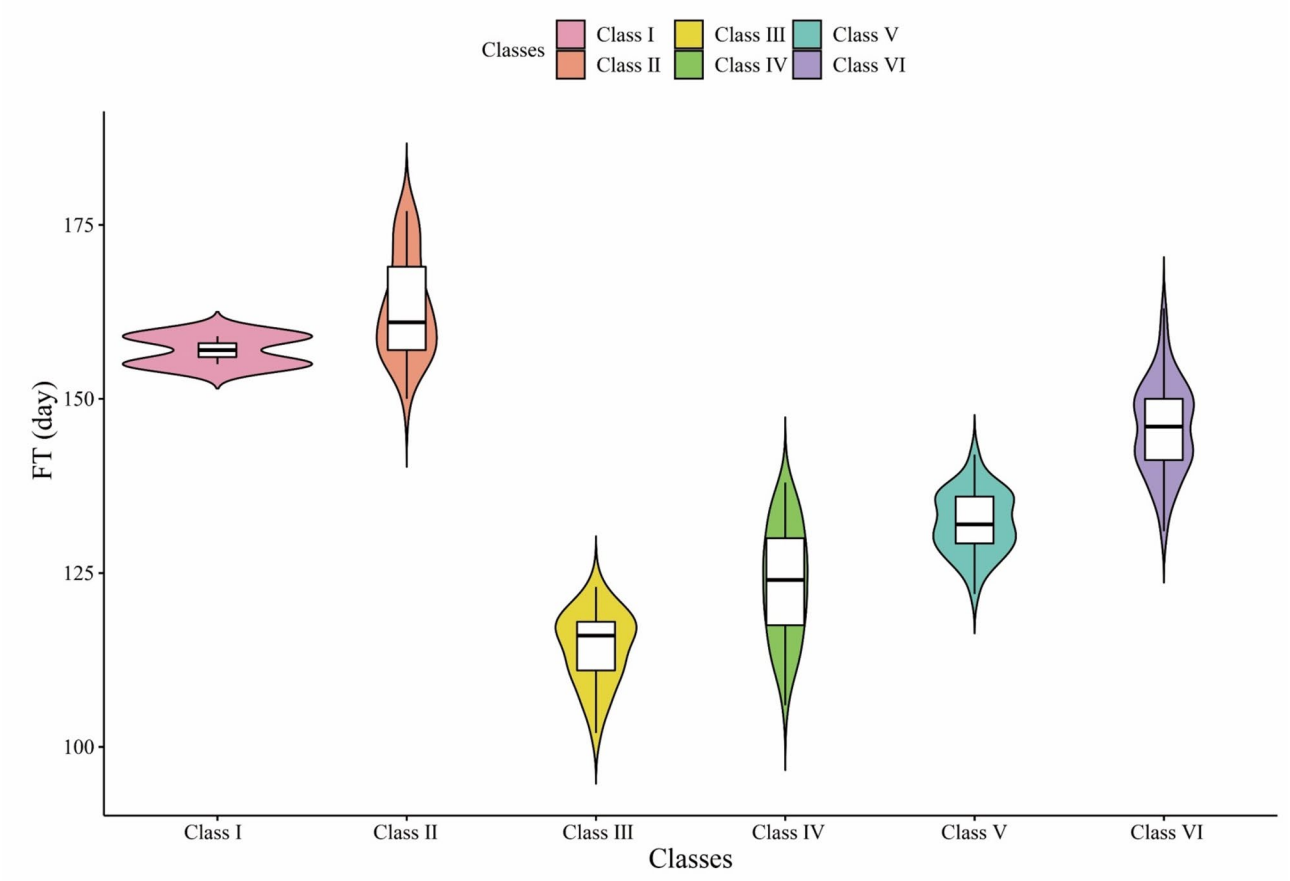

Fig. 2 A violin plot of the flowering time in six classes of orchardgrass accessions

are associated with flowering, whereas the remaining two genes, *DG1C00225.1* (*DgFH18*) and *DG7C02942.1* (*DgCMO-like*), have not been documented as playing a role in flowering. The expression patterns of 12 candidate flowering genes were examined in the root, stem, leaf, spike and flower tissues (Fig. 4). The majority of candidate genes were highly expressed in the spike and flower. In particular, the genes of *DG1C00225.1*, *DG7C00835.1*, *DG1C01069.1* and *DG1C01667.1* had the highest expression levels in the flower tissue compared to other tissues. These findings indicate that these candidate genes could play a role in the development of spikes and flowers in orchardgrss. In the year 2021, an effective SNP (Chr1_5910149) variation was identified in the 5'UTR segment of the *DgFH18* gene. Another effective SNP (Chr7_135386259) locus variation was found in the upstream promoter region (2094 bp) of the *DgCMO-like* gene. Variations in base sequences in the 5'UTR and promoter regions potentially influence gene expression. Therefore, *DgFH18* and *DgCMO-like* are likely to be significant contributors to the regulation of flowering time in orchardgrass.

