Title page:

Title: Diurnal Expression of Arabidopsis Gene Homologs during Daylength-Regulated Bulb Formation in Onion (***Allium cepa*** L.)

Running title: *Clock genes control daylength-regulated bulbing in onion*

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Abstract:

Bulb initiation in long-day onion is regulated at the physiological level in a similar way to the photoperiodic regulation of flowering in Arabidopsis. This study establishes in onion, the diurnal time-course expression, in onion, of key genes particularly linked to circadian regulation in Arabidopsis. The long-day onion variety '*Renate*' and the short-day (SD) onion variety '*Hojem*' were used for these experiments. Onion plants were grown under natural LD conditions in the Phytobiology Glasshouse and immediately after bulbing they were transferred to two SANYO 2279 controlled environment cabinets for 10 d providing constant LD (16 h photoperiod including 8 h fluorescent followed by 8 h incandescent light) and constant short days (8 h photoperiod with fluorescent light). Five *FLOWERING LOCUS T (FT)* and three *CONSTANS-LIKE* (*COL)* genes were identified in onion, including two novel *COL* sequences through RNA-Seq analysis. The new *AcCOL2* shows a diurnal pattern of expression similar to Arabidopsis *CONSTANS* (*CO*). *Allium cepa FLAVIN-BINDING, KELCH REPEAT, F-BOX PROTEIN 1* (*AcFKF1*), *Allium cepa GIGANTEA* (*AcGI*) and *AcCOL2* showed good diurnal expression patterns consistent with photoperiod sensing and regulation of *AcFT1*. All *FT* genes exhibited different diurnal expression patterns peaking at different times of the day. Notably, *AcFT1* was expressed in the later part of the day which is very similar to the expression of Arabidopsis *FT*, while *AcFT4* was expressed late in the night and the early morning in both *Renate* and *Hojem* varieties of onion, with the caveat that, *AcFT4* is under less stringent daylength control in *Hojem* than in *Renate*. The timing of the peaks and expression pattern in both *Renate F1* and *Hojem* suggest that *AcFT5* may be under circadian or diurnal regulation under LD conditions and *AcFT6* might not be circadian or diurnally regulated. These findings will help to understand the basis of the difference between

responses of onions adapted to different latitudes, which is important for developing new varieties.

Keywords: *AcFKF1*, *AcGI*, *AcCOL*, *AcFT*, Circadian clock genes, LD, SD, RNA-seq

1. Introduction

Onion (*Allium cepa* L.) belongs to the family Alliaceae, is one of the most important vegetable and spice crops cultivated (Brewster, 1994; McCallum, 2001). Numerous onion cultivars have been developed for size, form, colour, pungency, storability, resistance to pests and pathogens, and climatic conditions (Griffiths et al., 2002). Onion is a monocotyledonous bulbous perennial (often biennial), outcrossing and highly heterozygous crop plant, which is propagated by seeds, bulbs or sets (Eady, 1995). An onion plant is composed of photosynthetic leaf blades, which arise alternately from a base plate, or small-flattened scales (bulb), which is the vegetative overwintering stage in the life cycle of the plant (Lancaster et al., 1996). Bulb formation in onions from different global regions is adapted to local environmental conditions, particularly the daylength (Cardoso and da Costa, 2003). Onions are classified as long-day, intermediate-day or short-day, depending on the minimum daily duration of light required for bulbing, also known as the critical daylength (Albert, 2016). Temperate onions require long days (LD) for bulbing whereas tropical onions will form bulbs in short days (SD) (Rashid et al., 2016). The life cycle of onion can be divided into three main stages, namely seedling growth and bulb formation in the first year and, following overwintering, flowering in the second year (Brewster, 1990). Bulb initiation will not occur during early seedling growth, sometimes referred to as the Juvenile phase, regardless of plants being exposed to favourable environmental conditions (Massiah, 2007). When the onion plant becomes mature and the daylength has reached a critical length, bulb formation is initiated (Lee et al., 2013). At this stage, onion leaves must be exposed continuously to an inductive photoperiod in order to initiate and complete bulbing (Brewster, 2008). The long-day onion cv. Renate requires at least 14 h of light to initiate bulbing (Rashid et al., 2016), whereas Hojem, a short-day cultivar requires at least 10 h to enable bulbing.

Arabidopsis flowering and onion bulb formation are both photoperiodically driven processes (Thomas et al., 2006), induced by LD, signal perception is in the leaf and response is at the apex. Sepals, petals, stamens and anthers are produced as the end product in Arabidopsis, whereas, a storage scale leaves are produced as the end product in onion (Summerfield, 1991). Arabidopsis flowering and onion bulb formation can be compared in terms of the involvement of phytochrome, and both processes are promoted by far-red light, through PHYA (Brewster, 1977). Flowering in Arabidopsis has been characterised at the molecular and genetic level and is regulated by 6 major separate pathways viz., photoperiodic, convergent autonomous, sucrose, gibberellin, temperature and light quality pathway (Jack, 2004; Thomas et al., 2006). For onion, the main environmental stimuli are photoperiod and temperature (Brewster, 1990), but these are mainly based on physiological rather than genetics analyses (Khokhar, 2017).

