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RESEARCH PAPER

Fragaria vesca CONSTANS controls photoperiodic flowering and vegetative development

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Abstract

According to the external coincidence model, photoperiodic flowering occurs when CONSTANS (CO) mRNA expression coincides with light in the afternoon of long days (LDs), leading to the activation of FLOWERING LOCUS T (FT). CO has evolved in Brassicaceae from other Group Ia CO-like (COL) proteins which do not control photoperiodic flowering in Arabidopsis. COLs in other species have evolved different functions as floral activators or even as repressors. To understand photoperiodic development in the perennial rosaceous model species woodland strawberry, we functionally characterized FvCO, the only Group Ia COL in its genome. We demonstrate that FvCO has a major role in the photoperiodic control of flowering and vegetative reproduction through runners. FvCO is needed to generate a bimodal rhythm of FvFT1 which encodes a floral activator in the LD accession Hawaii-4: a sharp FvCO expression peak at dawn is followed by the FvFT1 morning peak in LDs indicating possible direct regulation, but additional factors that may include FvGI and FvFKF1 are probably needed to schedule the second FvFT1 peak around dusk. These results demonstrate that although FvCO and FvFT1 play major roles in photoperiodic development, the CO-based external coincidence around dusk is not fully applicable to the woodland strawberry.

Key words: CONSTANS, FLOWERING LOCUS T, Fragaria, photoperiod, reproduction, runner, strawberry.

Introduction

Plants use various environmental cues, such as light and temperature, to synchronize their life cycles according to local climate (Yanovsky and Kay, 2003). The external coincidence model indicates how environment is linked to flowering in Arabidopsis (Salomé and McClung, 2004; Nozue et al., 2007). According to this model, flower induction takes place when external stimuli such as photoperiod meet with the active phase of an internal oscillator (Sawa et al., 2008). A small transcription factor CONSTANS (CO) is at the heart of the external coincidence model (Suárez-López et al., 2001; Valverde et al., 2004): photoperiodic flowering occurs when the CO mRNA expression peaks at the end of the light period in long days (LDs) and CO activates the expression of FLOWERING LOCUS T (FT) that encodes a mobile flowering-inducing signal (Corbesier et al., 2007; Tamaki et al., 2007).

The circadian clock indirectly generates the rhythmic expression of CO. The basic mechanism of this clock involves
feedback loops of genes that are expressed in different phases of the daily cycle (McClung, 2009). The core feedback loop, formed by TIMING OF CAP EXPRESSION (TOC1) and LATE ELONGATED HYPOCOTYL/CIRCADIAN CLOCK ASSOCIATED1 (LHY/CCA1) (Yanovsky and Kay, 2003; Más, 2008; McClung, 2008), generates the rhythmic expression of several flowering time regulators including FLAVIN-BINDING, KELCH REPEAT, AND F-BOX 1 (CDF1), GIANTANEA (GI), and CYCLING DOF FACTOR1 (CDF1) (Huq et al., 2000; Mizoguchi et al., 2005; Fornara et al., 2009). In the morning, CDF1 directly binds to the promoter of CO to suppress the transcription of the gene (Imaizumi et al., 2005). CDF is degraded by the GI–FKF1 protein complex during the day, leading to a peak in CO expression in the afternoon (Sawa et al., 2007).

Along with transcriptional regulation, CO protein concentration is also strictly regulated by light. CO is unstable in darkness and in the morning, when E3 ubiquitin ligase HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 (HOS1) and phytochrome B (PHYB) activated by red light together destabilize CO (Valverde et al., 2004; Lazaro et al., 2015). In the afternoon, however, phytochrome A and cryptochrome 2 (PHYA and CRY2), which respond to far-red and blue light, respectively, stabilize CO. Therefore, CO protein only accumulates under LD conditions when CO mRNA expression peaks during the light period. This results in the activation of FT and thus flowering. On the other hand, under short-day (SD) conditions, CO mRNA expression peaks in the middle of the night, when CO protein cannot accumulate sufficiently to activate FT (Blázquez and Weigel, 1999; Suárez-López et al., 2001; Izawa et al., 2002; Valverde et al., 2004; Endo et al., 2005).

CO has two B-box type zinc finger domains which have been proposed to function in protein–protein interaction (Putterill et al., 1995; Robson et al., 2001). It also has one CCT (CO, CO-like, TOC1) domain on its C-terminus which mediates protein–protein interaction and nuclear localization (Robson et al., 2001; Ben-Naim, 2006; Wenkel et al., 2006). A total of 16 CO homologous genes, all of them with at least one B-box domain and one CCT domain, have been isolated from Arabidopsis and designated as CO-like (COL) 1–16 (Robson et al., 2001; Griffiths et al., 2003). These genes were allocated to Groups I–III, according to the degree of conservation and number of B-box domains (Robson et al., 2001). Group I, which includes the CO gene, was subdivided into 1a–1d according to the extent of conservation of four highly conserved regions in the middle (Griffiths et al., 2003). A recent study has provided evidence that COL1 and COL2, that do not encode floral promoters, are ancestral Group 1a COL genes; the floral promoter CO evolved within the Brassicaceae after the family split from the Cleomaceae (Simon et al., 2015). In addition to distinct functions, these COL genes show the highest expression at dawn, in contrast to CO which peaks in the afternoon (Ledger et al., 2001; Simon et al., 2015).

