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FLOWERING HTH1 is involved in CONSTANS-mediated flowering regulation in *Arabidopsis*

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Abstract

Flowering at the right time is essential for maximum reproductive fitness. In *Arabidopsis thaliana*, the CONSTANS (CO) protein facilitates the transition from the vegetative phase to the reproductive phase under long-day conditions. The formation of heterodimeric complexes between CO and DNA binding domain-containing transcription factors is important for the induction of day length-dependent flowering. Here, we report a myb-like helix turn helix (HTH) transcriptional regulator family protein as a new modulator of floral transition, which we have named FLOWERING HTH1 (FHTH1). We isolated FHTH1 as a CO-interacting protein by a yeast two-hybrid screen using an *Arabidopsis* transcription factor library. Our analysis showed that FHTH1 presented in the nucleus and the FHTH1-CO complex was formed in the same subcellular location. We also observed the expression of a *FHTH1:GUS* construct in the leaf vasculature, where CO exists. Transgenic plants overexpressing FHTH1 fused with the plant-specific repression domain SRDX showed a delayed flowering phenotype in long days, resembling the phenotype of the *co* mutant. Our results suggest that FHTH1 may contribute to CO-mediated photoperiodic flowering regulation.

Keywords: Flowering time, CONSTANS, Photoperiod, Myb-like helix turn helix transcriptional regulator, Chimeric repressor

Introduction

Plants sense seasonal changes and align their development with the surrounding environment to maximize reproductive fitness [1, 2]. One of the most important environmental factors controlling the transition from the vegetative phase to the reproductive phase is photoperiod [3]. In *Arabidopsis*, day length-dependent regulation of the floral promoter CONSTNAS (CO) plays a central role in seasonal flowering [1]. *CO* gene expression levels are relatively high under light in long days, but are very low during the daytime in short days [4]. This differential diel expression profile of *CO* is known to be the day length measurement mechanism [5, 6]. Additionally, post-translational regulation of CO by the function of photoreceptors is essential for day length-dependent induction of the *FLOWERING LOCUS T* (*FT*) gene, which accelerates flowering [7–10].

CO protein is the primary transcriptional activator of the *FT* gene, which encodes the mobile florigenic signal that is synthesized in the leaf phloem tissues and transmitted to the shoot apical meristem [11–14]. In long days, CO directly binds to the promoter region of the *FT* gene and activates its transcription [8, 15, 16]. CO appears to be recruited to CO-responsive elements (COREs) in the *FT* promoter through its C-terminal CCT (CONSTANS, CONSTANS-like, and TOC1) motif [15]. However, the function of CO in the induction of *FT* expression largely depends on other DNA-binding transcription factors [2, 17–21]. The CCAAT-box-binding

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nuclear factor Y (NF-Y) proteins form a trimeric complex with CO and affect its binding to the CORE element of the *FT* promoter [21]. This NF-Y-CO complex regulates the dynamic changes in the structure of the *FT* promoter region that occur throughout the day in long days by binding to a CCAAT box in the enhancer region [2, 18]. The myb-like helix turn helix (HTH) transcription factor family protein ASYMMETRIC LEAVES 1 (AS1) physically interacts with CO and is involved in photoperiodic flowering by contributing to CO-dependent *FT* induction in long days [20]. In contrast to NF-Y and AS1, TARGET OF EAT (TOE) proteins bind to CO and inhibit its activity [22]. In addition, the bZIP transcription factor TGACG (TGA) MOTIF-BINDING FACTOR 4/*ocs* element binding factor 4 (TGA4/OBF4) directly binds to the *FT* promoter interacts with CO in yeast and in vitro, although the biological relevance of this interaction is not fully understood [23]. Hence, the heterodimeric complexes formed between CO and DNA-binding transcription factors are a crucial component of photoperiodic flowering by regulating *FT* expression.

In this study, we report the identification of another CO-interacting protein, FLOWERING HTH 1 (FHTH1). A complex containing CO and FHTH1 was observed in the nucleus. Moreover, overexpression of a chimeric repression motif-fused FHTH1 significantly delayed flowering in long days. Our results suggest that FHTH1 may positively regulate flowering by binding to CO.