In this study we focus on the photoperiodic pathway, which is mediated by the circadian clock, an autonomous mechanism that generates endogenous rhythms in a 24-hour period in the leaf (Jackson, 2009) and is controlled by various feedback loops (Hayama and Coupland, 2003). Light plays an important role in the photoperiodic response in Arabidopsis and interacts with the circadian clock as part of the photoperiodic flowering pathway (Michael et al., 2003). In the leaf, light is perceived by different photoreceptors, both cryptochromes in blue light and phytochromes in red/ far-red light and inputs into the circadian clock (Devlin and Kay, 2000; Lin, 2002). Numerous key genes are involved in circadian regulation, where the clock derives the rhythmic expression of key genes *FLAVIN-BINDING, KELCH REPEAT, F-BOX* (*FKF1*), *GIGANTEA* (*GI*) and *CONSTANS* (*CO*). *FKF1* and *GI* promote *CO* expression (Sawa et al., 2007) and this *CO* positively regulates *FLOWERING LOCUS T* (*FT*) (Jung et al., 2007). The FT protein is then translocated to the apical meristem through the phloem and forms a FT/FD (FLOWERING LOCUS D) complex (Abe et al., 2005; Pnueli

et al., 2001; Purwestri et al., 2009; Taoka et al., 2011; Wigge et al., 2005). This complex activates the *APETALA 1* (*AP1*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) genes, which trigger *LEAFY* (*LFY*) gene expression and cause flowering at the floral apical meristem in Arabidopsis (Greg et al., 2015; Nakamichi, 2011; Yoo et al., 2005). In a previous study the expression of onion GI, FKF1 and ZTL homologs under SD and LD conditions was examined using quantitative reverse transcription-PCR (qRT-PCR), where the results showed that key genes namely *GI*, *CO* and *FT* controlling photoperiodic flowering in Arabidopsis are conserved in onion, and a role for these genes in the photoperiodic control of bulb initiation is predicted (Taylor et al., 2010). Also, Lee at al (2013) identified 6 members of the FT family (FT1-6) in onion. They proposed that two of them, FT1 and FT4 acted to regulate bulbing, being promoter and inhibitor respectively, although they did not look at their circadian expression. This raised the question of how these genes are linked to the daylength-sensing system to establish the critical daylength in long-day and short-day onions. To address this question, experiments were designed to quantify the diurnal expression of FT, CO and other key genes in two onion cultivars with contrasting daylength responses, namely a long-day type cv. *Renate* and a short-day type cv. *Hojem*.

2. Materials and methods

This work has been conducted at the School of Life Sciences, the University of Warwick, Coventry, CV4 7AL, UK during the period from July 2013 to September 2016 to investigate the diurnal expression of Arabidopsis gene homologs during daylength-regulated bulb formation in onion (*Allium cepa* L.). The plant physiological experiments including growing of onion plants have been performed at the Phytobiology Facility and all laboratory analyses have been done at the School of Life Sciences Plant Lab of the University of Warwick.

72 *2.1. Plant materials*

73 The long-day onion (*Allium cepa* L.) variety *'Renate F1'* (also called *Renate*) (Elsoms Seeds
74 Ltd., Spalding, UK) and the short-day onion variety '*Hojem*' were used for these experiments.
75 Seeds of *Hojem* were collected from the Vegetable Genetic Improvement Network (VeGIN,
76 UK) project Diversity Set.

77

78 *2.2. Diurnal time-course experiment to study gene expression in long-day cv. Renate*

79 For the LD diurnal time-course, onion plants were grown under natural conditions in the
80 Phytobiology Facility during the period from 26th July to 16th September 2013 when daylight
81 ranged from 15 h 42 min initially to 12 h 35 min at the end of the experiment. Supplementary
82 illumination with HPS lamps was provided to maintain a minimum 16 h daylength. Initially,
83 *Renate* seeds were sown in modular trays and after 4 weeks plants were potted up into 9 cm
84 pots containing Levington M2 compost (Gro-Well, Cherry Tree Cottage Farm, 210 Peasehill
85 Road, Ripley, Derbyshire, DE5 3JQ, UK). At 52 d from sowing, at the time of expected bulb
86 initiation, all plants were transferred to two SANYO 2279 controlled environment cabinets
87 (SANYO Electric Co., Ltd., Biomedical Division, Gunma Factory, Japan) for 10 d providing
88 constant LD (16 h photoperiod including 8 h fluorescent followed by 8 h incandescent light).
89 Both SANYO cabinets were set at a constant 22°C day/night with 60% relative humidity and
90 ambient CO_2 concentration (405 ± 0.1 ppm), and provided with a Photosynthetic Photon Flux
91 Density (PPFD) of 460 umol.m-2.s-1. Timing of ZT 0 (lights on) was offset by 8 h in the two
92 cabinets and harvesting of leaf materials was scheduled to provide continuous samples at 2-h
93 intervals over two consecutive 24-h cycles from ZT 0. Three plants were harvested each time
94 point and pooled together. Plants were selected for harvesting using a random number
generator (Haahr, 2006). Sampling involved removing the middle part of the first newly