DgFH18 overexpression promotes early flowering

To investigate the evolutionary relationships between different grass species, we performed a phylogenetic analysis using the *DgFH18* gene. The results demonstrated that DgFH18 protein had the closest evolutionary relationship with LrFH18 protein (Fig. 5a). Since gene function can be preliminarily determined by observation of the encoded protein's distribution within the cell, we investigated the subcellular distribution of DgFH18 by expressing a *DgFH18* gene fused in tobacco. The result showed that DgFH18 was located in the nucleus and cytoplasm (Fig. 5b). Overexpressing *DgFH18* in *Arabidopsis* plants further verified the biological function of *DgFH18* since the overexpressing lines exhibited earlier flowering phenotypes than the WT (Fig. 6a and c). Additionally, the well-known flowering-promoting genes *AtAP1*, *AtFUL* and *AtFT* were significantly upregulated in *DgFH18*-overexpressing plants than the WT (Fig. 6d). Accordingly, the negative flowering-regulator *AtFLC* was lowered significantly in the *DgFH18*_OE plants. These results demonstrate that *DgFH18* is capable of inducing the expression of flowering-related genes, promoting flowering in transgenic *Arabidopsis.*

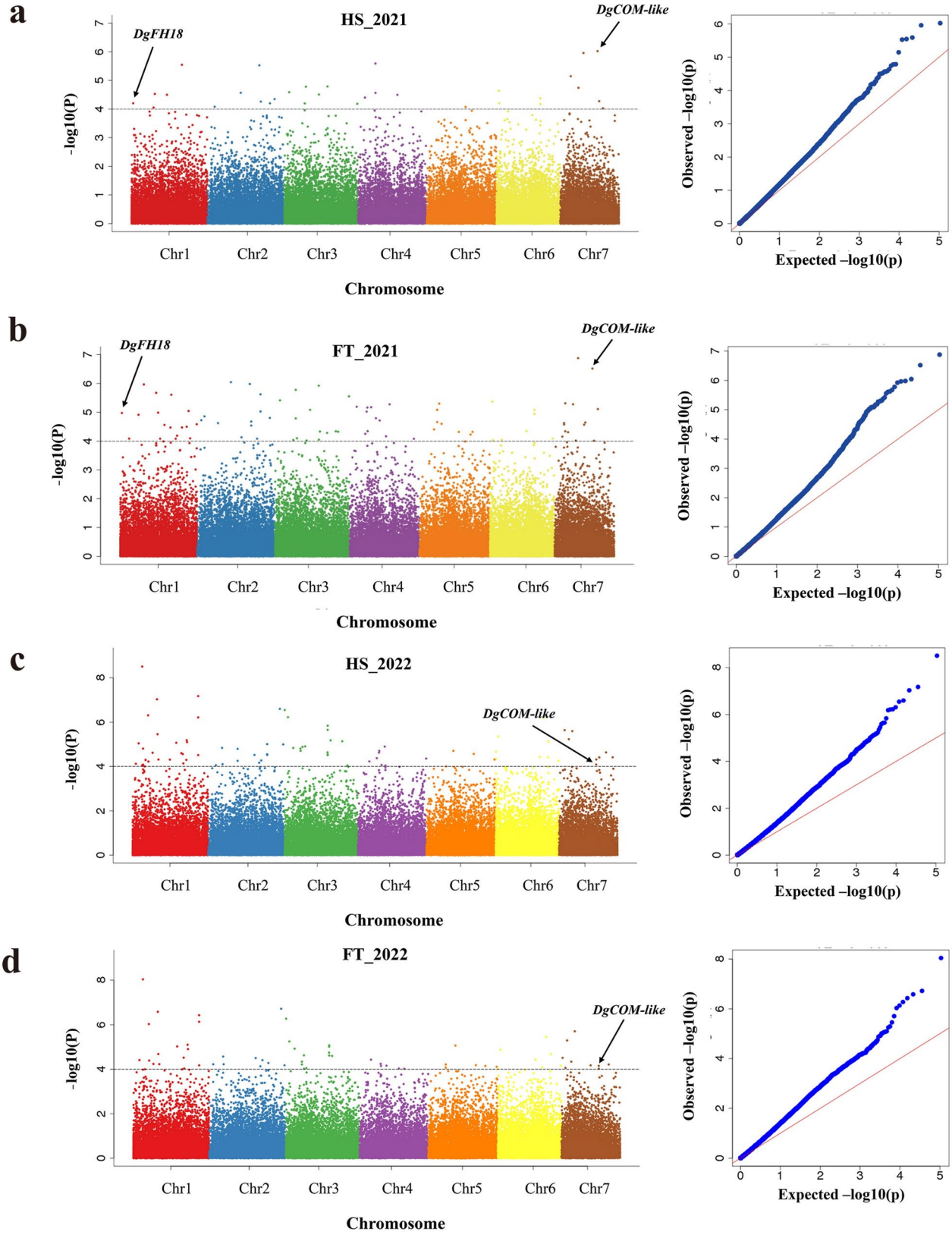

Fig. 3 Manhattan and quantile-quantile (Q-Q) plots. (**a** and **c**) Genome-wide *P*-values of the associations between single nucleotide polymorphism (SNP) markers and heading stage in 2021 and 2022. (**b** and **d**) Genome-wide *P*-values of the associations between SNP markers and flowering time in 2021 and 2022. Different colors represent different chromosomes of orchardgrass in the X-axis. The horizontal gray line represents the threshold for significant associations -log10(p) value of ≤ 4