CO homologues have been isolated from other plants including woody plants, monocotyledons, and even single-celled Chlamydomonas (Song et al., 1998; Lagercrantz and Axelson, 2000; Yuceer et al., 2002; Griffiths et al., 2003; Nemoto et al., 2003; Chia et al., 2008; Holefors et al., 2009; Serrano et al., 2009). In the SD plant rice (Oryza sativa), the CO homologue Heading date1 (Hd1) promotes expression of the FT homologue Hd3a under inductive SDs (Izawa et al., 2002; Ishikawa et al., 2005). Other CO homologues, OsCO3 (OsB) and OsCOL10, have a negative effect on the expression of Hd3a under these conditions (Kim et al., 2008; Tan et al., 2016). Several CO-like genes have also been identified in Chrysanthemum spp., and one of these was found to promote FT expression and flowering (Fu et al., 2015). However, studies in Pharbitis nil and Medicago truncatula indicated that their COL genes are not involved in the control of FT expression and flowering (Hayama et al., 2007; Wong et al., 2014).

FT has been shown to function as a floral activator in SD, LD, and day-neutral plants, while another member of the same gene family, TERMINAL FLOWER1 (TFL1), is a floral repressor (Wickland and Hanzawa, 2015). However, there are several independent examples about the evolution of FT homologues into floral repressors including BvFT1 in sugar beet, three FT homologues in tobacco, and a specific splicing variant of Brachypodium FT (Pin et al., 2010; Harig et al., 2012; Qin et al., 2017). In seasonal flowering commercial strawberry (Fragaria×ananassa Duch.) and the diploid model woodland strawberry (Fragaria vesca L.), which are both SD plants (Ito and Saito, 1962; Battey et al., 1998; Battey, 2000), TFL1 homologues are strong floral repressors. FvFT1 and FaTFL1 are highly expressed under LDs, and their repression under SDs and low temperature conditions enables flower induction to take place (Koskela et al., 2012; Nakano et al., 2015; Rantanen et al., 2015; Koskela et al., 2016). Interestingly, FT homologues, FvFT1 and FaFT1, are expressed specifically under LDs and correlate negatively with flower induction, indicating that they may also repress flowering in SD strawberries (Koskela et al., 2012; Nakano et al., 2015). A natural mutant of woodland strawberry (F. vesca semperflorens) lacks functional FvTFL1 and is an LD plant which flowers perpetually after flower induction (Koskela et al., 2012). In this mutant, FvFT1 is also expressed under LDs and functions as a promoter of flowering (Koskela et al., 2012; Rantanen et al., 2014). FvFT1 is normally expressed diurnally with peaks 4 h and 16 h after dawn; its expression is most effectively induced artificially by FR daylength extension in the mutant (Koskela et al., 2012; Rantanen et al., 2014).

A close CO homologue (FvCO) has been previously identified in woodland strawberry (Shulaev et al., 2011), but its function has not been tested. Here, using transgenic overexpression and RNAi lines of woodland strawberry, we demonstrate that FvCO has a major role in the photoperiodic development of this species. We show that, although the gene expression rhythms of FvCO and FvFT1 do not coincide, FvCO is needed to activate FvFT1 that controls reproductive and vegetative development in response to photoperiodic signals.

Materials and methods

Plant material

Experiments were mostly performed with the LD-flowering accession ‘Hawaii-4’ (H4; National Clonal Germplasm Repository
acrossion number PI551572). Gene expression analyses were also carried out in a Finnish SD accession FIN56 (PI551792). Seedlings or plants clonally propagated from runner cuttings were used for the experiments as indicated in the text and figure legends.

**Growth conditions and phenotypic observations**

Plants were raised in a growth chamber or a greenhouse under a non-flower-inductive photoperiod at 20–22 °C, under SDs (12/12 h light/dark) for H4 and LDs (16/8 h light/dark) for FIN56. Fluorescent tubes (Warm white 30W/32-930, Osram, Germany) or light-emitting diodes (LEDs; AP67, Valoya, Finland) were used as the white light source at a photosynthetic photon flux density (PPFD) of 200 µmol m⁻² s⁻¹ in growth chambers. High pressure sodium (HPS) lamps (Airam 400W, Kerava, Finland) at a PPFD of 120 µmol m⁻² s⁻¹ were used to supplement the natural light in the greenhouse. Seedlings were transplanted to 8 × 8 cm pots at the cotyledon stage, while runner cuttings were directly rooted in these pots. Fertilized peat (Kekkilä, Finland) supplemented with 25% (v/v) vermiculite (Ω2 mm) was used as a growing medium. Plants were fertilized with liquid fertilizer (Kekkilä; N-P-K: 17-4-25) biweekly.