95 expanded leaf, chopped into small pieces and freezing in liquid nitrogen before storing at -
96 80°C. The harvested materials were used for molecular analysis.
97 For the SD diurnal time-course, onion plants were grown in natural conditions in the
98 Phytobiology Facility during the period from 14th August to 13th October 2013 when daylight
99 ranged from 14 h 40 min to 10 h 50 min. Supplementary illumination with HPS lamps was
100 provided to maintain a minimum 16 h daylength. Plants were grown as for the LD experiment
101 and, at 61 d from sowing, when bulbing had been initiated, were transferred to two SANYO
102 2279 controlled environment cabinets (SANYO Electric Co., Ltd., Biomedical Division,
103 Gunma Factory, Japan) for 10 d providing constant SD (8 h photoperiod with fluorescent
104 light). Other environmental conditions were the same as for the LD diurnal time-course
105 experiment (Figure 1). Sampling, harvesting and storing were carried out as described for the
106 LD diurnal time-course.
107
108 *2.3. Diurnal time-course experiment to study gene expression in short-day cv. Hojem*
109 For the LD diurnal time-course, onion plants were grown in a photoperiod controlled
110 glasshouse compartment of Phytobiology Facility at 12 h daylight during the period from 17th
111 March to 27th May 2014. Initially, ***Hojem*** seeds were sown in modular trays and after 4 weeks
112 plants were potted up into 9 cm pots containing Levington M2 compost (Gro-Well, Cherry
113 Tree Cottage Farm, 210 Peasehill Road, Ripley, Derbyshire, DE5 3JQ, UK). At 71 d from
114 sowing, all plants were transferred to two SANYO 2279 controlled environment cabinets
115 (SANYO Electric Co., Ltd., Biomedical Division, Gunma Factory, Japan) for 10 d providing
116 the same environmental conditions as described for LD diurnal time-course in ***cv. Renate***.
117 Sampling, harvesting and storing were also carried out as described in the previous section.
The harvested materials were used for molecular analysis.

For the SD diurnal time-course, onion plants were grown in a photoperiod controlled glasshouse compartment of Phytobiology Facility at 12 h daylight during the period from 16th May to 23rd July 2014. At 68 d from sowing, all plants were transferred to two SANYO 2279 controlled environment cabinets (SANYO Electric Co., Ltd., Biomedical Division, Gunma Factory, Japan) for 10 d providing same environmental conditions as described for SD diurnal time-course in *cv. Renate*. Sampling, harvesting and storing were also carried out as described for LD. The harvested materials were used for molecular analysis.

2.4. RNA Sequencing

RNA-Seq analysis was performed to generate an onion transcriptome reference sequence and for more widespread identification of genes differentially expressed in response to photoperiod. Leaf and bulb material was harvested from ***Renate*** grown in long or short day and used to prepare libraries for Illumina sequencing in the Life Sciences genome centre. Leaf and bulb samples were then multiplexed to obtain differentiation between LD and SD samples and for biological replication (Supplementary Table S1). Two multiplex combinations were run: Multiplex 5 = Leaf (SD groups 3 & 4 and LD groups 5 & 6) and Multiplex 6 = Bulb (SD groups 3 & 4 and LD groups 5 & 6). All sequences obtained from RNA seq analysis were used for onion gene assembly with the assistance of the Life Sciences Bioinformatics support officer Mr. Siva Samavedam using Galaxy Biotinformatics Platform (http://galaxyproject.org/).

2.5 Gene identification and isolation

Arabidopsis sequences were obtained from National Center for Biotechnology Information (NCBI) database (NCBI., 2016 and blasted against the onion EST database (www.ncbi.nlm.nih.gov/nucest/?term=onion). The resulting EST sequences were aligned with

