Research

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Sorghum SbGhd7 is a major regulator of floral transition and directly represses genes crucial for flowering activation

Dimiru Tadesse[1,2], Estella F. Yee[1,3], Tezera W. Wolabu[2], Hui Wang[2,4], Jianfei Yun[2], Nicolas Grosjean[5], Desigan Kumaran[1], Kassandra Santiago[1], Wenqian Kong[6], Ankush Sharma[7], Jianghua Chen[8], Andrew H. Paterson[7], Meng Xie[1] and Million Tadege[2]

[1]Biology Department, Brookhaven National Laboratory, Upton, NY 11973, USA; [2]Department of Plant and Soil Sciences, Institute for Agricultural Biosciences, Oklahoma State University, Ardmore, OK 73401, USA; [3]National Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY 11973, USA; [4]College of Grassland Science and Technology, China Agricultural University, Beijing, 100193, China; [5]DOE Joint Genome Institute, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA; [6]Department of Soil and Crop Science, University of Georgia, Athens, GA 30602, USA; [7]Plant Genome Mapping Laboratory, University of Georgia, Athens, GA 30602, USA; [8]Key Laboratory of Tropical Plant Resources and Sustainable Use, Center for Excellence in Molecular Plant Sciences, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan, 650223, China

Authors for correspondence:
Meng Xie
Email: mxie@bnl.gov

Million Tadege
Email: million.tadege@okstate.edu

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Summary

- Molecular genetic understanding of flowering time regulation is crucial for sorghum development. *GRAIN NUMBER, PLANT HEIGHT AND HEADING DATE 7 (SbGhd7)* is one of the six classical loci conferring photoperiod sensitivity of sorghum flowering. However, its functions remain poorly studied.
- The molecular functions of SbGhd7 were characterized. The gene regulatory network controlled by SbGhd7 was constructed and validated. The biological roles of SbGhd7 and its major targets were studied.
- *SbGhd7* overexpression (OE) completely prevented sorghum flowering. Additionally, we show that *SbGhd7* is a major negative regulator of flowering, binding to the promoter motif TGAATG(A/T)(A/T/C) and repressing transcription of the major florigen *FLOWERING LOCUS T 10* (*SbFT10*) and floral activators *EARLY HEADING DATE* (*SbEhd1*), *FLAVIN-BINDING, KELCH REPEAT, F-BOX1* (*SbFKF1*) and *EARLY FLOWERING 3* (*SbELF3*). Reinforcing the direct effect of *SbGhd7*, *SbEhd1* OE activated the promoters of three functional florigens (*SbFT1*, *SbFT8* and *SbFT10*), dramatically accelerating flowering.
- Our studies demonstrate that SbGhd7 is a major repressor of sorghum flowering by directly and indirectly targeting genes for flowering activation. The mechanism appears ancient. Our study extends the current model of floral transition regulation in sorghum and provides a framework for a comprehensive understanding of sorghum photoperiod response.

Introduction

The transition from vegetative to reproductive growth is a major phase change in flowering plants that ensures continuity and survival. Plants synchronize the timing of their flowering with changes in environmental cues such as light and temperature to achieve reproductive success. Photoperiod (day length) is one of the major environmental factors that control reproductive competence and floral transition (Jackson, 2009). To initiate the floral transition, 'long-day' (LD) plants require a day length longer than a certain threshold, called critical daylength, '-short-day' (SD) plants require a day length shorter than the critical daylength, and 'day-neutral' plants flower independently of photoperiod (Jackson, 2009). The critical daylength varies greatly depending on the plant species and environmental conditions.

Sorghum, a multipurpose crop providing food, feed and biofuel feedstock, is well suited for sustainable agriculture in semiarid regions due to its ability to grow on marginal lands and resiliency to high temperatures. Sorghum is a typical SD plant and its cultivation for grain in temperate regions uses day-neutral genotypes produced either by natural mutation or introgression of one or more day-neutral recessive alleles (Stephens *et al.*, 1967). In the temperate summer, wild-type (WT; SD) sorghums often reach six meters in height, only transitioning to the reproductive phase near the end of the growing season as daylength gets shorter, producing few or no seed. By contrast, day-neutral lines initiate reproductive growth early, often at *c.* 1 m in height, producing less vegetation but more grain. Thus, while photoperiod-sensitive sorghums are cultivated for forage and biomass yield, photoperiod neutrality favors grain production in temperate regions.

Understanding of the critical importance of photoperiodic control of floral transition in sorghum cultivation, long recognized by the identification of six classical maturity loci named

Ma1-Ma6 in which dominant alleles confer photoperiod sensitivity (Quinby, 1966; Roy, 1974; Rooney & Aydin, 1999), is nonetheless in its infancy. Even, the molecular identities and/or functions of some of the classical maturity loci remain controversial. For example, *Ma1* is the strongest of these loci, replaced by introgression in 89% of 'converted' exotic lines and accounting for *c.* 85.7% of LD flowering time variation between SD and day-neutral genotypes (Lin *et al.*, 1995). First suggested to be *PSEUDORESPONSE REGULATOR 37* (*SbPRR37*), suppressing flowering in LDs (Murphy *et al.*, 2011), subsequent work reported several lines of evidence that *Ma1* is *FLOWERING LOCUS T* (*FT*) gene *SbFT12* (Cuevas *et al.*, 2016). *Ma6* corresponds to a CCT (CONSTANS, CO-like and TOC1) domain-containing protein-encoding gene *GRAIN NUMBER, PLANT HEIGHT AND HEADING DATE 7* (*SbGhd7*; Murphy *et al.*, 2014), whose rice homolog (*Ghd7*) represses flowering in LDs by suppressing the expression of the transcription activator *EARLY HEADING DATE 1* (*Ehd1*; Itoh *et al.*, 2010). *SbGhd7* was found to have a circadian clock-regulated expression pattern and was associated with LD-induced late flowering in photoperiod-sensitive sorghum accessions (Murphy *et al.*, 2014; Yang *et al.*, 2014). However, the molecular function of SbGhd7 has not yet been investigated.

In plants, the mobile florigens are the ultimate inducers of flowering (Putterill & Varkonyi-Gasic, 2016). Sorghum has three putative florigens activating flowering: SbFT1/SbCN15, SbFT8/SbCN12 and SbFT10/SbCN8. *SbFT8* and *SbFT10* are strongly induced by SD photoperiods in sensitive genotypes, and all the three genes are capable of activating flowering in transgenic Arabidopsis, with *SbFT10* having the strongest effect (Yang *et al.*, 2014; Wolabu *et al.*, 2016). The FT protein belongs to the phosphatidylethanolamine-binding (PEBP)-related kinase inhibitor family. Our previous phylogenetic analysis of plant PEBP proteins revealed that SbFT1 is clustered with rice HEADING DATE 3a (Hd3a), and SbFT8 and SbFT10 are in a separate subclade containing maize ZEA MAYS CENTRORADIALIS 8 (ZCN8; Wolabu *et al.*, 2016). Hd3a and ZCN8 are well-documented FT/FT-like genes that activate flowering (Kojima *et al.*, 2002; Lazakis *et al.*, 2011; Meng *et al.*, 2011), suggesting that SbFT1, SbFT8 and SbFT10 are possible functional florigens in sorghum. However, this assertion has not been confirmed in sorghum.