Materials and methods

Plant materials and growth conditions

All *Arabidopsis thaliana* plants used in this study, including wild type, *fhth1* (SALK_005547), *fd-4* (SALK_11848C), and *35S:FHTH1-SRDX #3*, are Columbia (Col-0) ecotype. The *fhth1* mutant used in this study was obtained from ABRC and was identified using the LBb1 primer (SALK) to verify the T-DNA insertion and *FHTH1* gene-specific primers. For flowering experiments, all plants were grown on soil in plant incubators at 22 °C under cool white fluorescent lights in long days (16-h light/8-h dark). Flowering time was measured by recording the number of rosette leaves when plants were bolted.

Yeast two-hybrid analysis

For the yeast two-hybrid screening, the *CO* cDNA encoding a deletion derivative of CO (designated COΔ133-174, with a deletion of amino acids 133–174) was constructed by overlap extension polymerase chain reaction (OE-PCR) and was introduced into pDEST32 to yield the bait construct BD-COΔ133-174. After transforming the construct into the yeast strain PJ69-4A, yeast cells harboring the BD-COΔ133-174 construct were grown on SD agar medium lacking leucine (Leu) and histidine (His) with various concentrations of 3-amino-1,2,4-triazole (3-AT; 0.1, 0.5, 1, and 5 mM) or lacking Leu, His, and Adenine (Ade) to prove that the construct is a suitable candidate as a bait. The yeast two-hybrid screening and assay procedures were described previously [20, 23]. Briefly, an *Arabidopsis* transcription factor (TF) library carrying about 1400 TF cDNAs in the pDEST22 AD vector was introduced into yeast cells carrying the BD-COΔ133-174 construct. Then, the transformants were selected on SD agar medium lacking tryptophan (Trp), Leu, His, and Ade. After removing false positives, one clone, containing *FHTH1* cDNA, was obtained. To validate the interaction, bait and prey vectors harboring the *COΔ133-174* and *FHTH1* cDNAs, respectively, were co-transformed into pJ69-4A. The transformants containing the vectors were selected on SD agar medium lacking Trp, Leu, His, and Ade.

Histochemical GUS assay

For the *FHTH1:GUS* reporter construct, the 5′ upstream sequence of the *FHTH1* gene (− 2009 to − 9; the translation initiation site was counted as + 1) was amplified from wild-type genomic DNA and introduced into the pMDC163 vector [24]. The procedures used for plant growth and GUS staining were described previously [23].

Intracellular distribution and BiFC experiments

The tobacco (*Nicotiana benthamiana*) transient expression system was used for the subcellular localization and bimolecular fluorescence complementation (BiFC) assays. For subcellular localization experiments, *FHTH1* and *CO* cDNAs lacking a stop codon were amplified by PCR and introduced into the pMDC43 vector to generate C-terminal GFP fusions [24]. The pMDC43 plasmid carrying either the *35S:FHTH1-GFP* or *35S:CO-GFP* construct was introduced into agrobacterium GV3101, and agrobacteria cultured overnight were individually infiltrated into 3-week-old tobacco leaves. For BiFC assay, the *FHTH1* and *CO* cDNAs were cloned into the pDEST-VYNE(R)GW and pDEST-VYCE(R)GW vectors [25], respectively, to generate N- and C-terminal fusions to enhanced yellow fluorescent protein (eYFP) or Venus, generating VN-FHTH1 and VC-CO, respectively.

Agrobacteria carrying either the *35S:VN-FHTH1* or *35S:VC-CO* construct were co-infiltrated into tobacco leaves. GFP and YFP were visualized by a fluorescence microscope (AX70, Olympus).