142 Arabidopsis sequences using MegAlign™. Onion ESTs and transcriptome sequences obtained
143 from RNA seq analysis were used to design primers (Forward and Reverse) for each gene
144 amplification using Primer3Plus (http://www.bioinformatics.nl/cgi-
145 bin/primer3plus/primer3plus.cgi) and synthesised by Invitrogen Ltd and Sigma-Aldrich®
146 (UK). Primers used for obtaining the full-length of key genes in onion are presented in
147 Supplementary Table S2 and Supplementary Table S3. cDNA was also synthesised from
148 DNase treated RNA and used for RT-PCR. Small amounts of cDNA from 4 individual samples
149 (LD leaf, LD bulb, SD leaf & SD bulb) were pooled together for preliminary isolation of the
150 genes. Primers used for reference genes were designed from the full-length sequences obtained
151 from onion transcriptome sequences (Supplementary Table S4). qRT-PCR primers for other
152 key genes (Supplementary Table S5) were designed from the full-length cDNA obtained from
153 gene isolation together with the EST sequences.
154
155 *2.6. RNA extraction, DNase treatment and cDNA synthesis*
156 Total RNA was extracted from leaf and bulb material from onion grown under LD and SD
157 using the Z6 buffer method (Rashid et al., 2016), following the manufacturer's (Roche
158 manufacturing Ltd., Republic of Ireland) guidelines. Samples were ground using pestle and
159 mortar and then approximately 100 mg of frozen plant tissue was homogenised using a
160 Dremel drill. In this step, Z6 buffer reagent and b-Mercaptoethanol were added which act to
161 remove RNase. Two extra reagents, 3M Sodium acetate (NaOAC) and 7.5M Lithium
162 chloride, which remove carbohydrates and polysaccharides, respectively, were included in
163 this method to obtain high quality RNA. After isolation, the quality and quantity of total RNA
164 was measured with the Thermo Scientific NanoDropTM 1000 Spectrophotometer (NanoDrop
Technologies, Inc., USA).

PCR products were purified following PCR and agarose gel electrophoresis using QIAquick PCR Purification Kit (QIAGEN) and QIAquick Gel Extraction Kit (QIAGEN), respectively, following the manufacturer's guidelines and samples were eluted in 30-50 µl of SDW. For gel purification, bands were cut out under UV light with a wavelength of 302 nm (Bio-Rad UV Transilluminator 2000) using a scalpel blade. A volume of 1 µl purified DNA was quantified using a NanoDrop™ ND-1000 spectrophotometer (Thermo Scientific).

A total amount of 10 µl (Premix 5 µl template of 20-80 ng/µl conc. + 5 µl Primer of 5 pmol/µl conc.) purified PCR products were sent to GATC Biotech for sequencing. Sequence files were viewed and edited using the EditSeq package of DNAStar Lasergene (DNAStar Inc.). Chromatograms where analysed and interpreted using 4Peaks Chromatogram and edited using SeqMan™, SeqBuilder™ and MegAlign™ of DNAStar Lasergene (DNAStar Inc.).

The TURBO DNA-*free treatment* kit (Ambion, USA) was used to eliminate the genomic DNA contamination following the manufacturer's guidelines. A PCR was set up to check for genomic DNA contamination using primers for *ALLINASE* (*ALL*) gene and visualized on RNA gel electrophoresis. Sequencing of PCR products from genomic DNA confirmed that the primers contained no mismatches.

cDNA was synthesised using 2 µg total RNA using ThermoScript™ Reverse transcription polymerase chain reaction (RT-PCR) System (Invitrogen by Life Technologies, Cat. No. 11146-016) for RT-PCR using oligo(dT) following the manufacturer's guidelines and subsequently treated with RNase H.

2.7. Analysis of gene expression using qRT-PCR

The extraction of total RNA and synthesis of cDNA was carried out following manufacturer's guidelines. The expression of reference genes and genes of interest was analysed by qRT-PCR using the CFX384 Touch™ Real-time PCR machine from BioRad (Bio-Rad

Laboratories Ltd., UK). The protocol and primer details are provided in Supplementary Tables S3, S4, S5 and S6. At the end of PCR run, the qRT-PCR data were normalised against expression levels of the house keeping genes such as *PP2AA3*, *PP2A1, TIP41* and *UBL* for each sample (Supplementary Table 4) were achieved by using Biogazelle qBase+ software (www.biogazelle.com). qbase+ software based on the geNorm (Vandesompele et al., 2002) and qBase technology (Hellemans et al., 2007). Forty-eight hour averages of expression were calculated and standard errors included. Standard curves (using 10-fold serial dilutions) were plotted using cDNA synthesised from approximately 2 µg of total RNA extracted from leaf material harvested at various time-points (0-48) in a 48-hour period as used for cDNA synthesis. The significance of the differences in gene expression between treatments were assessed by using two-way analysis of variance (ANOVA), which was carried out using statistical software package Prism 7.