To gain a comprehensive understanding of the molecular mechanism that controls the flowering pathway and uncover the quantitative significance of the three florigens in sorghum, we performed phenotypic analysis on transgenic sorghum and characterized the molecular functions of SbGhd7 using genome-wide and molecular approaches. Here, we report that overexpression (OE) of *SbGhd7* delayed sorghum flowering indefinitely, whereas OE of each of *SbEhd1*, *SbFT1*, *SbFT8* and *SbFT10* induced early flowering. In agreement with our previous studies on transgenic Arabidopsis (Wolabu *et al.*, 2016), *SbFT10* exhibited the strongest effect on flowering activation. By identifying and experimentally validating regulatory targets of SbGhd7 and SbEhd1, we discovered that SbGhd7 directly represses *SbEhd1* and *SbFT10*, and indirectly regulates *SbFT1* and *SbFT8* by repressing *SbEhd1* expression. Besides *SbEhd1* and *SbFT10*, SbGhd7 directly represses *FLAVIN-BINDING, KELCH REPEAT, F-BOX1* (*SbFKF1*) and *EARLY FLOWERING 3* (*SbELF3*), which promote flowering in SD plants (Yang *et al.*, 2013; Han *et al.*, 2015; Lu *et al.*, 2017). Furthermore, we defined that SbGhd7 binds to the DNA motif TGAATG(A/T)(A/T/C). This motif is widely distributed in flowering-related gene promoters in sorghum, suggesting that SbGhd7 may be a major regulator of floral transition. Overall, our study provides insight into the molecular function of SbGhd7 and its regulatory role in sorghum floral transition.

Materials and Methods

Plant materials and growth conditions

Seeds were surface sterilized and planted in soil for growth in the glasshouse or growth chamber in LD conditions with a 16 h : 8 h, 27°C : 30°C, light : dark cycle. In SD condition, plants were grown with an 8 h : 16 h, 24°C : 27°C, light : dark cycle with 70–80% relative humidity and 150 lmol m^2 light intensity.

Sorghum bicolor (L.) Moench genotypes, BTx623 (photoperiod insensitive, *ma1*) and Tx430 (photoperiod insensitive, *ma1*) were generously provided by Bill Rooney, and 100 M (photoperiod-sensitive, *Ma1*) and Wheatland (photoperiod insensitive, *ma1*) were obtained from USDA-ARS, GRIN. For OE under the maize ubiquitin (ZmUbi) promoter, *SbGhd7*, *SbEhd1*, *SbFT10*, *SbFT8* and *SbFT1* cDNAs were amplified from sorghum 100 M genotype. cDNAs were then cloned into the Gateway entry vector pDONR207 via BP reaction (Invitrogen) and subcloned into the binary vector pYPQ203 (pMDC32-Ubi1; Tang *et al.*, 2017) by LR recombination by LR Clonase II enzyme (Invitrogen, 11791020). These constructs were then introduced into the Agrobacterium strain AGL1. Sorghum transformation was performed as described (Liu & Godwin, 2012). *SbGhd7* OE plants were generated in the background of both Tx430 and Wheatland. *SbEhd1*, *SbFT10*, *SbFT1* and *SbFT8* OE plants were generated in Wheatland background. Positive transformation events were selected using 25 mM of hygromycin and confirmed using reverse transcription (RT)-PCR with primers listed in Supporting Information Table S1.

Flowering phenotyping

Transgenic lines were phenotyped in glasshouses and growth chambers. For flower heading, days were recorded from planting to heading. Leaf numbers were counted weekly until heading or senescence for delayed flowering lines.

RNA extraction, RNA-Seq and data analysis

Total RNAs were isolated using the TRIzol Reagent (Invitrogen) from leaves of 4-wk-old sorghum plants (T0 transgenic plants at 4 wk postsowing out of tissue culture) and treated with DNase I (Invitrogen) to remove genomic DNA contamination. RNA quality was evaluated by 2100 Bioanalyzer (Agilent). For quantitative PCR (qPCR) and semiquantitative RT-PCR, cDNAs were synthesized using the oligo(dT) primer and SuperScript IV reverse

transcriptase (Invitrogen) according to the manufacturer's instructions. Primers for qPCR and RT-PCR are listed in Table S1.

RNA-Seq library construction and sequencing was performed by the Beijing Genomics Institute (BGI) to obtain paired-end reads of 150 bp. After removing the adaptor and low-quality sequences, clean reads were aligned to the *Sorghum bicolor* genome v.3.1.1 (PHYTOZOME) using HISAT2 (v.2.2.1). Transcripts were then assembled using STRINGTIE (v.2.2.1). Differentially expressed genes (DEGs) were identified using DESEQ2 (v.2.11.40.7) with the criteria of fold change ≥ 2 and $P < 0.05$.

Protoplast isolation and transfection

The middle region of leaf blades from 12 to 15 d-old BTx623 sorghum plants grown in soil was cut into 1–1.5 mm strips and digested in enzymatic solution (0.4 M mannitol, 20 mM of KCl, 20 mM of MES, 1.5% cellulose R10, 0.4% of Macro enzyme R10, 10 mM of CaCl2, 5 mM 2-ME and 0.1% BSA) in the dark at room temperature for 2.5 h without shaking and 1 h with gentle shaking. The released protoplasts were collected by 200 ***g*** centrifuging and resuspended in 'MMg' solution (0.4 M mannitol, 15 mM of MgCl2 and 4 mM of MES) to the final concentration of 1×10^6 cells ml^{-1} for transfection. The Polyethylene glycol-calcium method was used for protoplast transfection (Xie *et al.*, 2020).

Subcellular localization and transient transactivation assays in protoplasts

SbGhd7 and *SbEhd1* cDNAs were cloned into a transient expression vector (Xie *et al.*, 2020) for C-terminal YFP fusion, respectively. Eight micrograms of this plasmid was co-transfected with 2 μg of mCherry-VirD2NLS plasmid (Lee *et al.*, 2008) into 100 μl of protoplast suspension (*c.* 100 000 cells). After 16 h incubation under weak light at room temperature, protoplasts were collected and imaged using a Leica TCS SP5 confocal microscope, equipped with 488 and 543 nm laser lines for excitation of YFP and mCherry, respectively. The emission bandwidth for YFP and mCherry was 500–530 nm and 580–620 nm, respectively. Images were processed using LAS X software (Leica, Deerfield, IL, USA).

Transient transactivation assays were performed as described (Xie *et al.*, 2020). A total of 10 μg of effector, reporter and/or transactivator plasmids were co-transfected into 100 μl of protoplast suspension (*c.* 100 000 cells). One hundred nanograms of 35S: luciferase plasmid was co-transfected for each reaction to normalize β-glucuronidase (GUS) activity. After 18–20 h incubation in the dark at room temperature, protoplasts were collected and lyzed for measuring GUS and luciferase activities using a Synergy Neo2 multimode plate reader (BioTek). GUS and luciferase activities were measured as described (Yoo *et al.*, 2007). GUS activity in individual samples was normalized against luciferase activity (GUS/-LUC). Three replicates were performed for statistical calculation.

Transient ChIP-Seq experiment and data analysis

SbGhd7 was fused with 10×Myc tag in a transient expression vector and transfected into 200 μl of sorghum protoplast suspension (*c.* 200 000 cells). After 14 h incubation at room temperature, protoplasts were collected for Western blot to measure protein expression and μChIP experiment as described previously (Xie *et al.*, 2020). ChIPed DNA and input DNA were cleaned and concentrated using the MinElute PCR Purification Kit (Qiagen). Sequencing libraries were prepared using ThruPLEX DNA-Seq Kit and DNA Unique Dual Index Kit (Takara, San Jose, CA, USA) following the manufacturer's manual. Sixteen libraries were pooled for paired-end 100-bp sequencing using the DNBSEQ instrument (BGI). Approximately 20–30 M reads were obtained for each sample.