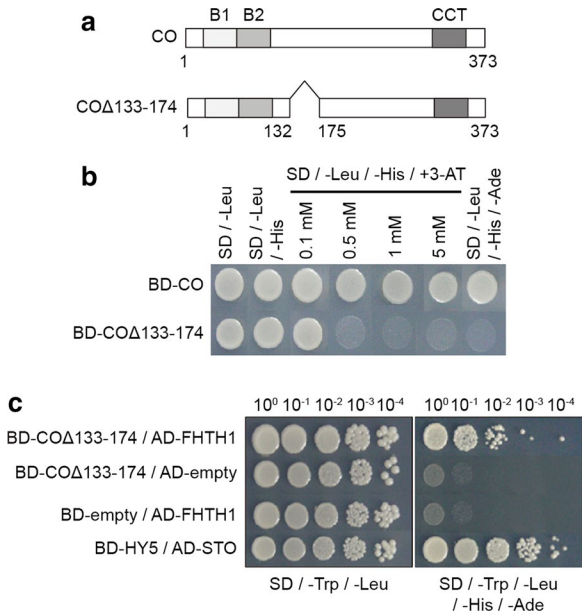

Fig. 1 Yeast two-hybrid assay to isolate CO-binding proteins. **a** Schematic representation of the CO deletion derivative, COΔ133-174. **b** Gal4 DNA binding domain (BD) fusions of CO and COΔ133-174 were expressed in yeast. Different concentrations of 3-AT (0.1, 0.5, 1, 5 mM) and Adenine (Ade) were used to validate elimination of the transcriptional activity for the reporter genes in the COΔ133-174 protein-expressing yeast cells. **c** After isolating FHTH1 as a CO-interacting protein in the yeast two-hybrid screen, co-transformation and serial dilution assays using the BD-COΔ133-174 and AD-FHTH1 constructs were performed to confirm the protein–protein interaction. Yeast cells were grown on SD agar medium lacking Trp and Leu (left panel) or lacking Trp, Leu, His, and Ade (right panel). The HY5-STO interaction was included as a Ref. [37]

Generation of transgenic plants

To generate *35S:FHTH1-SRDX* transgenic plants, the *FHTH1* cDNA lacking a stop codon was introduced into the pH35GEAR vector [26]. The pH35GEAR vector carrying the *35S:FHTH1:SRDX* construct was transformed into wild-type plants.

Results and discussion

Isolation of FHTH1 as a CO-interacting protein

CO is the main floral promoter in *Arabidopsis* under long-day conditions [27]. Although many proteins that form heterodimer complexes with CO have been identified [8–10, 16, 17, 19, 20, 22, 23, 28–30], the molecular mechanisms by which the transcriptional activity of CO regulates day length-dependent flowering still remain to be understood. Therefore, a yeast two-hybrid screen was performed to isolate additional unidentified CO-interacting DNA-binding proteins. First, we constructed a deletion derivative of the *CO* gene that lacks the coding sequence for the glutamine-rich region (amino acids 133–174) to eliminate its intrinsic transcriptional activation potential (Fig. 1a) [15]. As expected, the mutant protein (COΔ133-174) expressed from the deletion construct showed no self-activation on the reporter genes, which encode proteins required for the biosynthesis of histidine and adenine (Fig. 1b). Next, we introduced an *Arabidopsis* transcription factor library into yeast cells expressing Gal4 DNA-binding domain (BD)-COΔ133-174, the bait peptide. One clone containing a cDNA encoding a DNA-binding transcription factor was obtained as a potential interactor with COΔ133-174, which was confirmed by co-transformation with the bait and prey vectors and subsequent serial dilution experiments (Fig. 1c). The encoded transcription factor (At5g06800) belongs to the myb-like helix turn helix (HTH) transcription factor family and has not been previously characterized. As this gene encodes a HTH protein that regulates the timing of floral induction (as shown later), we named it *FLOWERING HTH 1* (*FHTH1*). Our data suggests that FHTH1 can physically bind to CO not only in yeast but also *in planta*.