Table 1 Basic information of the 12 selected candidate genes

Year	Gene_ID	Chr	Peak_value	Distance	Annotation	Function
2021	DG1C00224.1	Chr1	4.9767212	5703	Protein MRG1	Mutants of *mrg1/mrg2* can inhibit flowering time [33]
	DG1C00225.1	Chr1	4.9767212	0	Formin-like protein 18 (FH18)	unkonwn
	DG1C01069.1	Chr1	4.0859378	-10,771	HUA2-like protein 2 (HUA2)	*hua2* mutations suppress late flowering [34]
	DG5C01795.1	Chr5	5.0855299	26,208	25.3 kDa vesicle transport protein (Sect. 22)	*Sect. 22* mutant pollen becomes abnormal [35]
	DG6C01454.1	Chr6	4.0452596	44,798	Protein ROS1	*dme; ros1;dml2;dml3* mutants display early flowering [36]
	DG7C00835.1	Chr7	4.1562084	49,676	BTB/POZ and MATH domain -containing protein 1 (BPM1)	*BPM1*-overexpressing plants show early flowering [37]
	DG7C02155.1	Chr7	4.5691769	-6969	Protein TOPLESS (TPL)	mutants of *tpl* display an early flowering [38]
	DG7C02942.1	Chr7	6.5182556	2094	Choline monooxygenase (CMO-like)	unknown
2022	DG1C01667.1	Chr1	4.3898488	12,742	MADS-box transcription factor 18 (MADS18)	Overexpression of *MADS18* promotes flowering [39]
	DG2C01610.1	Chr2	4.2224971	-48,334	HVA22-like protein i (HVA22)	Deletion of *AtHVA22* impairs flower development [40]
	DG3C01722.1	Chr3	4.3361541	-29,790	Acetyl-coenzyme A synthetase (ACS)	*acs1* mutant delays flowering [41]
	DG6C00366.1	Chr6	4.8750519	33,843	FRIGIDA-like protein 3 (FRL3)	*frl3* mutant delays floral timing [42]

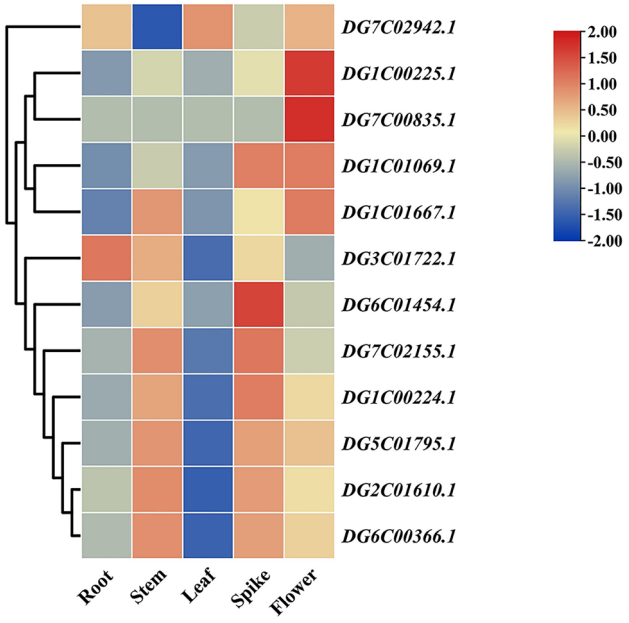

Fig. 4 Heatmaps of 12 selected candidate genes in different tissues. The x-axis represents different tissues. From left to right: Root, Steam, Leaf, Spike and Flower

Overexpressing *DgCMO-like* induces early flowering and regulates sucrose signaling responses

The *DgCMO-like* gene encodes choline monooxygenase. The DgCMO-like-GFP fusion vector was localized in cytoplasmic particles (Fig. 7b). The phylogenetic tree constructed based on 17 species showed that DgCMO-like protein had the closest evolutionary relationship with BdCMO protein (Fig. 7a). However, these two proteins have different conserved domains, suggesting their distinct biological functions. In order to conduct a more in-depth analysis of the function of *DgCMO-like*, we generated transgenic *Arabidopsis* plants overexpressing *DgCMO-like* (Fig. 8a and c). The *DgCMO-like* transgenic *Arabidopsis* plants showed early flowering phenotypes, with significantly increased expression levels of *AtCAL*, *AtFT*, *AtFUL*, *AtLFY*, *AtAP1*, and *AtSPL3* (Fig. 9a). The promoter region of the *DgCMO-like* gene contains *cis*-elements associated with the regulation of flavonoid biosynthetic genes (Fig. 8b). Sucrose not only induces flavonoid biosynthesis, particularly anthocyanin biosynthesis, but it also serves as a signaling molecule modulating early reproductive processes [42, 43]. In this view, we evaluated whether the *DgCMO-like* gene is responsive to sucrose.

Compared to normal conditions, the exogenous addition of 1% sucrose caused stress to WT and *DgCMO-like*-overexpressing *Arabidopsis* but did not affect their flowering phenotypes (Fig. 8a and c). Moreover, the levels of MDA, total flavonoids, and anthocyanins in *DgCMO-like*-overexpressing *Arabidopsis* plants were elevated compared to WT under sucrose treatment (Fig. 8e – 8 g). Correspondingly, the expression levels of *AtTT4*, *AtTT5*, *AtTT7*, *AtCHIL*, *AtF3H*, *AtDFR*, and *AtBAN* associated with flavonoid biosynthesis pathway were also higher in *DgCMO-like*-overexpressing lines than in the WT under 1% sucrose treatment (Fig. 9b). These results confirmed that *DgCMO-like* positively influences flowering time under normal conditions. Moreover, the application of 1% sucrose did not affect the flowering phenotype of the *DgCMO-like*-overexpressing *Arabidopsis* plants