Both flowering time and vegetative development were studied in the experiments. To observe flowering time differences between H4 and transgenic lines, either the number of leaves in the primary leaf rosette before the terminal inflorescence or the number of days before the first open flower was recorded. In addition, the differentiation of axillary buds into either axillary leaf rosettes called branch crowns or runners (stolons) was observed.

**Gene expression analysis**

Leaf and shoot apex samples were frozen in liquid nitrogen and stored at −80 °C before total RNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method as described in Koskela et al. (2012). cDNAs were synthesized from 1 µg of total RNA using Superscript III reverse transcriptase (Invitrogen). SYBR Green I master mix (Roche) was used for real-time PCRs which were performed in the Light Cycler 480 instrument (Roche) as described previously (Mouhu et al., 2009). Real-time PCR reactions were performed with three technical replicates and two or three biological replicates as mentioned in the figure legends. Relative expression levels were calculated by the ∆∆Ct (cycle threshold) method, with FvMSI1 as the normalization gene as described previously (Mouhu et al., 2013). Primers used in the real-time PCR are listed in Supplementary Table S1 at JXB online. Primer efficiencies were almost equal for all primer pairs (Rantanen et al., 2014).

**Plasmid constructs**

Plasmid constructs for overexpression and RNAi silencing lines were created according to Gateway technology with Clonase II (Invitrogen). For FvCO overexpression and RNAi constructs, cDNA from F. vesca H4 was amplified with primer pairs 5’TACGAGTGAGGAGGAAAACAACA-3’ and 5′-(attB1)-TTGCTGAAAAGGTTGAACT-3’, and 5′-(attB1)-ACAAATCGTAGTCTCAG-3’ and 5′-(attB2)-AGGAACAATGCCGTATCAG-3’, respectively. The destination vectors were pK7WG2D1 for overexpression and pK7GW1WG2D(II) for RNAi silencing (Karimi et al., 2002). Both vectors contain green fluorescent protein as a positive selection marker.

**Transformation**

Vectors carrying overexpression and RNAi constructs were electroporated into Agrobacterium tumefaciens strain GV3101 and transformed into H4 as described previously (Oosumi et al., 2006). Several transgenic lines were generated for both constructs. Transgenic lines were selected for the experiments based on their phenotypes and FvCO expression levels.

**Sequence alignment and phylogenetic analysis**

Amino acid sequence alignment was conducted using the ClustalW program with the BLOSUM62 matrix. MrBayes 3.2.2 was used to construct a Bayesian estimation of a phylogeny of CO-like proteins. Two independent runs were performed, the averaged. WAG (Whelan and Goldman) matrix was used as a substitution model, and gamma distribution was set for among-site rate variation with the rate category of 4. The Markov chain Monte Carlo algorithm was run with chain length of 1 000 000 with four heated chains (heated chain temperature=0.2). Subsampling was performed every 200 generations and burn-in length was set to 10%. CrCO from Chlamydomonas reinhardtii was used as the outgroup.

**Statistical analyses**

ANOVA was conducted on the averages using the general linear model, and differences between means were analysed by Tukey–Kramer test. All statistical analyses were conducted using the R package (ver. 3.3.2).

**Accession numbers**

Sequence data from this article can be found in the GenBank/ National Center for Biotechnology Information data library under the following accession numbers: FvSOC1 (FJ531999) and FvFT1 (JN172098). Predicted gene models (Hybrid V2) can be found in the Genome Database for Rosaceae (http://www.rosaceae.org): FvCO (gene04172), FvMSI1 (gene03001), gene02008, gene03742, gene14015, gene14981, gene15552, gene24941, gene25039, gene25171, and gene27383. Accession numbers of the protein sequences used in the phylogenetic analysis are listed in Supplementary Table S2.

**Results**

**Isolation, structure, and phylogenetic analysis of Fragaria CO**

One woodland strawberry homologue of CO, FvCO (gene04172), was previously annotated in the F. vesca whole-genome v1.1 assembly (Shulaev et al., 2011). To explore the strawberry CO-like gene family, a BLASTx database search was performed using the full-length sequence of FvCO against the whole-genome assembly. In total, nine additional putative CO-like protein sequences longer than 200 amino acids were identified. These protein sequences were subjected to a phylogenetic analysis to identify putative regulators of flowering time.