3. Results

3.1. Transcriptome analysis and sequence comparison in Renate

An objective of the study was to identify and isolate a range of key genes hypothesised to be involved in bulbing in response to daylength. A combination of approaches was used, including identifying genes from EST databases, sequences from published work and through a transcriptome assembly. For the latter, RNA-seq analysis provided 12604 differential expressed transcripts in LD leaf vs bulb, 13665 in SD leaf vs bulb, 484 in SD leaf vs LD leaf and 964 in SD bulb vs LD bulb of onion. Differentially expressed sequences included both upregulated and downregulated genes (Figure 2). The data in Table 1 shows the summary of the genes of study in onion and their degree of homology to Arabidopsis gene sequences (NCBI, 2016) at the nucleotide and amino acid levels. It was found that all of the sequences used in this study had at least 46% identity with Arabidopsis homology with E-values <0.001.

have been presented in the result (Table 1). New sequences of onion homologs of genes that have known function in the daylength regulation of flowering e.g. *FT* and *CO* were obtained from the transcriptome assembly.

Prior to this study, only one CO-like gene (*AcCOL*) had been identified in onion (Taylor et al., 2010). Sequence analysis revealed that this gene contains both a B-Box and CCT domain, which are found in all *CO* and *CO*-like genes (Robson et al., 2001; Taylor et al., 2010). Three *COL* genes including two novel sequences (*AcCOL2* & *AcCOL3*) were identified in the transcriptome assembly. *AcCOL2* (Accession number KY012331) showed 52.5% nucleotide and 23.1% amino acid sequences similarity with Arabidopsis *CO* (Accession number X94937.1) (Table 1). *AcCOL3* showed 46.5% nucleotide and 30.9% amino acid sequences similarity with Arabidopsis *CO*. Both *AcCOL2* and *AcCOL3* contain B-Box and CCT domain regions, the conserved domains, which are present in all *CO* and *CO*-like genes.

Lee et al. (2013) published a paper in which the authors identified 6 FT-like genes (*AcFT1-6*). Five out 6 *FT* genes were identified in *Renate*, with the exception of *FT2*, which was, however, detected in *Hojem*. Sequencing of PCR products confirmed the identity of the *FT* genes. Further analysis revealed that *AcFT5* is identical to the previously identified *FT-LIKE PROTEIN 2* and *AcFT6* is identical to *FT-LIKE PROTEIN 1*. RNA-Seq analysis also supports those results.

3.2. Diurnal time-course expression of the genes in onion by qRT-PCR

3.2.1. Expression of clock genes

In *Renate*, *AcFKF1* showed a clear diurnal expression pattern peaking at around ZT8 in both LD and SD conditions (Figure 3a). This result is slightly different to Arabidopsis *AcFKF1*, which showed peaks at around ZT10 in LD and ZT7 in SD (Imaizumi et al., 2003). In *Hojem*,

AcFKF1 also showed clear diurnal expression pattern peaking at around ZT8 in both LD and SD conditions (Figure 3b).

AcGI showed a clear diurnal expression pattern peaking at around ZT8 in both LD and SD (Figure 3c), which is quite similar to the expression of Arabidopsis *AcGI*, where it peaks at ZT10 in LDs and ZT8 in SDs (Taylor et al., 2010). In *Hojem*, *AcGI* also showed a clear diurnal expression pattern peaking at around ZT8 in both LD and SD conditions (Figure 3d).

3.2.2. Expression of *COL* genes

For both *AcCOL1 and AcCOL3* there was no indication of a consistent diurnal pattern of expression in LD and SD in either *Renate* or *Hojem* (Figure 4a-b, 4e-f). In contrast, in both *Renate* and *Hojem*, *AcCOL2* showed a distinct diurnal expression pattern, peaking at around ZT10-12 in LD and later in SD (Figure 4c-d).

3.3. Expression of *FT* genes

All *FTs* showed different diurnal expression patterns peaking at different times of the day. In *Renate*, *AcFT1* showed a distict and repeatable diurnal pattern of expression in LD, being expressed in the later part of the day and during the dark period in both cycles (Figure 4a). In contrast, there was no expression of *AcFT1* in SD (Figure 5a). The pattern of expression of *AcFT1* in *Hojem* was similar to that of the expression in *Renate*, peaking in the later part of the day during the dark period at both cycles in LD but showing no detectable expression in SD (Figure 5b).

The expression of *AcFT2* was initially investigated in *Renate*, a long-day onion variety but it was not expressed in either LD or SD conditions. The expression of *AcFT2* was further investigated in *Hojem*, a short-day onion variety. Expression was detected in these plants with some indication of a diurnal pattern, at least in LD. It was expressed in the early part of the

day peaking at about ZT2-4 during the light period in both cycles in LD but otherwise showed no obvious pattern in SD (Figure 5c).

In *Renate*, *AcFT4* showed a clear diurnal expression pattern peaking at the end of the dark period and in the early part of the day in SD, but, in contrast, showed limited expression with no obvious trend in LD (Figure 5d). The high expression in the early part of the day only in SD is consistent with the proposal that *AcFT4* is inhibitory for bulbing. In *Hojem*, *AcFT4* was expressed under both LD and SD conditions. It showed a clear diurnal expression pattern, peaking at the end of the dark period and in the early part of the day in SD, as seen in *Renate*. The expression in LD was higher in *Hojem* than seen in *Renate* but there was a less obvious pattern in LD than in SD for *Hojem* although expression tended to be higher in the early part of the day compared to the later period (Figure 5e). However, *AcFT4* showed a consistent pattern of expression in both long-day (*Renate*) and short-day (*Hojem*) varieties of onion in SD conditions. Therefore, it was confirmed that *AcFT4* shows distinct circadian or diurnal regulation under SD conditions.