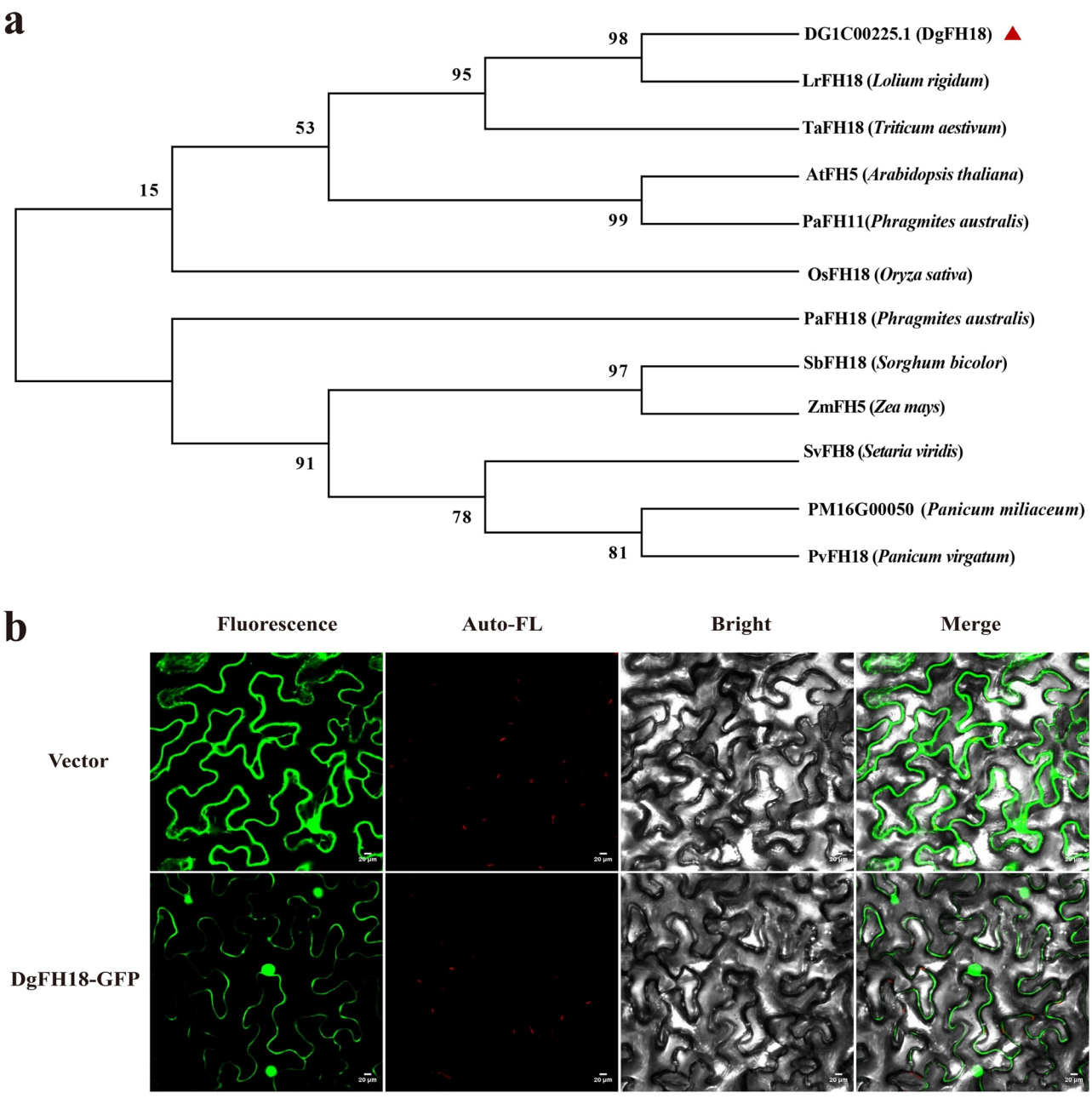

Fig. 5 The basic information of *DgFH18* gene. (**a**) Phylogenetic tree among DgFH18 and other homologous genes. (**b**) The subcellular localization of DgFH18 protein in tobacco leaves. Scale bar = 20 μm

but significantly affected the total flavonoid content and anthocyanin content.

Discussion

Orchardgrass plays a crucial role as forage for meat and dairy production in temperate zones [44]. The transition to flowering represents a critical physiological event in the ontogeny of crop plants and constitutes a significant agronomic characteristic influencing both crop productivity and breeding strategies. It is regulated by a sophisticated system involving various external stimuli and internal signals. Therefore, understanding the natural variations in flowering time and identifying SNPs associated with those variations are prerequisites for the improvement of yield by molecular breeding technology. Genome-wide association studies are an effective method for exploring intricate agricultural traits [4, 45]. The present work evaluated the flowering time of 249 orchardgrass accessions and conducted GWAS analysis to explore and validate the crucial candidate genes related to flowering time.