A phylogenetic tree of COL proteins was constructed using CrCO from C. reinhardtii as the outgroup. FvCO was placed in the same clade with CO homologues of eastern cottonwood, morning glory, and tomato, and with Arabidopsis Group Ia proteins CO, COL1, and COL2 (Fig. 1; Supplementary Fig. S1). The predicted protein for gene14981 was placed in the clade comprised of Malus domestica CO-like proteins, BvCOL2 of sugar beet, and Arabidopsis COL3 and COL4, which are categorized as CO Group Ib proteins (Griffiths et al., 2003; Chia et al., 2008); the predicted protein for gene27383 was close to COL5 (Fig. 1; Supplementary Fig. S1). Other predicted proteins clustered in Group II (gene03742 and gene25171) or Group III (gene14015, gene15552, and gene24941); gene02008 and gene25039 made up an isolated clade of their own (see Supplementary Fig. S1).
As the phylogenetic tree indicated that FvCO, gene14981, and gene27383 belong to Group I, conserved domains of the corresponding protein sequences were subjected to further analysis (Supplementary Fig. S2a). Predicted protein sequences of these genes were aligned with other CO-like proteins by ClustalW. The alignment showed that two B-box domains (Griffiths et al., 2003) and the CCT domain (Wenkel et al., 2006) were highly conserved in these three Fragaria CO-like sequences (Supplementary Fig. S2b, c). FvCO showed the highest level of conservation in the M1–M4 conserved regions. Gene27383 had glutamate to aspartate, tryptophan to leucine, and leucine to isoleucine substitutions in the M1 region found in Group Ia–Ic (Glu-X2-Ser-Trp-Leu-Leu), while the other two have a conserved sequence (Supplementary Fig. S2d). The M2 region (Leu-Val-Asp/Gly-Tyr) of FvCO had an aspartate/glycine to glutamate substitution similarly to PnCO (Group Ia), and the other two were lacking valine similarly to COL3 and COL4 (Group Ic) (Supplementary Fig. S2e). The M3 region (Gly-X-Asp/Glu-X-Ile/Val-Val-Pro) of gene14981 had a substitution of the first glycine residue to alanine (Supplementary Fig. S2f), and the M4 region (Ser-X-Glu/Asp-X-Val-Pro) of gene14981 had a substitution of the first serine to proline (Supplementary Fig. S2g). As the phylogentic tree and further analyses on conserved domains indicated that FvCO was the only Group Ia COL protein encoded by the accessible woodland strawberry genome, functional analysis was mainly focused on FvCO.

FvCO expression peaks at dawn

The diurnal expression patterns of FvCO and FvFT1 were investigated in woodland strawberry accessions with contrasting photoperiodic responses. In the perpetual flowering LD accession H4 and the seasonal flowering SD accession FIN56, FvCO exhibited a single mRNA expression peak at dawn under both LD and SD conditions, and its expression stayed low during the day regardless of the accessions examined (Fig. 2A; Supplementary Fig. S3). In H4 under LDs, the expression of FvFT1 peaked 4–8 h after dawn and again in the evening (ZT16–ZT20), the second peak being slightly higher than the first (Fig. 2B). A similar diurnal rhythm of FvFT1 mRNA expression was observed in FIN56 under LDs, but the morning peak was higher than the evening peak (Supplementary Fig. S3). The morning peak of FvFT1 followed that of FvCO, but the evening peak of FvFT1 did not coincide with high FvCO mRNA levels. In H4 under SDs, the FvFT1 mRNA level was very low or undetectable throughout the 24 h cycle (Fig. 2B). In addition to FvCO, we explored the rhythmic expression of two COL genes showing the highest homology with FvCO. The gene27383 exhibited rhythmic expression, peaking at the same time as FvCO, whereas the expression of gene14981 did not show a clear rhythm (Supplementary Fig. S4).

Our data indicated that FvCO expression peaked at dawn under different photoperiods, so we tested whether the dawn signal was critical for the timing of its expression. LD-grown plants were transferred to darkness (DD) and FvCO mRNA levels measured. Under DD conditions, in contrast to the LD control, FvCO expression continued to rise after the subjective dawn (the beginning of the light period in the LD control) and stayed high during the next 8 h (Fig. 2C). These results suggest that the up-regulation of FvCO takes place in darkness and the dawn signal is needed for its down-regulation.

FvCO controls vegetative and generative development

To test the role of FvCO in the photoperiodic control of vegetative and reproductive development, we generated transgenic plants of the H4 accession with FvCO overexpressed [driven by the Cauliflower mosaic virus (CaMV) 35S promoter] or RNAi silenced. The expression levels of FvCO mRNA were clearly altered in these transgenic lines (Fig. 3A, B). In the overexpression lines, strong up-regulation of FvCO was observed especially in the evening (ZT16) when its expression
FvCO controls photoperiodic development

level in the wild-type H4 is low. In RNAi lines, in contrast, clear down-regulation of FvCO was observed, but no silencing of two other Group I COL genes was detected, confirming the specificity of our RNAi construct (Supplementary Fig. S4).