Complex formation between CO and FHTH1 *in planta*

The nuclear B-box protein CO regulates the expression of its target gene in response to long days [8]. Based on the amino acid sequence of its myb-like HTH domain, FHTH1 was predicted to be a DNA-binding protein. To determine the intracellular localization of FHTH1, we expressed the protein as an N-terminal green fluorescent protein (GFP) fusion under the control of the cauliflower mosaic virus (CaMV) 35S promoter in *Nicotiana benthamiana* leaves. GFP-FHTH1 fluorescence was observed in the nucleus of tobacco epidermal cells, resembling the localization of CO (Fig. 2a). This finding suggests that FHTH1 forms a protein complex with CO in the same subcellular localization. We then investigated where the FHTH1-CO interaction occurs in plant cells using BiFC. The FHTH1 and CO proteins were respectively tagged with the N- and C-terminus of enhanced yellow fluorescent protein (eYFP, also called Venus) and co-expressed in tobacco leaves. A strong signal for reconstituted eYFP fluorescence, which requires the association of the N- and C-termini, was only detected in the nucleus (Fig. 2b), confirming the interaction observed in yeast (Fig. 1c). These results indicate that FHTH1 and CO colocalize in the nucleus and form a complex (Fig. 2b). The *CO* gene is expressed in the vasculature, especially phloem tissues [31, 32]. As FHTH1 physically interacts with CO *in planta* (Fig. 2b), the tissue-specific expression of the *FHTH1* gene in leaves might overlap with that of *CO*. Accordingly, an expression cassette bearing the *GUS* marker gene under the control of a 2.0 kb *FHTH1*

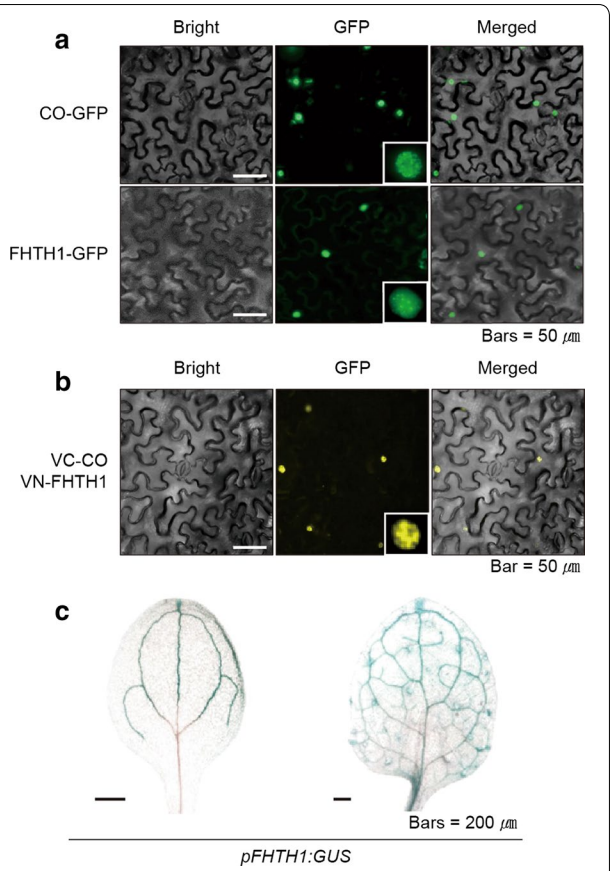

Fig. 2 Subcellular and vasculature expression of FHTH1. **a** Nuclear localization of CO-GFP and FHTH1-GFP in tobacco (*Nicotiana benthamiana*) leaf epidermal cells. Scale bars = 50 µm. **b** *In planta* interaction between FHTH1 and CO. The N-terminal fragment of Venus (VN) and C-terminal fragment of Venus (VC) were fused to FHTH1 and CO, respectively. Constructs constitutively expressing VN-FHTH1 and VC-CO were introduced into 3-week-old *N. benthamiana* leaves. Scale bar = 100 µm. **c** *FHTH1* expression pattern in the *FHTH1:GUS* transgenic plant. A 2.0 kb *FHTH1* promoter fragment was cloned in front of the *GUS* coding sequence. The transgenic plants were grown for 10 days in long days. A detached cotyledon (left panel) and first true leaf (right panel) are shown. FHTH1:GUS activity was detected in the leaf vasculature. Scale bars = 200 µm