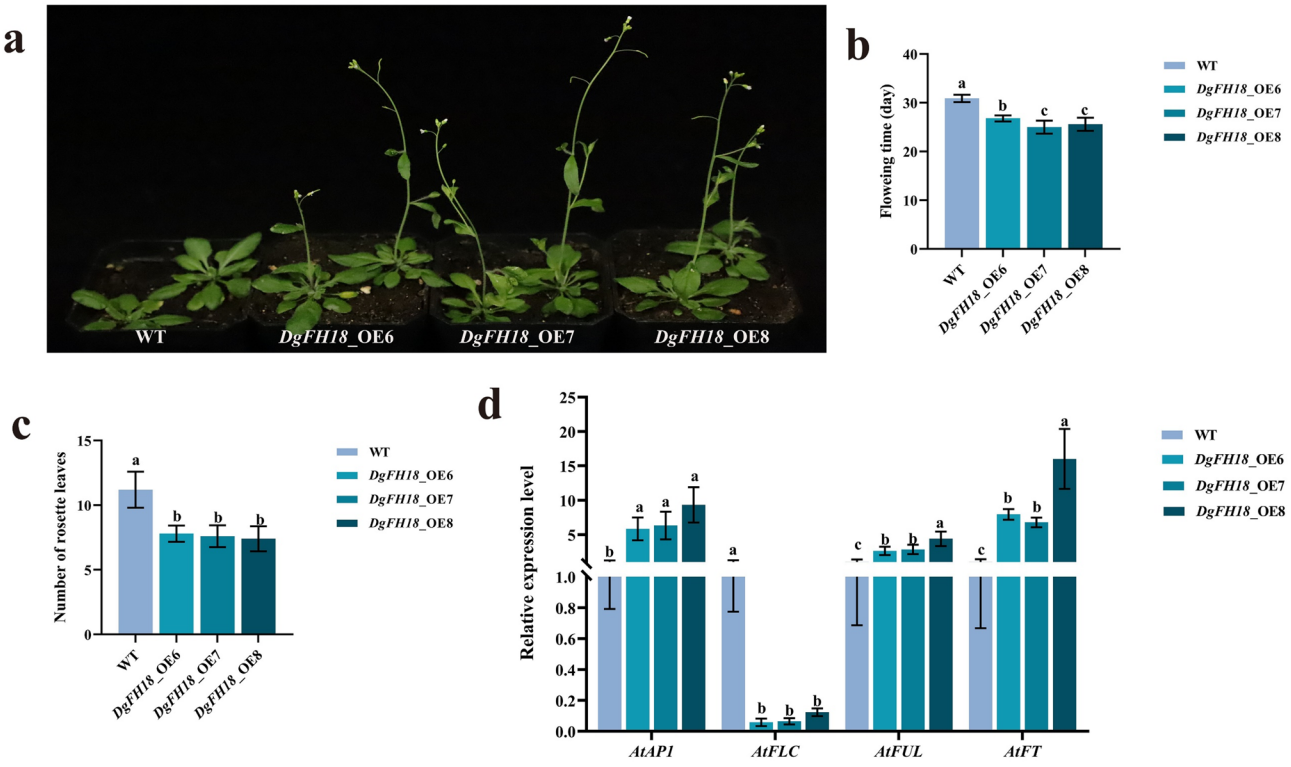

Fig. 6 Effect of *DgFH18* overexpression on the flowering time. (**a**) The flowering phenotypes of the *DgFH18*-overexpressing plants and wild type (WT). (**b**) The flowering time in the *DgFH18*-overexpressing plants and WT. (**c**) The number of rosette leaves in the *DgFH18*-overexpressing plants and WT. (**d**) The relative expression level of flowering time-related genes at 21 days. Bars represent the mean ± SD of ten biological replicates in flowering time and number of rosette leaves analysis, while three biological replicates in qRT-PCR analysis. The different lowercase letters above the bars indicate statically significant differences at $P < 0.05$

The effects of climatic conditions on flowering time has been extensively studied, for instance, 61.4% of the SNPs linked to altitude were concurrently found to be related to the flowering time in maize [46]. In *Arabidopsis*, the flowering time for high-altitude populations was prolonged but shortened for those at low and medium altitudes [47]. The heading date represents flowering initiation in wheat. Spring temperature is a dominant factor in wheat heading induction compared with other climate factors [31]. Here, the flowering time of orchardgrass accessions ranged from 73 days to more than 190 days, with a large variation. However, the orchardgrass population in the YA region consistently flowered earlier than that in DY. The higher altitude associated with lower temperature in the DY region might have caused the late flowering of the orchardgrass population in the DY region compared to the YA region. In addition to climate factors, we also identified effective SNP markers related to flowering time in orchardgrass through GWAS analysis. Twelve genes closely linked to the flowering time of orchardgrass were identified (see Table 1). Among these, ten genes have been identified as being linked to flowering time in other plant species. The genes were annotated as homologs of *MRG1* [32], *BPM1* [36], *HUA2* [33], *Sect. 22* [34], *ROS1* [35], *TPL* [37], *MADS18* [38], *HVA22* [39], *ACS* [40], and *FRL3* [41], respectively, which are mainly involved in regulating flowering time, flower development and pollen development.

The effective SNPs were identified in the 5'UTR segment of the *DgFH18* and the upstream promoter region of the *DgCMO-like.* Therefore, *DgFH18* and *DgCMO-like* may also be critical novel genes affecting the flowering time of orchardgrass. Numerous developmental processes in plants necessitate the interaction between actin filaments and microtubules [48, 49]. Formins are large multidomain proteins involved in various action-dependent activities, including cell division and cell polarity maintenance [50]. There are few studies on plant formin proteins, with most mainly focusing on rice and *Arabidopsis* [51, 52]. Overexpression of *Arabidopsis AtFH1* in pollen showed that the actin polymerization regulated by *AtFH1* is essential for the polar growth of pollen cells [53]. The rice mutants of formin-like gene *FH5/RMD* rice exhibited retardation and aberrant inflorescence and seed shape [52]. Nevertheless, the effect of *DgFH18* and its homologs on flowering time have not been reported. The findings revealed that overexpressing *DgFH18* displayed a notably earlier flowering phenotype than WT