We recorded the number of leaves in the primary leaf rosette before the terminal inflorescence in plants that had been subjected to LDs or SDs. Overexpression lines produced slightly fewer leaves before the terminal inflorescence compared with wild-type plants under LDs, whereas a strong promotion of flowering was observed in overexpression lines under SDs (Figs 3C, D, 4A; Supplementary Table S3). In FvCO RNAi lines, in contrast, flowering was significantly delayed compared with non-transgenic control plants under LDs, while under SDs, both H4 and FvCO RNAi lines remained vegetative or flowered very late, depending on the experiment (Figs 3E, F, 4A; Supplementary Table S3). An additional experiment revealed that FvCO overexpression plants flowered within 4 weeks and wild-type H4 after 5 weeks in LDs, whereas FvCO RNAi lines flowered ~1 month later (Supplementary Fig. S5). Comparison of FvCO RNAi lines with the previously published FvFT1 RNAi lines (Koskela et al., 2012) showed that both constructs had a similar effect on flowering time in H4 (Fig. 4B; Supplementary Table S3).

Flower-inducing conditions promote the differentiation of axillary buds to axillary leaf rosettes called branch crowns, while in non-inductive conditions vegetative reproduction through runners takes place. To gain insight into the effect of FvCO and FvFT1 on vegetative development, we studied the differentiation of axillary buds of the primary leaf rosette. In H4, most axillary buds differentiated to runners in SD conditions and only a few branch crowns were observed, whereas the effect of LDs was opposite (Fig. 4C–F). A clear photoperiodic response was also observed in FvCO overexpression lines, although they tended to produce fewer runners and more branch crowns than the wild type. In both FvCO and FvFT1 RNAi lines, in contrast, axillary buds did not show a clear photoperiodic response (Fig. 4C–F; Supplementary Fig. S5). In all RNAi lines, roughly two-thirds of axillary buds differentiated to runners and only very few buds produced branch crowns in both photoperiods. Moreover, in H4 and all transgenic lines, ~20–30% of axillary buds remained dormant (data not shown).

To explore further the effect of FvCO on the balance between generative and vegetative development, we observed the cumulative number of inflorescences and runners in generative plant materials. FvCO overexpression plants produced slightly more new inflorescences than the wild type (Fig. 5A). In FvCO and FvFT1 RNAi plants, however, inflorescence production was reduced so that by the end of the experiment they had almost 50% fewer inflorescences than the H4 accession. In contrast to the intense flowering, runner production was strongly suppressed in overexpression and wild-type plants, whereas all RNAi lines continuously produced new runners at the rate of approximately one runner per week (Fig. 5B).

FvCO up-regulates FvFT1 in light

Next, we examined the expression of flowering time genes in FvCO transgenic lines. First, leaf samples were collected 4 h or 16 h after dawn (ZT=4 or 16) under LD conditions, as the FvFT1 mRNA level peaks at these times in wild-type plants (Fig. 2B). The up-regulation of FvFT1 was observed at both time points in FvCO overexpression lines (Supplementary Fig. S6). In RNAi lines, however, FvFT1 mRNA expression...
was not detected. To understand the regulation of FvFT1 by FvCO in more detail, we explored diurnal expression patterns in H4 and FvCO transgenic lines grown under LD and SD conditions. Overexpression of FvCO induced expression of FvFT1 under both LD and SD conditions, but the normal diurnal expression cycle was lost (Fig. 6). Under LDs, up-regulation of FvFT1 was observed during the light period from ZT0 to ZT16 in overexpression plants (Fig. 6A, B), while under SDs a strong up-regulation was observed only at ZT4 and another minor peak was present 4 h after dusk at ZT12 (Fig. 6C, D). In FvCO RNAi plants, in contrast, FvFT1 mRNA levels remained extremely low or undetectable during the whole diurnal cycle under SD and LD conditions (Fig. 6B, D), even under continuous light which strongly increased FvFT1 mRNA levels (Supplementary Fig. S7). These data indicated that FvCO affected both morning and evening peaks in FvFT1 expression, even though FvCO expression was high only around dawn. Moreover, FvFT1 expression is dependent on the light/dark cycle also in FvCO overexpression lines that highly express FvCO mRNA throughout the day.