After filtering to remove adaptor sequences and low-quality reads, clean reads were mapped to the reference genome *Sorghum bicolor* v.3.1.1 (PHYTOZOME) using BWA-MEM package (v.0.7.17.2). Mapped data of chromatin immunoprecipitation (ChIP) samples and input samples from two biological replicates with > 50% correlation were then pooled for peak calling using MACS2 (v.2.2.7.1). The CHIPSEEKER package (v.1.18.0) was then used to annotate narrow peaks identified by MACS2.

DNA motif analysis

To predict DNA motifs bound by SbGhd7, 100-bp flanking sequences around the top 100 peak summits before the peak annotation and target identification were analyzed using the Multiple Em for Motif Elicitation (MEME)-CHIP and Simple Enrichment Analysis (SEA) functions of the MEME suite (Bailey *et al.*, 2015).

The occurrence of the TGAATG(A/T)(A/T/C) motif in promoters of floral genes was searched using the FIMO function of the MEME suite (v.5.5.2). 2-kb promoter sequences (−2001 to −1 bp) of floral genes were obtained from PHYTOZOME (https://phytozome-next.jgi.doe.gov/) *Sorghum bicolor* genome (v.3.1.1).

In vitro protein expression and purification

SbGhd7Δ (aa184–231) was codon optimized and cloned into a modified pET11e expression vector containing a C-terminal tobacco etch virus (TEV) cleavage site followed by a Strep-tag II. Protein was then overexpressed in *Escherichia coli* BL21 (DE3) cells by autoinduction at 17°C for 18 h (Studier, 2005). Cells were pelleted and resuspended in lysis buffer (50 mM sodium phosphate, pH 7.6, 1 mM TCEP, 1 mg ml^{-1} lysozyme and cOmplete Protease Inhibitor Cocktail; Roche). The mixture was then homogenized on ice using a cell disruptor. Insoluble detritus was removed by centrifuging and the desired proteins were purified by cation exchange chromatography (Mono S 5/50 GL; Cytiva, Marlborough, MA, USA; elution with a linear gradient of 0–500 mM NaCl). Strep-II was then cleaved by TEV protease digestion, and tag-free proteins were polished by size-exclusion chromatography (Superdex Increase 75 10/300 GL; Cytiva; 50 mM sodium phosphate, pH 7.6, 150 mM NaCl). Fractions containing SbGhd7Δ were determined by Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), pooled and concentrated. To assess the identity and quality of SbGhd7Δ, its molecular weight was approximated with small angle x-ray scattering (National Synchrotron Light Source II; 16-ID) and by size-exclusion chromatography (Superdex 30 Increase 3.2/300 GL with protein standards).

Electrophoretic mobility shift assay

DNA probes were end-labeled with biotin using DNA 3' End Biotinylation Kit (Thermo Scientific, Waltham, MA, USA) in accordance with the manufacturer's manual. The electrophoretic mobility shift assay (EMSA) was performed using LightShift Chemiluminescent EMSA Kit (Thermo Scientific). 0.5 μg of purified protein and 1 μM of biotin-labeled probe were used and incubated at room temperature for 20 min. For competition assays, 50× or 200× unlabeled DNA probe was added. The reaction mixtures were then resolved in 6% DNA retardation gel (Novex, Waltham, MA, USA) by electrophoresis at 100 V for 1–2 h and transferred to nylon membranes. Biotin signals were detected using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) as suggested by the manufacturer.

Computational simulation of protein-DNA interaction

AlphaFold2 (Jumper *et al.*, 2021) was used to predict the structure of the CCT DNA-binding domain of SbGhd7 (Ser 174 to Arg 220). To simulate its DNA binding and define residues involved in the interaction, the helix of TGAATGAA DNA motif and the CCT domain of SbGhd7 were docked manually using COOT (Emsley & Cowtan, 2004) and PYMOL.

Accession numbers

Sequence data can be found under the following Phytozome accession nos: (*Sorghum bicolor* v.3.1.1): SbGhd7 (Sobic.006G004400), SbEhd1 (Sobic.001G227900), SbFT1 (Sobic.010G045100), SbFT8 (Sobic.003G295300), SbFT10 (Sobic.009G199900), SbFKF1 (Sobic.005G145300), SbELF3 (Sobic.009G257300) and SbActin (Sobic.005G047100).

Results

SbGhd7 overexpression in photoperiod neutral sorghum delayed flowering indefinitely

By generating and characterizing transgenic sorghums overexpressing *SbGhd7* (Fig. S1), we found that OE of *SbGhd7* resulted in a nonflowering phenotype in both the Tx430 and wheatland genotypes. In contrast to the empty vector control (Ctrl), which flowered in *c.* 67 d ($n = 8$) after transfer to soil, *SbGhd7* OE lines never flowered in over 3 yr, including the SDs of winter in the glasshouse but underwent senescence after prolonged vegetative growth ($n = 5$; Figs 1a,b, S2). The long duration of vegetative growth in *SbGhd7* OE lines resulted in more than two-fold biomass accumulation compared with controls as indicated by increases in plant height and leaf number (Fig. 1c,d). RT-qPCR results showed that expressions of functional florigens *SbFT1*, *SbFT8* and *SbFT10* were significantly downregulated in *SbGhd7* OE lines (Fig. 1e). In addition, the expression of *SbEhd1*, whose homolog in rice was found to be negatively controlled by Ghd7 (Nemoto *et al.*, 2016), was also downregulated in *SbGhd7* OE lines (Fig. 1e). These results demonstrate that SbGhd7 is a key regulator of flowering time in sorghum and negatively controls the floral transition pathway.

SbGhd7 is a transcriptional repressor

The SbGhd7 protein is localized in the nucleus in sorghum protoplasts (Fig. 2a). Furthermore, our protoplast-based transient transactivation assays using the GUS and luciferase reporter system (Xie *et al.*, 2020) revealed that SbGhd7 has transcriptional repressor activity, but not activator activity (Figs 2b, S3). SbGhd7 was fused with the Gal4 DNA-binding domain (GD-SbGhd7) and recruited to the promoter of the *GUS* reporter gene via the interaction between GD and the Gal4 DNA sequence upstream of *GUS*. A plasmid overexpressing luciferase (LUC) was co-transfected as an internal standard. To test repressor activity, an additional transactivator plasmid overexpressing LexA DNA-binding domain (LBD) fused herpes simplex virus VP16 transactivator (VP16) was co-transfected to constitutively activate *GUS* expression through the interaction between LexA and LBD. The normalized GUS activity (GUS/LUC) was used to evaluate the transcriptional activity of SbGhd7. As shown in Fig. 2(b), SbGhd7 has a significantly lower GUS/LUC value than the green fluorescent protein (GFP) negative control, indicating transcriptional repressor activity.

Noting that its rice homolog represses the maturity gene *Ehd1* (Itoh *et al.*, 2010), we inserted the *SbEhd1* promoter between the 35S promoter and the *GUS* reporter gene and co-transfected it with a plasmid overexpressing SbGhd7 or the GFP negative control (Fig. 2c). As indicated by the GUS/LUC values, we found that SbGhd7 can specifically repress the activity of the *SbEhd1* promoter (Fig. 2c).