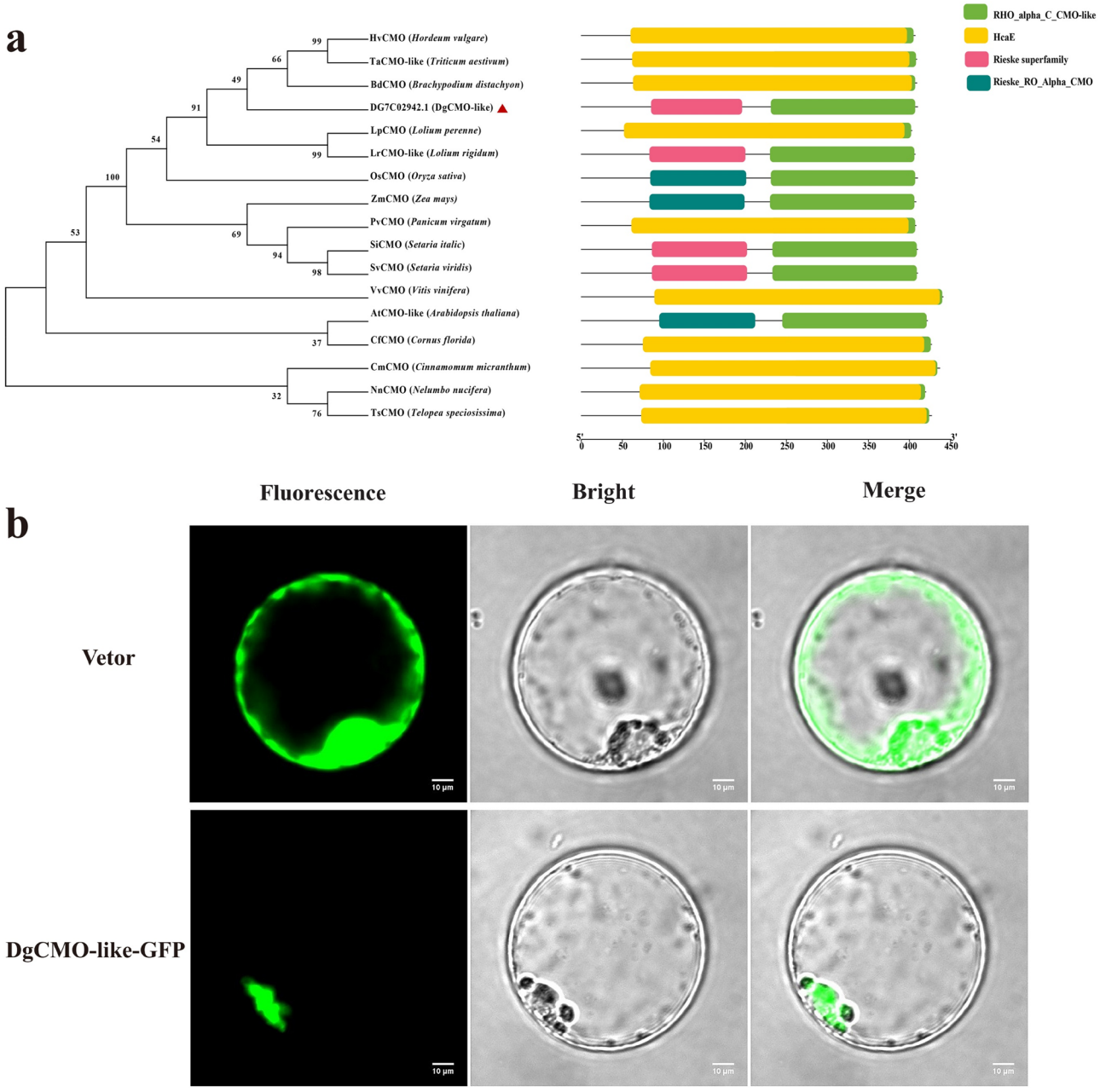

Fig. 7 The basic information of *DgCMO-like* gene. (**a**) Phylogenetic tree and conserved domain among DgCMO-like and other homologous genes. (**b**) The subcellular localization of DgCMO-like protein in rice protoplasts. Scale bar = 10 μm

plants, suggesting that *DgFH18* may be instrumental in regulation of flowering time in orchardgrass.

The *CMO* gene is involved in glycine betaine synthesis, however, previous research on this gene has mainly focused on abiotic stress resistance [54]. Limited information is available about the biological role of the *CMO* gene in the growth and development of plants. In this study, an effective SNP (Chr7_135386259) locus variation was found in the upstream promoter region of the *DgCMO-like* gene, which may be involved in flowering time regulation in orchardgrass. The transgenic *Arabidopsis* plants exhibiting *DgCMO-like* characteristics demonstrated an accelerated flowering phenotype, accompanied by elevated expression levels of flowering-related genes, including *AtCAL*, *AtFLC*, *AtFT*, *AtFUL*, *AtLFY*, *AtAP1*, and *AtSPL3*. Furthermore, the promoter region of *DgCMO-like* gene contained *cis*-elements related to the regulation of flavonoid biosynthetic genes. Flavonoids can regulate plant development, pigment deposition and environmental stresses [55, 56]. Anthocyanins are also a type of flavonoids [57]. Sucrose is an effective inducer of flavonoid biosynthesis,