To explore further the downstream flowering gene pathway, we studied the expression of FvSOC1, that is activated by FvFT1 in shoot apices in LDs (Mouhu et al., 2013), and the expression of the floral meristem identity gene FvAP1. FvSOC1 was strongly activated in FvCO overexpression lines compared with H4 especially under SD conditions (Fig. 7A). In RNAi lines, however, the FvSOC1 mRNA level was reduced in LD conditions and, in contrast to wild-type H4, no clear photoperiodic regulation of the gene was observed. Consistent with the observed differences in flowering time, FvAP1 was down-regulated in RNAi lines and highly activated in the stronger SD-grown FvCO overexpression line compared with H4 at 3 weeks after the beginning of the treatment (Fig. 7B). However, an equally high FvAPI expression level was detected in wild-type and overexpression lines in LDs at this time point, but in another experiment, at a 1 week earlier time point, an elevated FvAPI expression level was detected in overexpression lines compared with H4 in LDs (Supplementary Fig. S8).

FvGI and FvFKF1 expression peaks precede the up-regulation of FvFT1 towards evening in LDs

Although FvCO is clearly required for the activation of FvFT1 mRNA expression, additional factors are probably needed to schedule its diurnal cycle, especially towards evening (Fig. 2). Therefore, we studied the diurnal expression patterns of strawberry homologues of GI and FKF1, genes which encode regulators of FT expression in Arabidopsis (Sawa et al., 2007; Sawa and Kay, 2011). In the H4 accession under 12 h SDs, the expression of FvGI increased rapidly in the morning and stayed high until ZT12, after which time there was a rapid drop in expression (Fig. 8A). Slightly slower up-regulation was observed under 16 h LD conditions, and FvGI expression remained high until dusk at ZT16; a similar expression pattern was also observed in FIN56 (Supplementary Fig. S9A). The expression of FvFKF1 began to increase in the morning and peaked 8–12 after dawn (Fig. 8B; Supplementary Fig. S9B). The up-regulation was slower under LDs, where the strong activation took place between ZT4 and ZT8. The peak of expression of both FvGI and FvFKF1 therefore preceded...
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the up-regulation of *FvFT1* that takes place after ZT12, in the evening.

**Discussion**

Plants typically contain a large *COL* gene family; for example Arabidopsis and rice have 17 and 16 genes, respectively, while 26 genes have been identified in soybean (Griffiths et al., 2003; Wu et al., 2014). A few of these genes encode floral activators, but also repressors as well as regulators, with no effect on flowering (Putterill et al., 1995; Wong et al., 2014; Cao et al., 2015; Mulki and von Korff, 2016; Tan et al., 2016). Here, we have identified 10 *COL* genes in woodland strawberry and shown that, based on phylogenetic analysis (Fig. 1; Supplementary Fig. S1), the previously identified *FvCO* is the only Group Ia *COL* gene in the *F. vesca* genome (Shulaev et al., 2011). We have also shown that it plays a major role in the photoperiodic control of reproductive and vegetative development in this species. Although *FvCO* mRNA is expressed at different phases during the day compared with Arabidopsis *CO*, it is nevertheless required to generate the evening expression peak of *FvFT1* (a feature similar to the expression pattern of Arabidopsis *FT*; see Fig. 6; Suárez-López et al., 2001), as well as an additional peak in the morning.

Previous studies suggested that the LD-activated *FvFT1–FvSOC1–FvTFL1* pathway represses flowering in woodland strawberry, and flower induction occurs after the silencing of this pathway by SDs and cool temperature in autumn. However, the characterization of the LD-flowering mutant H4, that is lacking the functional floral repressor *FvTFL1*, revealed a relic function of *FvFT1* and *FvSOC1* as floral activators in this accession (Koskela et al., 2012; Mouhu et al., 2013; Rantanen et al., 2014, 2015). We show here that, similarly to the RNA silencing of *FvFT1*, the silencing of *FvCO* delays flowering in H4, while *FvCO* overexpression has an opposite effect (Figs 3, 4; Koskela et al., 2012; Rantanen et al., 2014). In agreement with these phenotypic observations, the silencing of *FvCO* strongly reduces *FvFT1* mRNA level in leaves, whereas *FvCO* overexpression leads to the activation of *FvFT1*. As previously observed in *FvFT1* RNAi lines (Mouhu et al., 2013), in our *FvCO* transgenic...
lines, FvSOC1 and FvAP1 mRNA levels in shoot apices correlated positively with FvFT1 expression in leaves. This indicates that in H4, FvCO has a major role in regulating FvFT1 and FvSOC1 expression to advance flowering under LD conditions. Also SD genotypes of woodland strawberry and cultivated strawberry may contain the relic flowering-promoting FvCO–FvFT1–FvSOC1 pathway, but the activation of FvTFL1 by FvSOC1 probably reverses the developmental outcome, namely the photoperiodic flowering response (Mouhu et al., 2013; Koskela et al., 2016). Direct functional analyses of FvCO and FvFT1 in an SD genotype, however, are needed to confirm this model.