SbGhd7 directly targets SbFKF1, SbELF3 and SbFT10

To seek genome-wide targets of SbGhd7 repression, the transcriptomes of *SbGhd7* OE and Ctrl were compared by RNA-Seq, identifying 3880 upregulated and 2639 downregulated genes (Fig. S4; Dataset S1). In transient ChIP-Seq experiments, 1874 genes showed SbGhd7 binding peaks localizing to the 3-kb proximal promoter regions (Fig. S5; Dataset S2), of which 129 were also downregulated in the RNA-Seq (Fig. 2d,e; Dataset S3). These 129 putative SbGhd7 target genes were highly enriched for flowering time genes including blue-light photoreceptor *FLAVIN-BINDING, KELCH REPEAT, F-BOX1* (*SbFKF1*), *EARLY FLOWERING 3* (*SbELF3*) and *SbFT10* (Fig. 2e; Dataset S3).

Noting significant SbGhd7 binding peaks in the promoters of *SbFKF1*, *SbELF3* and *SbFT10* (Fig. 2f), inserting *SbFKF1*, *SbELF3* and *SbFT10* promoters between the 35S promoter and the GUS reporter gene, respectively, the transient transactivation assay confirmed that SbGhd7 directly represses the activity of all three promoters (Fig. 2g). While SbFT1, SbFT8 and SbFT10 all activated flowering in transgenic Arabidopsis (Wolabu *et al.*, 2016) and each was significantly downregulated in SbGhd7 OE lines (Fig. 1e), no SbGhd7 binding peaks or transcriptional repression were detected in the *SbFT1* or *SbFT8* promoters (Fig. 2f,g), suggesting that their control could be indirect through other regulators.

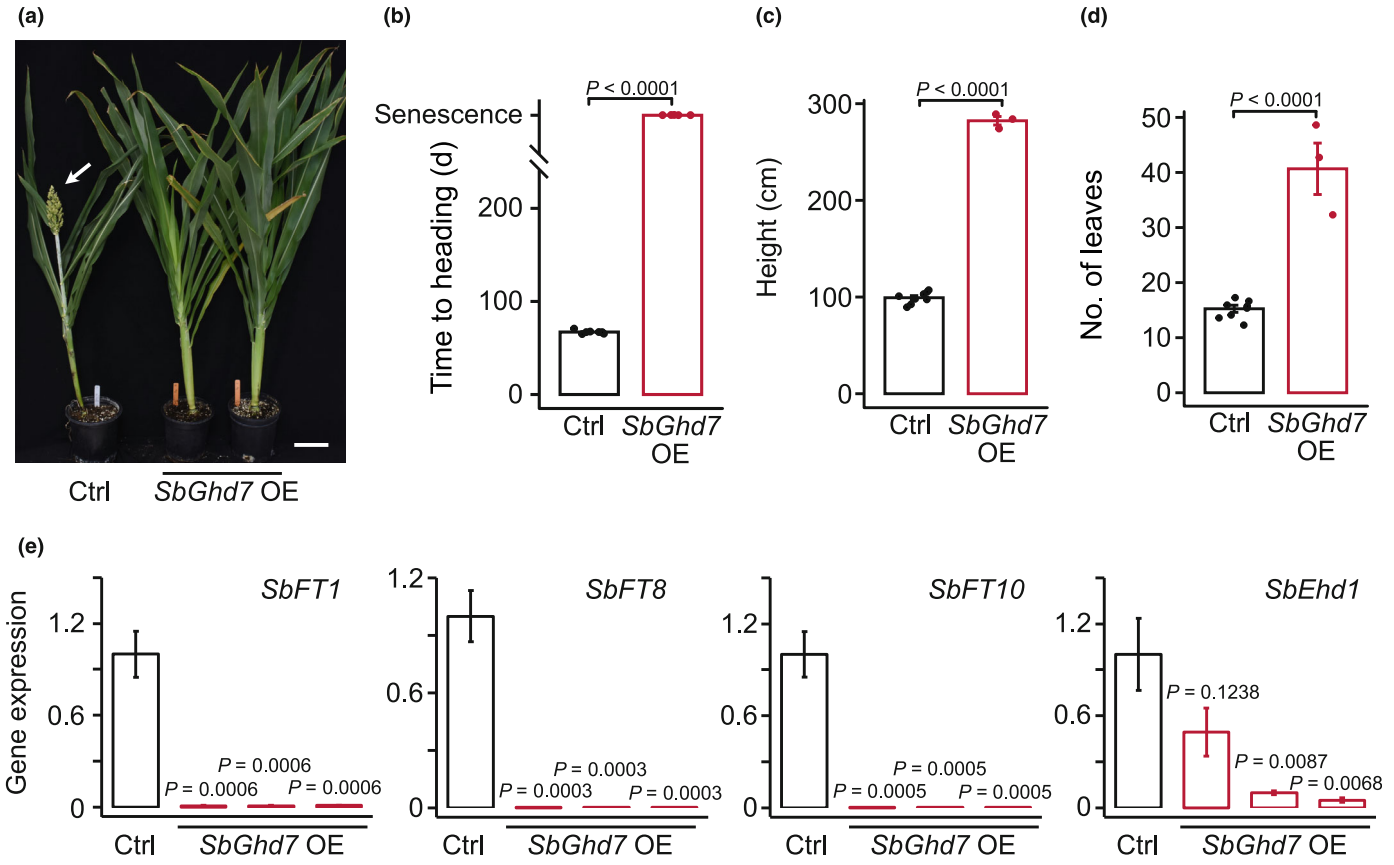

Fig. 1 *SbGhd7* overexpressing sorghum plants show delayed flowering phenotypes. (a) Images of 65-d-old empty vector control sorghum (Ctrl) and *SbGhd7* overexpressing sorghum (*SbGhd7* OE). White arrow indicates the emerging head. Bar, 10 cm. (b) Bar plot showing OE of SbGhd7 ($n = 5$; three in Tx430 background and two in Wheatland background) induces nonflowering compared with the empty vector control ($n = 8$). (c–d) Bar plots showing OE of *SbGhd7* ($n = 3$, Tx430 background) induces increased biomass compared with the empty vector control ($n = 8$). (e) qRT-PCR results showing *SbGhd7* OE represses the expression of *SbFT1*, *SbFT8*, *SbFT10* and *SbEhd1*. Gene expression was normalized against *SbActin*, with Ctrl set as 1. Values represent means + SE, $n = 4$. *P*-values were calculated by two-tailed Student's *t*-tests.

SbGhd7 binds to the DNA motif TGAATG(A/T)(A/T/C)

The 100-bp DNA sequences flanking the top 100 SbGhd7 peak summits identified from transient ChIP-Seq are significantly enriched for TGAATG(A/T)(A/T/C) sequence (*e*-value, 2.71×10^{-7}; Fig. 3a; Materials and Methods section). This motif is present in the 2-kb promoters of 13 out of 31 sorghum floral transition genes including *SbEhd1*, *SbFT10*, *SbELF3* and *SbFKF1* (Fig. 3a; Tables S2, S3). Computational simulation suggests that the nucleotides AT in the middle of the TGAATG(A/T)(A/T/C) motif may be essential for the protein-DNA interaction (Fig. S6).

Electrophoretic mobility shift assay confirmed that *SbGhd7* binds specifically to this DNA motif (Fig. 3b). We expressed and purified the DNA-binding domain of *SbGhd7* (SbGhd7Δ; amino acids 184–231; Fig. S7) and generated a biotin-labeled DNA probe containing three copies of the TGAATGAA sequence (Probe 1; Fig. 3b). EMSA and the competition assay using 50× and 200× unlabeled probe of the same sequence demonstrated that the binding was specific as indicated by reduction in the bound signal (protein–DNA complex) in the presence of the competitor (Fig. 3b). A mutated probe replacing AT with GG (Probe 2; Fig. 3b) showed no binding to SbGhd7, suggesting that the AT is critical for SbGhd7-DNA binding.