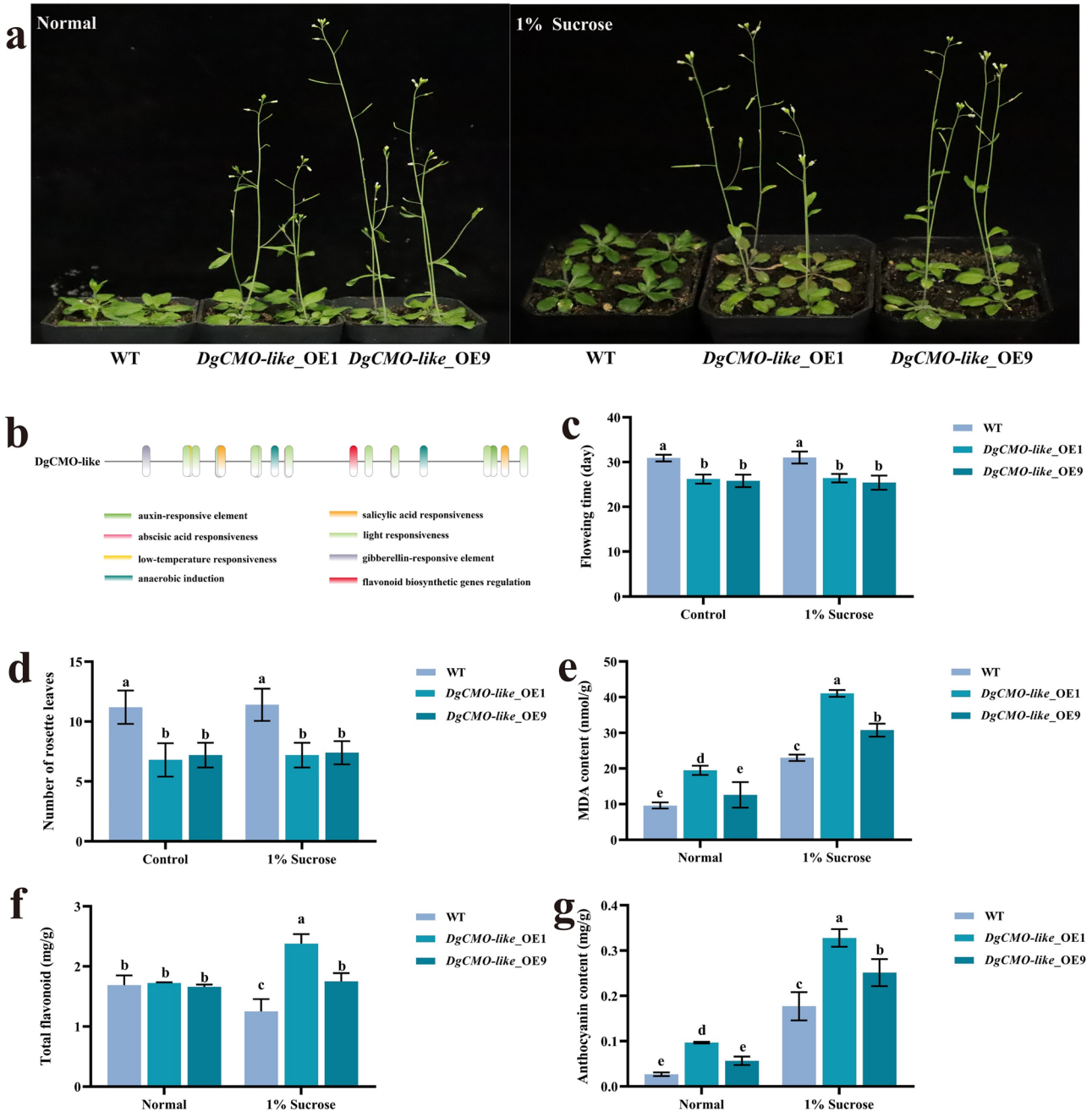

Fig. 8 Effect of *DgCMO-like* overexpression on flowering time and responses to sucrose signals. (**a**) The flowering phenotypes of the *DgCMO-like*-overexpressing plants and wild type (WT). The images on a and were photographed on the same day. (**b**) The distribution of *cis*-acting regulatory elements of *DgCMO-like* in orchardgrass. (**c**-**d**) The flowering time and number of rosette leaves of the *DgCMO-like*-overexpressing plants and WT under normal and 1% sucrose conditions. (**e**-**g**) The malondialdehyde (MDA) content, total flavonoid content, and anthocyanin content of the *DgCMO-like*-overexpressing plants and WT under normal and 1% sucrose conditions. Bars represent the mean ± SD of ten biological replicates in flowering time and number of rosette leaves analysis, while three biological replicates in MDA, total flavonoid content, and anthocyanin content analysis. The different lowercase letters above the bars indicate statically significant differences at $P < 0.05$

particularly anthocyanin biosynthesis. Sucrose not only supplies carbon skeletons, but also functions as signaling molecules or stimuli, thereby impacting metabolic processes and modulating the expression of pertinent genes [58]. Sucrose can repress the gibberellin-mediated degradation of DELLA proteins, thereby increasing anthocyanin biosynthesis. Compared with normal conditions, the exogenous addition of 1% sucrose induced stress to WT and *DgCMO*-like-overexpressing *Arabidopsis* plants but did not affect their flowering phenotype.