A recent study has suggested that another FT, FaFT3, is activated before FaAP1 and may induce flowering in cultivated strawberry under SDs (Nakano et al., 2015). A similar SD-specific activator may also function in the LD accession H4 which will eventually flower under SD conditions, when FvFT1 expression is undetectable (Fig. 6D; Rantanen et al., 2014). However, we found very low FvFT3 expression in both H4 and FvCO transgenic lines in both SDs and LDs (data not shown). Thus, our results do not support the role of FvFT3 in flower induction in H4.

Phylogenetic analysis grouped FvCO with other Group Ia COL proteins including Arabidopsis COL1 and COL2 that have no effect on flowering time (Ledger et al., 2001; Kim et al., 2013) and the major floral activator CO that has evolved from COL1 or COL2 by gene duplication in the Brassicaceae (Simon et al., 2015). Unlike FvCO, studies on Group Ia COLs of the SD plant P. nil and the LD plant M. truncatula suggested that they do not promote flowering (Hayama et al., 2007; Wong et al., 2014); in Glycine max, COL1 functions as a
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floral repressor under LDs (Cao et al., 2015). In the monocots rice and spring barley, however, the closest CO homologues Hd1 and HvCO2, respectively, activate flowering (Izawa et al., 2002; Mulki and von Korff, 2016). This indicates that the functions of Group Ia COLs are species specific. What causes these diverse functions of CO homologues in flowering time regulation is an interesting open question.

FvCO controls vegetative development in strawberry

Differentiation of strawberry axillary buds to runners and branch crowns is also regulated by photoperiod (Hytönen et al., 2004). Our data demonstrate the major role of the FvCO/FvFT1-mediated photoperiodic pathway in this response as well as in controlling the balance between vegetative and floral development. H4 produced far more runners under SDs than under flower-inducing LDs, while the silencing of either FvCO or FvFT1 caused continuous photoperiod-independent production of runners. FvCO overexpression plants, however, produced slightly fewer runners than H4 and, when these plants were moved from SDs to flower-inductive LD conditions, their runner production slowed down earlier than in H4. LD, in contrast, promoted the differentiation of axillary buds to branch crowns in H4 and FvCO overexpression lines, whereas RNAi lines did not show this response.

In contrast to runner production, generative FvCO RNAi plants produced fewer and overexpression lines slightly more inflorescences than H4 (Fig. 5). Such a balance between vegetative and generative growth is well documented in cultivated strawberries (e.g. Sonstebey and Heide, 2007), and it may be caused by competition for resources in clonal plants (Loehle, 1987). Furthermore, FvCO and its counterpart in cultivated strawberry can affect the expression/function of the gene at the PFRU locus that has been reported to control the balance between vegetative and generative growth (Gaston et al., 2013; Samad et al., 2017).

In the SD accession of woodland strawberry, FvSOC1 promotes runner formation in LDs (Mouhu et al., 2013), and studies in non-flowering FvTFL1 overexpression plants and in a non-transgenic SD cultivar of cultivated strawberry confirmed that direct photoperiodic regulation of axillary bud differentiation can occur (Hytönen et al., 2009; Koskela et al., 2012). In H4 and FvCO transgenic lines, however, we found a negative correlation between the FvSOC1 expression level and the number of runners. Therefore, our data suggest that in this accession, which flowers perpetually after flower induction, axillary bud differentiation is primarily controlled...
by flowering, and FvSOC1 may have a minor role. Runners are formed from axillary buds at the vegetative stage and, upon flower induction, the uppermost axillary buds differentiate into new branch crowns instead of runners, which leads to a reduction in runner formation and increases the number of meristems that can produce inflorescences (Hytönen et al., 2004). Taken together with this information, our study indicates that the photoperiodic pathway affects the balance between vegetative and generative development in strawberries; further studies are needed to uncover how this balance is regulated in LD and SD genotypes.

The diurnal FvFT1 expression is under control of FvCO

In Arabidopsis, the CO mRNA level increases towards evening and, according to the external coincidence model, FT is activated under LDs when CO expression coincides with light (Suárez-López et al., 2001). Similarly to Arabidopsis FT, FT homologues in woodland strawberry and cultivated strawberry (FvFT1 and FaFT1, respectively), exhibited a major mRNA expression peak in the evening at ZT16–ZT20 (Fig. 6; Koskela et al., 2012, 2016). However, an additional peak was observed between 4 h and 8 h after dawn; other work shows that the height of this peak depends on the light conditions (Rantanen et al., 2014). FvCO is expressed in a different phase from Arabidopsis CO in both LD and SD accessions (Supplementary Fig. S2; Kurokura, 2009). It exhibits a sharp expression peak towards dawn, similar to COL1, COL2, and COL5, BvCOL1 of Beta vulgaris, and PnCO of P. nil (Ledger et al., 2001; Liu et al., 2001; Chia et al., 2008; Hassidim et al., 2009). The dawn signal (dark to light) causes the down-regulation of the COL gene in the SD plant Chenopodium rubrum (Draběšová et al., 2014), and this is also likely to be the case in woodland strawberry, because the transfer of plants to dark-gradual up-regulation of FvFT1 mRNA after subjective dawn (Fig. 2c).