SbEhd1 induces early flowering by activating *SbFT* expression

Since SbGhd7 directly targets and represses SbEhd1 expression, we hypothesize that indirect regulation of SbGhd7 on *SbFT1* and *SbFT8* may occur via repression of SbEhd1 expression. In contrast to the nonflowering of *SbGhd7* OE lines, *SbEhd1* OE (Fig. S1) dramatically accelerated flowering (*c.* 27 d, $n = 3$; Fig. 4a), also significantly upregulated the expression of *SbFT1*, *SbFT8* and *SbFT10* (Fig. 4b).

Tests with sorghum protoplasts revealed that SbEhd1 is localized in the nucleus and acts as a transcription activator (Fig. S8). Using the transient transactivation assay, each *SbFT* promoter was inserted in front of the *GUS* reporter gene in the reporter constructs and co-transfected with the construct overexpressing SbEhd1 or GFP control. SbEhd1 activated the promoters of *SbFT1*, *SbFT8* and *SbFT10*, but not *SbGhd7* (Fig. 4c), indicating direct downstream activation of *SbFT* expression.

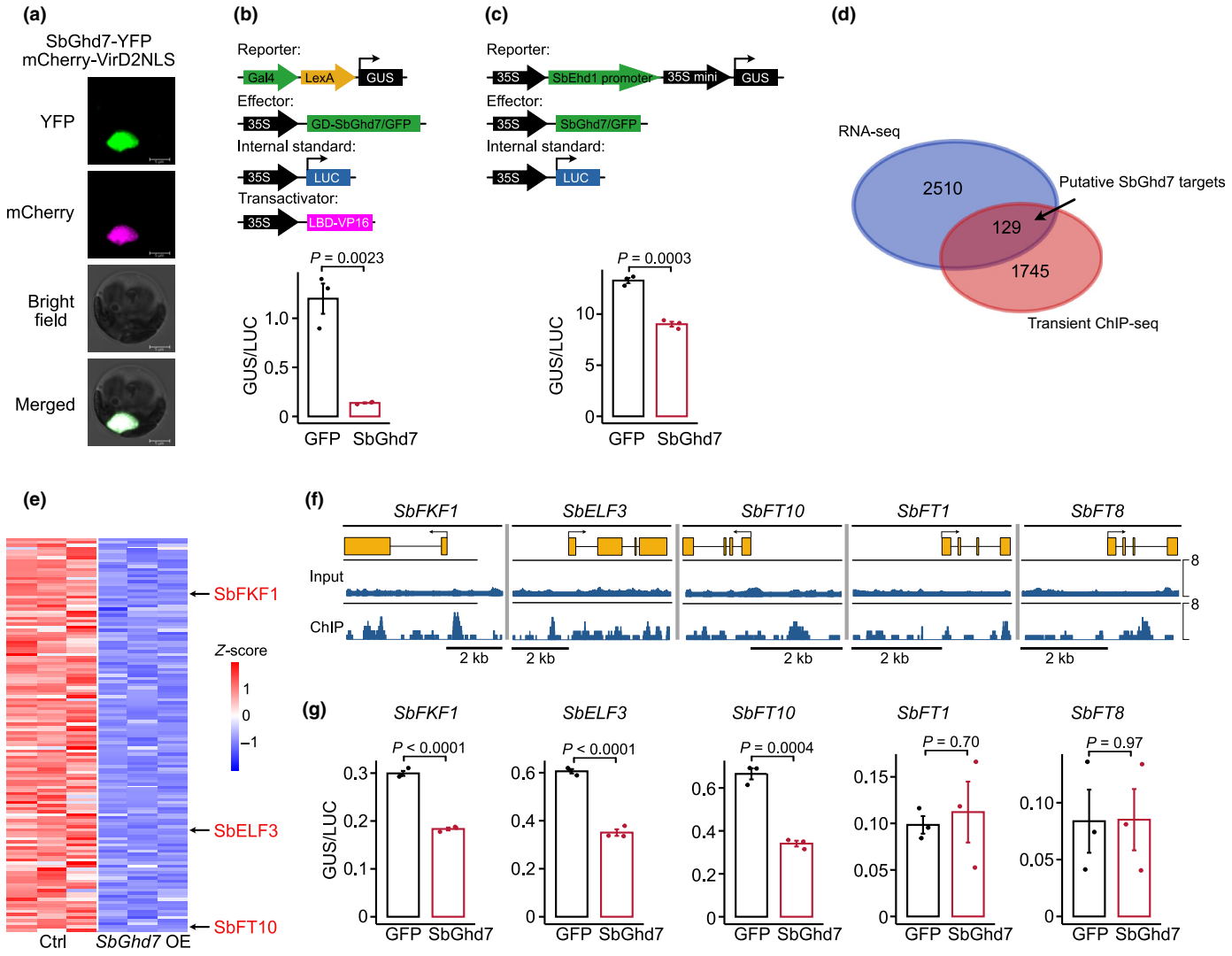

Fig. 2 SbGhd7 directly represses sorghum *SbFT10*, *SbEhd1*, *SbFKF1* and *SbELF3*. (a) SbGhd7 subcellular localization in sorghum protoplasts. Yellow fluorescence protein-tagged SbGhd7 (SbGhd7-YFP; green) and the nuclear marker (mCherry-VirD2NLS; magenta) were coexpressed in protoplasts, and their fluorescent signal overlap is indicated in yellow. Bar, 5 μm. (b) Transient transactivation assay for SbGhd7 repressor activity. Top scheme displays the four types of vectors used. The transactivator construct overexpressing herpes simplex virus VP16 transactivator fused with the LexA DNA-binding domain (LBD) was used to constitutively activate *GUS* expression through the interaction between LexA and LBD. (c) Transient transactivation assay testing SbGhd7 transcriptional repression on *SbEhd1* promoter. Top scheme displays the three types of vectors used. (d) Venn diagram representing putative SbGhd7 targets identified by integrating RNA-Seq and transient chromatin immunoprecipitation-sequencing (ChIP-seq) data. (e) Heatmap comparing the expression of putative SbGhd7 targets in empty vector control (Ctrl) and *SbGhd7* overexpressing (*SbGhd7* OE) plants (three replicates). *SbFKF1*, *SbELF3* and *SbFT10* are indicated. (f) Transient ChIP-Seq read depths in *SbFKF1*, *SbELF3*, *SbFT10*, *SbFT1* and *SbFT8* loci. The 2-kb promoter regions are indicated. (g) Transient transactivation assays show that SbGhd7 targets and represses *SbFKF1*, *SbELF3* and *SbFT10*, but not *SbFT1* or *SbFT8*. Relative GUS activity (GUS/LUC) was calculated by normalizing GUS activity against luciferase activity (b, c, g). Values represent means ± SE, $n = 3$ (b, c, g). *P*-values were calculated by two-tailed Student's *t*-tests (b, c, g).