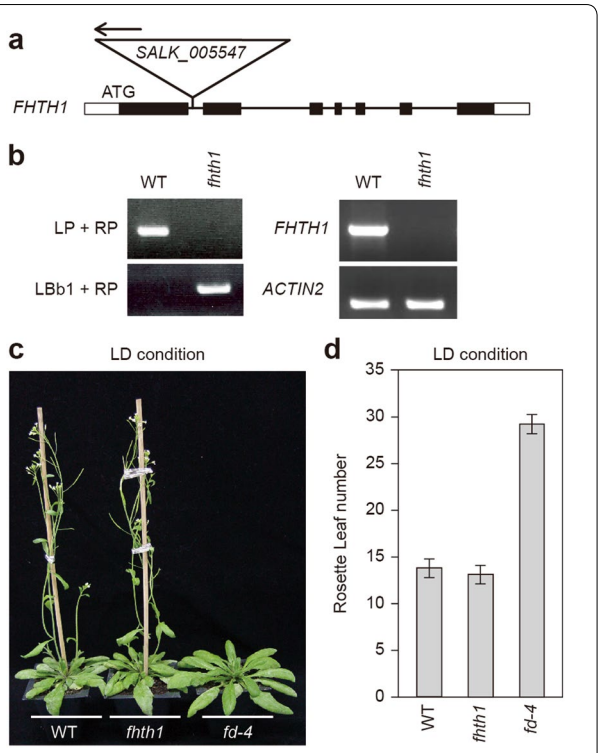

Fig. 3 Identification and flowering time of the *fhth1* mutant. **a** The position of the T-DNA insertion in the *FHTH1* locus. The T-DNA was located in the first intron of the *FHTH1* gene. Black boxes, lines, and white boxes represent exons, introns, and UTRs, respectively. **b** Genomic PCR (left panel) and RT-PCR (right panel) to identify the *fhth1* mutant. We characterized the homozygous mutant using combinations of the *FHTH1* left primer (LP), right primer (RP), and T-DNA-specific left board primer (LBb1) for genomic PCR and comparing *FHTH1* expression levels between wild-type and mutant plants. *ACTIN2* was used as an internal control. **c**, **d** Flowering time of *fhth1* plants grown in long days. d The number of rosette leaves were counted on the main stem. Mean ± SD

promoter was constructed and introduced into wild-type *Arabidopsis* plants to analyze the spatial expression pattern of *FHTH1*. FHTH1:GUS activity was observed in the vascular tissues of cotyledon and rosette leaves (Fig. 2c), where CO mainly exists, supporting the notion that FHTH1 regulates flowering through complex formation with CO in the phloem.

FHTH1 is involved in the regulation of flowering

Since CO functions as a floral promoter in long days, we examined whether FHTH1 regulates flowering. First, an *FHTH1* T-DNA insertion mutant (SALK_005547) was obtained from public collections, and the homozygosity of *fhth1* was verified by genomic PCR and RT-PCR analysis (Fig. 3a, b). We then analyzed the effect of the *fhth1* mutation on flowering regulation in long days and found that the time to flowering in the mutant plants was similar to that in wild-type plants (Fig. 3c, d). This suggests that FHTH1 functions redundantly with other proteins in the same flowering pathway. To overcome this potential genetic redundancy, we generated a dominant-negative version of FHTH1 that could repress the transcription of its target gene. To this end, a DNA sequence encoding the plant-specific EAR-repression domain, also called SRDX (LDLDLELRLGFA) [33], was fused in frame to the coding region of *FHTH1* (Fig. 4a). The resultant *FHTH1-SRDX* chimeric sequence was overexpressed driven by the CaMV 35S promoter in wild-type plants. Gene