Our studies on transgenic lines indicate that, although diurnal expression rhythms of FvCO and FvFT1 do not match in woodland strawberry, functional FvCO is needed to activate FvFT1 mRNA expression in both the morning and evening in LDs. FvCO RNAi lines exhibit very low FvFT1 mRNA levels during the whole diurnal cycle compared with the wild type, whereas overexpression of FvCO results in the induction of FvFT1 in a light-dependent manner with a broad peak during the light period under LDs. Under SDs, however, FvFT1 is highly activated only in the morning in overexpression plants, with an additional minor peak after dusk (Fig. 6). Our results in FvCO overexpression plants suggest that the FvCO protein is regulated by light, as has been observed in Arabidopsis (Valverde et al., 2004; Song et al., 2012). Although the FvCO expression pattern is different from that of CO (Suárez-López et al., 2001), light-regulated FvCO protein could form a part of the photoperiod measurement system that controls the gradual up-regulation of FvFT1 under increasing photoperiods (Rantanen et al., 2015). However, additional unknown factors are probably needed to schedule the evening peak of FvFT1. These factors may include CRYPTOCHROME-INTERACTING BASIC-Helix-LOOP-Helix and/or PHYTOCHROME-DEPENDENT LATE-FLOWERING proteins that activate FT specifically in the evening (Endo et al., 2013; Liu et al., 2013). Further studies on these regulators as well as on FvCO protein stability and activity are needed to understand the photoperiodic control of FvFT1 mRNA expression.

In Arabidopsis, GI and FKF1 interact in a blue light-dependent manner to activate CO and FT mRNA expression by removing the repressor protein CDF1 in the afternoon (Imaizumi et al., 2005; Sawa et al., 2007; Fornara et al., 2009; Song et al., 2012). In addition, FKF1 and GI can directly activate the expression of FT (Sawa and Kay, 2011). Since CO-independent FT regulation has also been suggested in other species (Doi et al., 2004; Hayama et al., 2007; Ridge et al., 2016), it is unlikely that FvFT1 expression is regulated only by FvCO in woodland strawberry, even though FvCO seems to play a major role.

To gain insight into the function of these genes in woodland strawberry, we investigated their diurnal expression rhythms and observed that FvGI was highly expressed during the day in both SDs and LDs (Fig. 8A). FvFKF1 exhibited a sharper expression peak in the afternoon, a few hours before the FvFT1 evening peak (Fig. 8B). Therefore, FvFKF1 and FvGI may control the expression of FvFT1 in the evening in LDs, but detailed gene functional studies are needed to confirm their roles in the photoperiodic flower induction of the woodland strawberry.

Conclusions

The CO homologue of the woodland strawberry, FvCO, has a diurnal expression rhythm with a sharp peak around dawn, regardless of photoperiodic conditions. FvCO plays a major role in the photoperiodic regulation of FvFT1 and thus flowering time, as well as in vegetative reproduction (i.e. the production of runners). The expression of FvCO is promoted under darkness, and light is required to suppress its expression in the morning. Partial coincidence of the expression pattern of FvCO and FvFT1 in the morning indicates that FvCO1 regulates FvFT1 expression in part, but other unknown factor(s) may be involved in the generation of the bimodal diurnal expression pattern of FvFT1. Woodland strawberry homologues of FvGI and FvFKF1 are good candidates for the factors that schedule FvFT1 expression in the evening because, as in Arabidopsis, corresponding genes are expressed during the day before the FvFT1 evening peak.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Full structure of the phylogenetic tree of COL proteins.

Fig. S2. The analysis of conserved motifs of Group I COL proteins.

Fig. S3. Expression patterns of FvCO and FvFT1 in FIN56.

Fig. S4. Expression patterns of COL genes 14981 and 27383 in H4 and FvCO RNAi lines.
Fig. S5. Vegetative and reproductive growth of \textit{FvCO} transgenic lines under LD conditions.
Fig. S6. Expression of \textit{FvFT1} in \textit{FvCO} transgenic plants.
Fig. S7. \textit{FvCO} and \textit{FvFT1} expression under continuous light.
Fig. S8. \textit{FvAPI} expression in \textit{FvCO} transgenic plants.
Fig. S9. Expression patterns of \textit{FvGI} and \textit{FvFKF1} in SD accession FIN56.

Table S1. List of primers used in quantitative real-time PCR.
Table S2. List of protein accession numbers used in the phylogenetic tree.
Table S3. Flowering time of Hawaii-4 and \textit{FvCO} transgenic lines.

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