In *Renate*, *AcFT5* was expressed throughout the day in LD, although the expression patterns were variable between the first and second 24 h cycles, while, showed very limited expression in SD (Figure 5f). It was difficult to explain the variable expression patterns of *Renate AcFT5* in LD, as repeating the qPCR revealed the same results. In addition to that, the same samples were used as for the other genes, including *AcFKF1* and *AcGI*, which show consistent patterns of expression in both LD and SD conditions and between first and second cycles. Therefore, while no circadian pattern of expression could be confirmed for *AcFT5* expression did seem higher in LD than in SD in *Renate*. In *Hojem*, *AcFT5* showed a clear diurnal rhythm peaking at the middle part of the day and around ZT8 during light period in LD, while, showed no obvious diurnal expression in SD where various peaks were seen between the first and second 24 h cycles (Figure 5g).

In *Renate*, *AcFT6* showed a clear diurnal expression pattern peaking at the early part of the day and during the light period in both LD and SD conditions (Figure 5h). In *Hojem*, *AcFT6* showed a clear diurnal expression pattern peaking at around ZT8 during light period in both LD and SD (Figure 5i).

4. Discussion

In this work we studied the expression patterns of putative onion homologs of Arabidopsis genes involved in the photoperiod regulation of flowering. Homology is the existence of shared common ancestry between a pair of structures, or genes, in different taxa (Pearson, 2013) and common rule of thumb is that two sequences are homologous if they are more than 30% identical over their entire lengths. Sequences that share more than 40% identity are very likely to be considered as high homology or functional similarity as judged by Enzyme Commission (E.C.) numbers (Pearson, 2013). In addition to percent identity, E-value is also very useful which reflect the evolutionary distance of the two aligned sequences, the length of the sequences, and the scoring matrix used for the alignment. The similarity scores for two sequences are always be statistically significant when E-value is <0.001 (Pearson, 2013).

In Arabidopsis, the circadian clock regulates *FKF1* and *GI* genes, which can mediate *CO* stability for the precise control of flowering time (Song et al., 2014). While the expression of *AcFKF1* and *AcGI* genes are not expected to be directly correlated with bulb initiation they should show a diurnal rhythm of expression if part of the daylength sensing system. Under both long-day (*Renate*) and short-day (*Hojem*) varieties of onion *AcFKF1* showed a clear diurnal expression patterns in LD and SD, consistent with a role in daylength sensing. The diurnal expression patterns of *AcFKF1* and *AcGI* can also be considered as internal controls for assessing diurnal rhythmicity for the other genes assayed in the experiment.

The diurnal expression pattern of *AcFKF1* also showed no distinct difference between the timing of expression in LD and SD conditions in onion varieties under study. We were unable to repeat the small difference of timing of peaks reported by (Taylor et al., 2010). However, it is clearly evident that *AcFKF1* shows a diurnal rhythm of expression, similar to that of Arabidopsis *FKF1*, consistent with *AcFKF1* being homologous to Arabidopsis *FKF1* (Nelson et al., 2000; Somers et al., 2000; Taylor et al., 2010). Similarly, the data showed that *AcGI* has a clear diurnal expression pattern, characteristic of genes involved in the photoperiod response (Mizoguchi et al., 2005; Sawa et al., 2007; Jackson, 2009). In Arabidopsis, *AcFKF1* interacts with *AcGI* through the LOV domain to form a complex in a blue-light dependent manner in the late afternoon and regulates the expression of *CO* and induction of flowering specifically under LD conditions (Mizoguchi et al., 2005; Sawa et al., 2007). In the LD conditions, sufficient *FKF1-GI* complex is formed to activate *CO* transcription during the daytime, and which is stabilized by light at the end of the day.

In Arabidopsis, *CO* is a direct output from the clock and functions at the site of perception in leaf (Thomas et al., 2006). It plays a central role in the mechanism of photoperiod measurement, integrating clock and light signals to provide photoperiod-specific induction of the mobile floral integrator, *FT* and thus controls flowering in Arabidopsis (Andres and Coupland, 2012; Song et al., 2013; Thomas, 2006). *AcCOL2* showed a diurnal expression in both LD and SD in both *Renate* and *Hojem*, peaking towards the end of the LD and slightly later, into darkness, in SD (Suarez-Lopez et al., 2001). This is very similar to the expression pattern of *CO*, reponsible for daylength regulation of flowering in Arabidopsis. The expression and sequence data suggest that *AcCOL2* is a CO-like gene that is under circadian regulation and which has a diurnal expression pattern consistent with a role in daylength regulation of bulb initiation. Therefore, it could be confirmed that *AcCOL2* is diurnally regulated and would be a good candidate for being a homolog of Arabidopsis *CO*.