Sorghum has three florigen encoding genes that activate flowering

Based on expression pattern and activity in transgenic Arabidopsis, we previously proposed that sorghum has three functional florigen encoding genes: *SbFT10*, *SbFT8* and *SbFT1* (Wolabu *et al.*, 2016). However, direct functional evidence in sorghum is still missing (Wolabu & Tadege, 2016). By generating and characterizing transgenic sorghums overexpressing each of *SbFT*s (*SbFT1* OE, *SbFT8* OE and *SbFT10* OE; Fig. S1), we found that all the three *SbFT*s accelerated flowering compared with the empty vector control (Fig. 5a,b), with *SbFT10* showing the shortest flowering time (*c.* 25 d, $n = 3$). Albeit less accelerated flowering of *SbFT8* (*c.* 31 d, $n = 4$) and *SbFT1* (*c.* 46 d, $n = 6$) OE lines indicate that all three proteins are functional florigens.

Discussion

The central importance of the floral transition to reproductive fitness may have conferred a sustained selective advantage to the

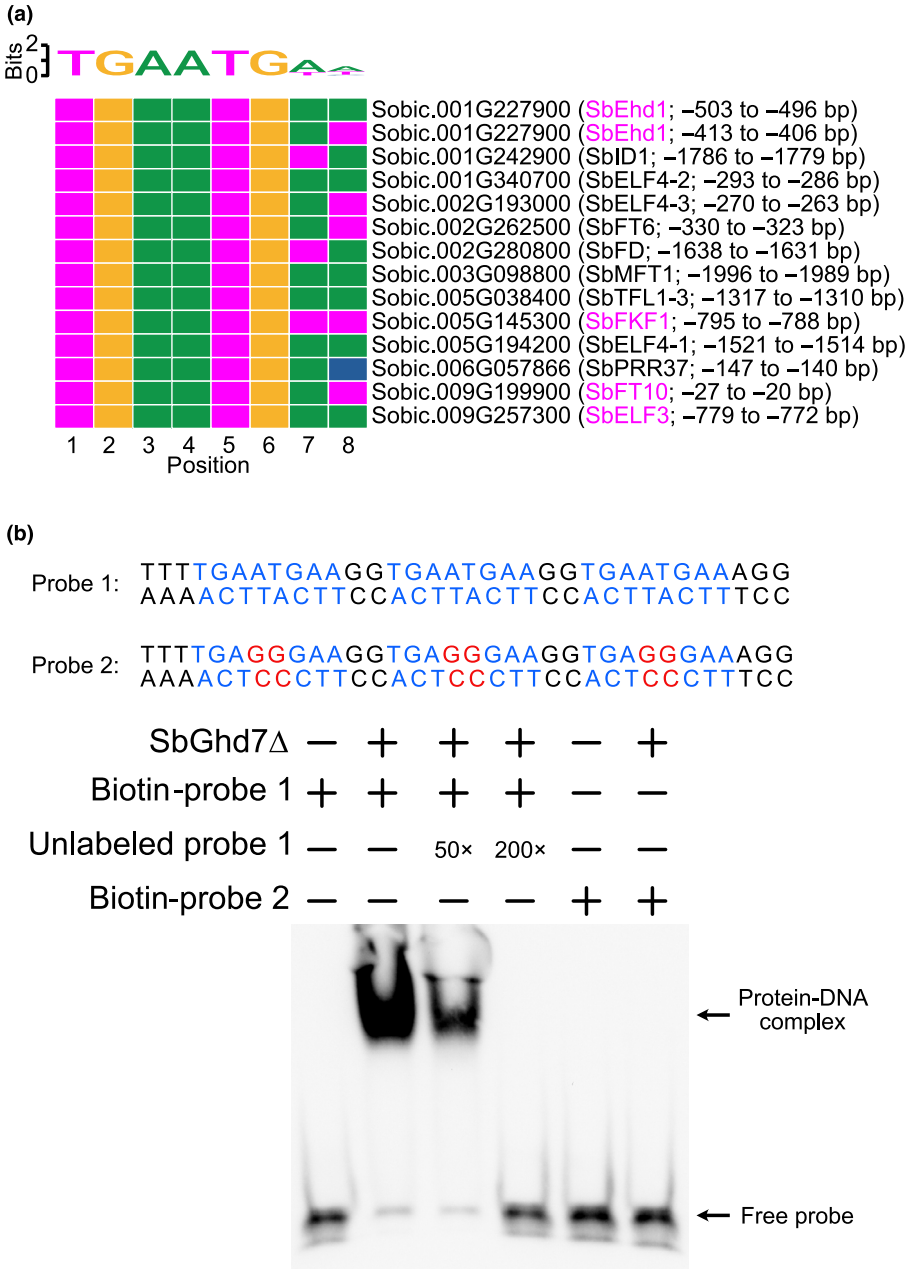

Fig. 3 Sorghum SbGhd7 binds to the TGAATG(A/T)(A/T/C) motif. (a) Heatmap depicting sequences of TGAATG(A/T)(A/T/C) motifs in 2-kb promoter regions of floral genes, with T (magenta), G (yellow), A (green) and C (blue). The validated SbGhd7 targets in this study are highlighted by magenta color. (b) Electrophoretic mobility shift assay (EMSA) assay showing that SbGhd7 specifically binds to the DNA probe containing three copies of TGAATGAA sequence (blue color; Probe 1), but not the mutated probe by replacing AT with GG (red color; Probe 2).

establishment or maintenance of multiple, seemingly redundant signals. The more striking phenotypic consequences of *SbGhd7* OE than its rice ortholog *OsGhd7* (Fig. 1) appear to reflect a combination of direct regulation of the major florigen *SbFT10*, reinforced by additional florigens *SbFT1* and *SbFT8* that are activated by *SbEhd1*, which in turn is under *SbGhd7* control (Fig. 5c). *SbFT8* and *SbFT10* are paleologs, and are among only 14.2% of genes for which both copies remain both in sorghum and in rice (OsFTL9 and OsFTL10; Paterson *et al.*, 2009) since duplicating in a common ancestor *c.* 98 million years ago (Wang *et al.*, 2015). Floral induction by *SbFT10* and its reinforcement by *SbFT8* (via *SbEhd1*) appears ancient, and its repression by *SbGhd7* may impart permanent vegetative growth. Instead, OsGhd7 is believed to be a key component of the rice external coincidence model that explains the relationship between photoperiod and flowering time control (Vicentini *et al.*, 2023). The mRNA and protein accumulation of OsGhd7 highly depends on photoperiod. SDs have been found to not only reduce *OsGhd7* transcription but also reduce OsGhd7 protein stability via post-translational mechanisms, even when it is overexpressed (Zheng *et al.*, 2019). Although *SbGhd7* transcription is controlled by SDs, *SbGhd7* OE completely blocked sorghum flowering, suggesting that the post-translational mechanism controlling Ghd7 protein accumulation in SDs may be absent in sorghum. This is possible considering the divergent evolution of these two monocot crops in very different environments. Another aspect of their divergent evolution is indicated by the differences in *cis*-element binding of OsGhd7 and SbGhd7. OsGhd7 was found to bind to