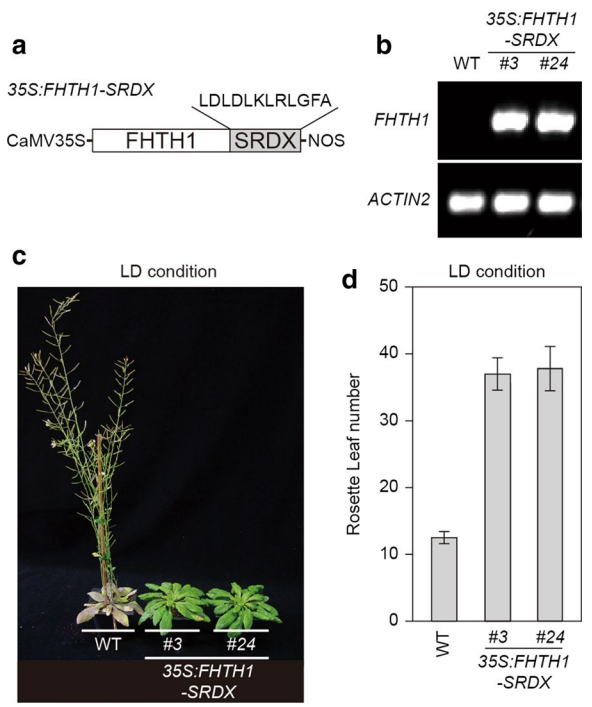

Fig. 4 FHTH1 regulates flowering. **a** Schematic representation of the chimeric repressor construct used to generate the transgenic plants. The gene encoding FHTH1 (white box) fused to the SRDX domain (grey box), and the amino acid sequences of the SRDX peptide are shown. **b** The results of the *FHTH1* expression analysis by RT-PCR in the two independent *35S:FHTH1-SRDX* transgenic lines. *ACTIN2* was used as an internal control. **c**, **d** Delayed flowering phenotypes of the *35S:FHTH1-SRDX* plants grown in long days. These data suggest that FHTH1 function as a floral regulator. Mean ± SD

expression analysis showed high levels of *FHTH1-SRDX* mRNA in two independent *35S:FHTH1-SRDX* lines, validating overexpression of the construct (Fig. 4b). Then, we analyzed the flowering phenotype of the two *FHTH1-SRDX* overexpressors in long days and observed a distinct late flowering phenotype (Fig. 4c, d), which resembles that of the *co* mutant [20], suggesting that FHTH1 likely regulates flowering time through the protein complex formation with CO.

Many plants in temperate regions have adapted the timing of their reproductive transition to the most favorable season of the year based on day length [27, 34]. In *Arabidopsis thaliana*, CO functions as the floral promoter by activating the expression of the florigen in response to inductive day length conditions [35]. In order to understand this photoperiod-dependent flowering, we aimed to isolate a CO-interacting transcription factor and demonstrated that FHTH1 is a new floral regulator. The formation of a nuclear complex containing FHTH1 and CO proteins and their overlapping tissue-specific expression (Fig. 2) imply that the two proteins function in the same flowering pathway. However, genetic analysis showed that a mutation in the *FHTH1* gene had little effect on flowering time. Since transcription factors are often organized into multi-subunit complexes carrying out their biological functions [36], it is not surprising that the loss of function mutation in the *FHTH1* gene did not alter the flowering phenotype (Fig. 3c, d). In contrast, the late flowering phenotype of transgenic plants overexpressing FHTH1-SRDX indicates the presence of functional redundancy between FHTH1 and other proteins in the regulation of flowering. In summary, as CO interacts with many DNA binding transcription factors in the regulation of its target gene [16, 17, 19, 20, 22], we suggest that FHTH1 might function in the CO-mediated flowering pathway by forming heterodimer complexes with CO and other CO-interacting transcription factors. Further biochemical and genetic studies are required to understand the functional relationships among FHTH1, CO, and other CO-related proteins.

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Authors' contributions
SAS and SGW performed experiments and collected data. DYH analyzed data. J-HK, SSL, and COL provided resources and interpreted data. JCH and YHS conceptualized the research, interpreted data, and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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