In contrast, *AcCOL1 and AcCOL3* showed no consistent diurnal expression in *Renate* or *Hojem* and the expression pattern of this gene is not similar to the expression pattern of Arabidopsis *CO*. This result is also consistent with the earlier study conducted in *Renate*, where the authors did not find a diurnal expression pattern for *AcCOL1* (Taylor et al., 2010). *AcCOL1* and *AcCOL3* may be CO-like genes but the expression patterns suggest that they do not have a role in the photoperiodic control of bulb formation. The literature reports the presence of CO-like genes in SD plants such as rice and *Pharbitis nil*, which suggests a conserved pathway that regulates flowering during an inductive daylength (Shrestha et al., 2014). Also overexpression of Arabidopsis *CO* in potato, impairs tuberisation in SD inductive conditions, indicating a wider role for *CO* in daylength regulation than just the control of flowering (Martínez-García et al., 2002). However, in both these instances, the CO-like genes show diurnal patterns of expression.

The previous study of Lee et al. (2013) proposed that *AcFT1* promoted bulb formation in onion and the data here are consistent with *AcFT1* being responsible for the correlation of bulbing under LD conditions. The diurnal expression pattern of *AcFT1* suggests that this gene could be a homolog of Arabidopsis *FT*, and might be positively regulated by *AcCOL2* and have an important role in the daylength regulation of bulb formation in onion (Lee et al., 2013). *AcFT2* was not expressed in *Renate* but was expressed in *Hojem*. Lee et al. (2013) reported that the flowering is promoted by vernalization and correlates with the upregulation of *AcFT2* and the expression of this gene was either not detected or at very low levels in seedlings and older plants before or after bulb formation. The precise timing of the peaks was not distinct, or consistent in the first cycle with that of the second cycle confirming that this gene is not fully under circadian or diurnal regulation under these non-flowering conditions.

In *Renate* and *Hojem*, *AcFT4* showed a clear diurnal expression peaking in the early part of the day in SD, but was expressed at a lower level in LD, particularly in *Renate*. Lee et al.

(2013) proposed that *FT4* inhibited bulb formation. The higher expression in SD is consistent with that proposal. The expression of *FT1*, which might induce bulbing and *FT4*, which might inhibit bulb formation show evidence of negative correlation. For example, *AcFT1* is expressed in the later part of the day in LD but shows very limited, or no, expression in SD, whereas *AcFT4* is expressed at the end of the dark period and in the early part of the day in SD but has more limited expression in LD. It is therefore possible that *AcFT1* may be negatively regulating *AcFT4* or vice versa. The timing of the expression also suggests that *AcFT4* could be contributing to the juvenile phase by inhibiting bulb formation at early stages of growth. For the other two *FT* genes, *FT5* and *FT6*, there was no obvious pattern that could be easily linked to the bulbing response to daylength. In *Renate*, *AcFT5* was expressed throughout the day in LD but showed no or very limited expression in SD. Lee et al (2013) reported in their supplementary information that *AcFT5* expression appears higher in LD than SD but there was no obvious effect of daylength on AcFT6. In *Hojem*, *AcFT5* showed a clear diurnal expression pattern in LD but no obvious trend in SD. *AcFT6* showed distinct expression pattern in the early (*Renate*) to middle (*Hojem*) part of the day in both LD and SD, which suggesting that *AcFT6* might be circadian or diurnally regulated. However, further work is required to understand the roles of these genes.

In summary, onion homologs of *CO*, *FT*, *GI* and *FKF1* genes showed diurnal patterns of expression in both long-day and short-day onions. The findings support their involvement in the daylength regulation of bulbing through a mechanism similar to that found in Arabidopsis flowering. Two new CO-like genes were identified from an RNA-seq library. One of these, *AcCOL2*, showed an expression pattern very similar to *CO* from Arabidopsis and is consistent with a role in daylength regulation. The patterns of mRNA expression presented in this paper support the proposal that *AcFT1* promotes bulbing in LD while *AcFT4* inhibits bulbing in SD

Lee et al. (2013). In addition, this paper shows that these genes are expressed at different times of the day, with *AcFT1* expressed in the evening and *ACFT4* in the morning.

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Conflicts of interest

Dr. Md. Harun Ar Rashid and Professor Brian Thomas designed the experiments and wrote the paper. Dr. Md. Harun Ar Rashid conducted the experiments. The authors declare that there is no conflict of interests regarding the publication of this paper.

Declaration

The authors declare that the manuscript report is unpublished work and it is not under active consideration for publication elsewhere, nor been accepted for publication, nor been published in full or in part.