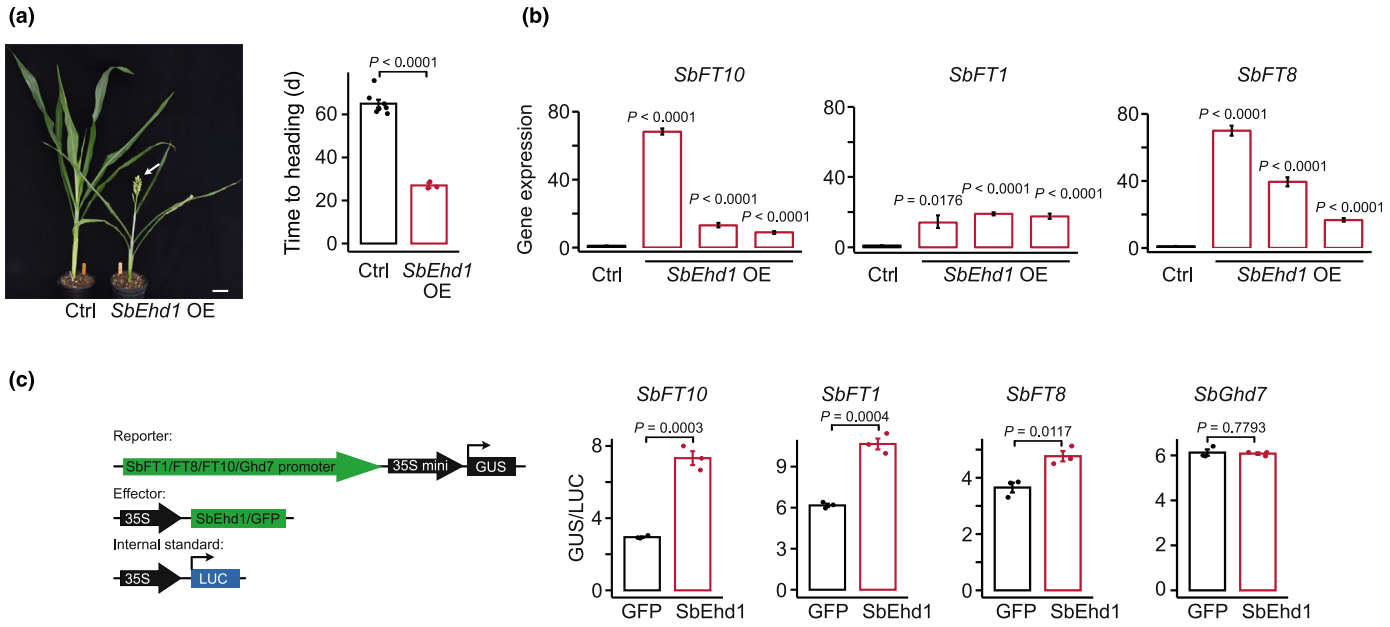

Fig. 4 SbEhd1 activates flowering and *SbFT*s expression in sorghum. (a) *SbEhd1* overexpression (*SbEhd1* OE, $n = 3$, Wheatland background) induces early flowering compared with the empty vector control (Ctrl, $n = 7$). White arrow indicates the emerging head. Bar, 10 cm. (b) qRT-PCR results showing *SbEhd1* OE activates the expression of *SbFT1*, *SbFT8* and *SbFT10*. Gene expression was normalized against the expression of *SbActin*, with Ctrl set as 1. Values represent means ± SE, $n = 4$. *P*-values were calculated by two-tailed Student's *t*-tests. (c) Transactivation assay testing SbEhd1's transcriptional activation on *SbFT10*, *SbFT1*, *SbFT8* and *SbGhd7* promoters. Left scheme displays the used three types of vectors. Relative GUS activity (GUS/LUC) was calculated by normalizing GUS activity against luciferase activity. Values represent means ± SE, $n = 3$. *P*-values were calculated by two-tailed Student's *t*-tests.

the CACA/TGTG sequence, which is absent from the *OsEhd1* promoter (Nemoto *et al.*, 2016). Therefore, it was hypothesized that OsGhd7 represses *OsEhd1* through the formation of a protein complex instead of direct promoter binding (Nemoto *et al.*, 2016). By contrast, we found that SbGhd7 binds to the TGAATG(A/T)(A/T/C) sequence, which is widely presents in promoters of sorghum flowering genes including *SbEhd1*. Since the CCT DNA-binding domain of OsGhd7 and SbGhd7 is highly conserved (Fig. S9), it is possible that other regions of the protein could be contributing to their target specificity. However, whether OsGhd7 is capable of binding to the TGAATG(A/T)(A/T/C) motif has not been tested.

Scrutiny of *SbGhd7* reveals new dimensions of floral regulation, perhaps reflecting botanical diversity or alternatively merely easier to resolve due to its striking effects. In the facultative LD plant Arabidopsis, the blue-light receptor FKF1 promotes flowering in LDs and provides timing information for CONSTANS (CO) stabilization (Imaizumi *et al.*, 2003, 2005; Song *et al.*, 2012). In the facultative SD plant rice, OsFKF1 promotes flowering independent of day length by activating *Ehd1*, *Ehd2* and repressing *Ghd7* (Han *et al.*, 2015). In seeming contrast, *SbGhd7* is an upstream negative regulator of *SbFKF1* (Figs 2f,g, 5c). An intriguing hypothesis is whether a feedback loop may exist whereby SbGhd7 represses *SbFKF1* in LDs and SbFKF1 represses *SbGhd7* independent of photoperiod to promote flowering, which might be tested by quantifying *SbGhd7* expression in *SbFKF1* OE and knockout lines.

Similarly, SbELF3, a homolog of Arabidopsis ELF3, was found to be directly regulated by SbGhd7 (Figs 2f,g, 5c). In Arabidopsis, ELF3 interacts with the red light photoreceptor PHYB to mediate photoperiodic flowering (McWatters *et al.*, 2000; Liu *et al.*, 2001; Kolmos *et al.*, 2011), together with EARLY FLOWERING 4 (ELF4) and LUX ARRHYTHMO (LUX) forming the evening complex (EC) that delays floral transition under noninductive SD photoperiods (Nusinow *et al.*, 2011). ELF3 homologs promote flowering of rice (OsELF3) under LD conditions by negatively regulating OsGI and OsGhd7 (Yang *et al.*, 2013) and of soybean (GmELF3) in SD photoperiods by directly repressing the legume-specific flowering repressor E1 (Lu *et al.*, 2017). QTL and genome-wide association studies have linked *SbELF3* to flowering time (Li *et al.*, 2018; Hu *et al.*, 2019), but how it affects floral transition remains to be elucidated.

The relationship between *SbGhd7* and *Ma1*, on the same chromosome and thought to be the major classical maturity locus inhibiting LD flowering, remains unknown. Although currently depicted downstream of PHYB (Yang *et al.*, 2014), it remains unclear whether LD upregulation of *SbGhd7* transcription is a direct response to light or an indirect response through photoreceptors and other light sensors. The SbGhd7-encoded CCT domain-containing protein is not known to bind to or be associated with chromophores, nor is it likely to perceive photons directly, as it lacks protein motifs common to known photoreceptors, including phytochrome, cryptochrome, phototropin,