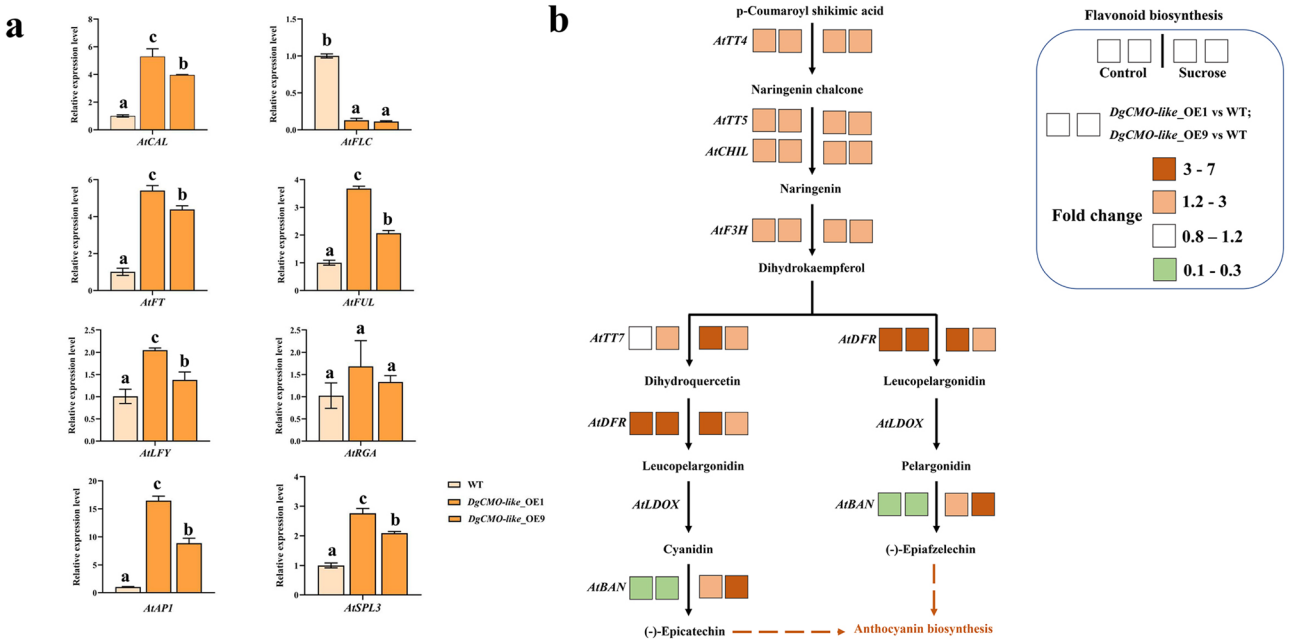

Fig. 9 The relative expression levels of flowering-related genes (**a**) and flavonoid biosynthesis pathway genes (**b**). Bars represent the mean ± SD ($n = 3$). The different lowercase letters above the bars indicate statically significant differences at $P < 0.05$

DgCMO-like-overexpressing plants had higher total flavonoid and anthocyanin contents under sucrose treatment. Consistently, overexpression of *DgCMO-like* enhanced the expression level of major flavonoid biosynthesis genes (such as *AtTT4*, *AtTT5*, *AtTT7*, *AtCHIL*, *AtDFR*, *AtBAN*, and *AtF3H*) under sucrose treatment. These findings indicate that *DgCMO-like* may be a positive regulatory factor in flowering time and sucrose-induced flavonoid biosynthesis. The integration of GWAS with transgenic data implied that the genes *DgFH18* and *DgCMO-like* may significantly contribute to orchardgrass flowering. This study provides clues about their influence on flowering; however, whether these genes affect each other in flowering is unknown. Further research on their regulatory mechanisms is needed to confirm whether there is a connection between them.

Conclusion

This study provides insights into the control of flowering time by integrating phenotypic, GBS, and transgenic data, and reveals the role of *DgFH18* and *DgCMO-like* genes in flowering time of orchardgrass. Overexpression of *DgFH18* and *DgCMO-like* stimulated the expression of flowering genes, resulting in early flowering in *Arabidopsis*. Understanding the functions of *DgFH18* and *DgCMO-like* in regulating flowering time offers new insights into exploring flowering time regulation in perennial grasses.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11708-5.

Supplementary Material 1: Table S1. The geographic information of the orchardgrass population.

Supplementary Material 2: Table S2. The primers for subcellular localization and plant transformation.

Supplementary Material 3: Table S3. The primers for qRT-PCR.

Supplementary Material 4: Table S4. The phenotypic data pertaining to the heading stage and flowering time in the GWAS population.

Supplementary Material 5: Table S5. Cluster analysis of 249 orchardgrass accessions.

Supplementary Material 6: Table S6. The information of total SNPs and related candidate genes.

Supplementary Material 7: Fig. S1. Statistics and Pearson correlation analysis of heading stage and flowering time in orchardgrass. (a) The distributional characteristics of heading stage and flowering time at Ya'An (YA) and Da'Yi (DY) in 2021 and 2022. (b) Pearson correlation between heading stage and flowering time.

Supplementary Material 8: Fig. S2. The analysis of candidate genes. (a) Venn diagram of candidate genes in heading stage and flowering time. (b) The GO enrichment of all candidate genes in 2021. (c) The GO enrichment of all candidate genes in 2022.

Supplementary Material 9: Fig. S3. qRT-PCR analysis of ***DgFH18*** gene in the overexpressed Arabidopsis plants.

Supplementary Material 10: Fig. S4. qRT-PCR analysis of ***DgCMO-like*** gene in the overexpressed Arabidopsis plants.

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Author contributions
MLW analyzed data and prepared the original draft. GYF and FYZ provided key guidance for experimental design. LC, XHX and FXH conducted part of experiments. GN revised and edited the manuscript. LKH revised the manuscript and provided project administration. XQZ provided project administration and funding acquisition.

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Data availability
The protein sequences of LrFH18 (XP_047068237.1), TaFH18 (XP_044329897.1), AtFH5 (NP_200276.1), PaFH11 (XP_062187170.1), SbFH18 (XP_021317914.1), ZmFH5 (ONM19156.1), SvFH8 (XP_034602488.1), PM16G0050, PvFH8 (XP_039819597.1), HvCMO (KAE8799846.1), TaCMO-like (XP_044424145.1), BdCMO (XP_003563491.1), LpCMO (XP_051198948.1), LrCMO (XP_047086524.1), OsCMO (XP_015643170.1), ZmCMO (ABI21840.1), PvCMO (XP_039844653.1), SiCMO (XP_004966128.1), SvCMO (XP_034589995.1), VvCMO (XP_019078540.1), AtCMO-like (AT4G29890.1), CfCMO (XP_059634565.1), CmCMO (RWR91481.1), NnCMO (XP_010276721.1), TsCMO (XP_043713700.1) were obtained from the NCBI protein database based on their gene ID (https://www.ncbi.nlm.nih.gov/protein/). All data generated or analysed during this study are included in this article and can be found in the supplementary information file.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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