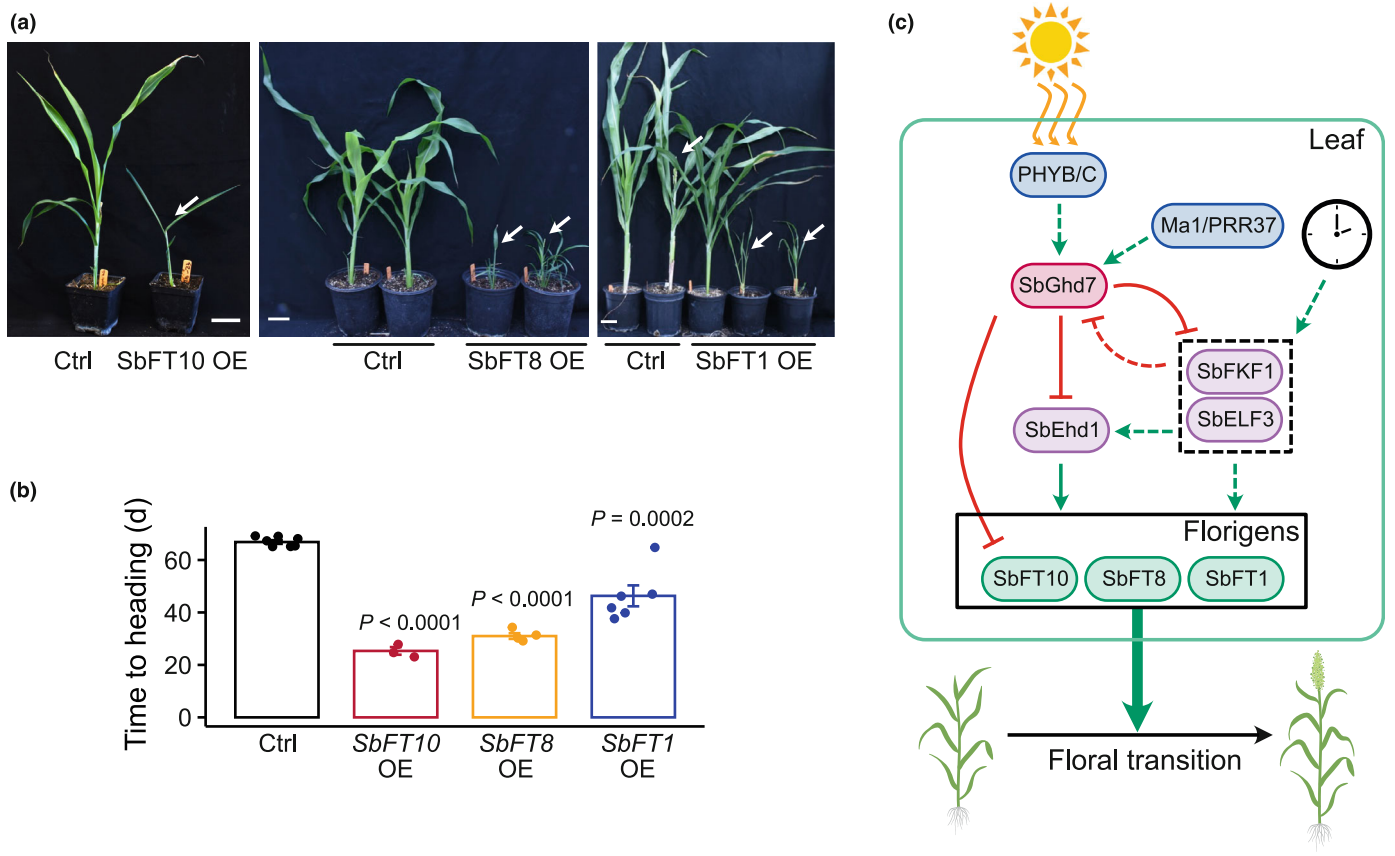

Fig. 5 Three florigens activate early flowering in sorghum. (a, b) Overexpression of *SbFT10* ($n = 3$, Wheatland background), *SbFT8* ($n = 4$, Wheatland background), *SbFT1* ($n = 6$, Wheatland background), respectively, induces early flowering compared with the empty vector control ($n = 7$). White arrow indicates the emerging head. Bars: 10 cm. Values represent means ± SE. *P*-values were calculated by two-tailed Student's *t*-tests by comparing with Ctrl. (c) Proposed model for how SbGhd7 may repress sorghum floral transition. Under long-day conditions, SbGhd7 expression activated in leaves represses the expression of the florigen *SbFT10*, the flowering activator *SbEhd1* and putative flowering activators *SbFKF1* and *SbELF3*. SbGhd7 may also indirectly repress the expression of other florigens (*SbFT1* and *SbFT8*) through the repression of *SbEhd1*. Under short-day conditions, *SbGhd7* must be repressed by as-yet uncharacterized floral activators (including SbFKF1 and SbELF3) to permit early flowering. Arrow heads indicate activation and solid heads show repression. Solid lines indicate data reported in this manuscript and broken lines indicate potential scenarios that need experimental verification.

xanthopsin, blue-light sensor using FAD (BLUF) and rhodopsin (Moglich *et al.*, 2010). It could amplify and transduce light signals by interactions downstream of photon perception, although this has not yet been investigated.

A direct relationship with rice phytochromes OsPHYA and OsPHYB stabilizes the Ghd7 protein post-transcriptionally and delays flowering (Zheng *et al.*, 2019) in WT genotypes but not in phytochrome mutants. The *Ghd7* OE effect was reduced by coexpression with rice GIGANTIA (OsGI), which destabilizes the Ghd7 protein (Zheng *et al.*, 2019). Studies of SbGhd7 interaction with these and other photoreceptors, the circadian clock and upstream transcriptional regulators (Fig. 5c) may further elucidate the molecular mechanism of photoperiod response in sorghum and other plants.

In summary, our study demonstrates that SbGhd7 is a major repressor of sorghum flowering and expands the molecular genetic understanding of the regulation of sorghum floral transition. The SbGhd7-centric gene regulatory network controlling sorghum floral transition provides opportunities to bioengineer this key trait for sorghum improvement and provides a fundamental framework for studying the regulation of sorghum flowering time in response to environmental cues.

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Competing interests

None declared.

Author contributions

MT, MX and DT conceived and designed the research. DT, MX, EFY, TWW, HW, JY, NG, DK and KS performed experiments. MT, MX, DT, WK and AS analyzed the data. JC and AHP contributed reagents. and MX, MT and DT wrote the manuscript. All authors reviewed and approved the final version of the manuscript for publication.

ORCID

Jianghua Chen https://orcid.org/0000-0003-0715-1859
Wenqian Kong https://orcid.org/0000-0002-4540-6165
Million Tadege https://orcid.org/0000-0002-3306-3695
Dimiru Tadesse https://orcid.org/0000-0002-5523-3704
Hui Wang https://orcid.org/0000-0002-1206-4533
Meng Xie https://orcid.org/0000-0003-0247-3701
Estella F. Yee https://orcid.org/0000-0001-7108-1387

Data availability

The data that support the findings of this study are openly available on GEO at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE238095, reference no: GSE238095.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 Significantly downregulated and upregulated genes by comparing transcriptomes of *SbGhd7* OE and Ctrl.

Dataset S2 Targets of SbGhd7 that were identified in transient ChIP-Seq experiment.

Dataset S3 TPM counts representing the gene expression of 129 putative targets of SbGhd7.

Fig. S1 RT-PCR analysis of overexpression lines.

Fig. S2 Nonflowering phenotype of *SbGhd7* overexpression lines.

Fig. S3 SbGhd7 has no transcriptional activator activity in transient transactivation assay.

Fig. S4 RNA-Seq data analysis to identify differentially expressed genes (DEGs) by *SbGhd7* overexpression.

Fig. S5 Identification of SbGhd7 binding peaks using transient ChIP-Seq.

Fig. S6 Computational modeling of the interaction between the CCT domain of SbGhd7 and the TGAATGAA DNA helix.

Fig. S7 The purification of SbGhd7Δ protein for EMSA assay in Fig. 3.

Fig. S8 SbEhd1 has transcriptional activator activity in transient transactivation assay.

Fig. S9 Protein sequence alignment of rice OsGhd7 (E5RQA1.1, NCBI) and sorghum SbGhd7.

Table S1 Primers used in this study on sorghum flowering.

Table S2 Sorghum floral genes used for searching the TGAATGAA motif.

Table S3 Motif sequences used for generating the sequence heatmap of the motif in promoters of sorghum floral genes in Fig. 3(a).

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