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Table

Table 1. Summary of the genes of study in onion and their degree of homology to Arabidopsis gene sequences (NCBI, 2016) at the nucleotide and amino acid levels. The homology was compared over the entire region of the genes. The similarities of the sequences are statistically significant when E-value is <0.001.

Gene name	GeneBank ID for Arabidopsis	Degree of homology to Arabidopsis (%)		E-value
		Nucleotide level	Amino acid level	
AcFKF1	NM_105475.3	66.1	66.7	<0.001
AcGI	NM_102124.3	67	60.9	<0.001
AcCOL1	X94937.1	47.9	41.6	<0.001
AcCOL2	X94937.1	52.5	23.1	<0.001
AcCOL3	X94937.1	46.5	30.9	<0.001
FT-LIKE PROTEIN 1	AB027504.1	60.2	84.9	<0.001
FT-LIKE PROTEIN 2	AB027504.1	61.1	65.7	<0.001
AcFT1	AB027504.1	90.1	72.4	<0.001
AcFT2	AB027504.1	64.7	49.1	<0.001
AcFT3	AB027504.1	69.7	67.4	<0.001
AcFT4	AB027504.1	65.3	58.5	<0.001
AcFT5	AB027504.1	69.7	67.4	<0.001
AcFT6	AB027504.1	56.5	55.2	<0.001

Legends: *AcFKF1: Allium cepa FLAVIN-BINDING KELCH REPEAT PROTEIN; F-BOX 1 PROTEIN, AcGI: Allium cepa GIGANTEA, AcCOL: Allium cepa CONSTANS LIKE 1FT-LIKE PROTEIN: FLOWERING LOCUS T-LIKE PROTEIN, AcFT: Allium cepa FLOWERING LOCUS T.*

Figures

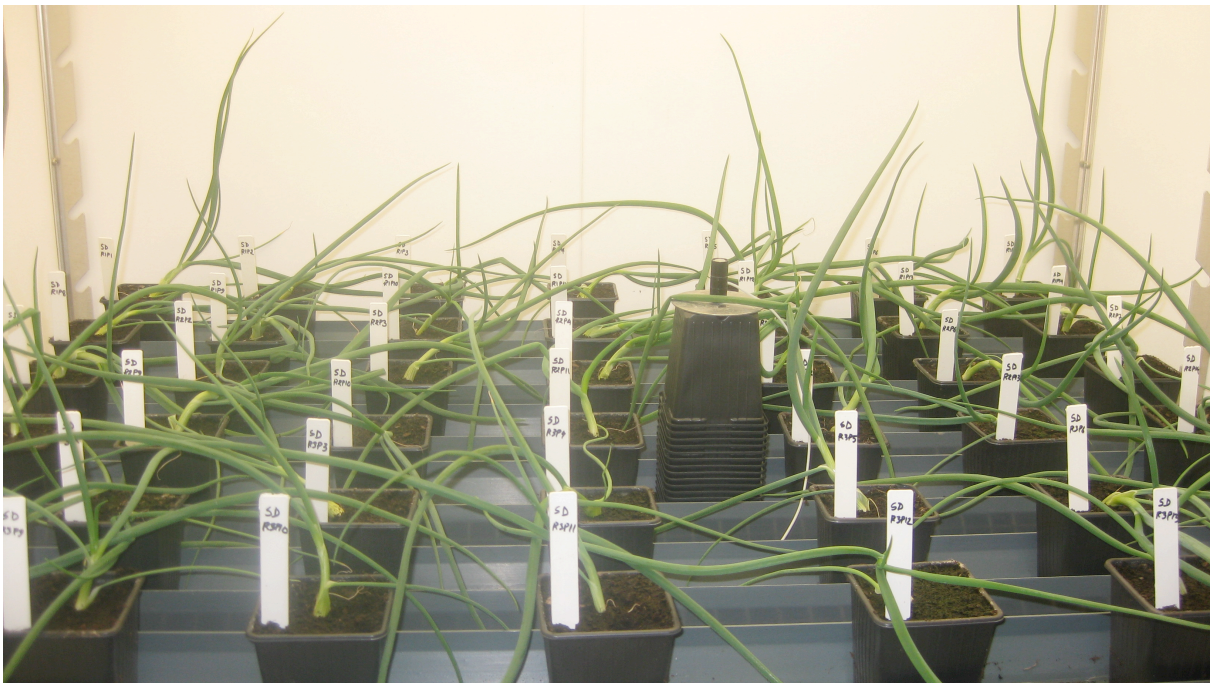


Figure 1. Growth of *Renate F1* plants under SD conditions (8 h light) in the Controlled Environment SANYO Cabinet to generate material for molecular analyses in SD diurnal experiment. A similar design was employed for plants grown in LD and other diurnal experiments. White coloured labels represent different replications in completely randomised design (CRD).

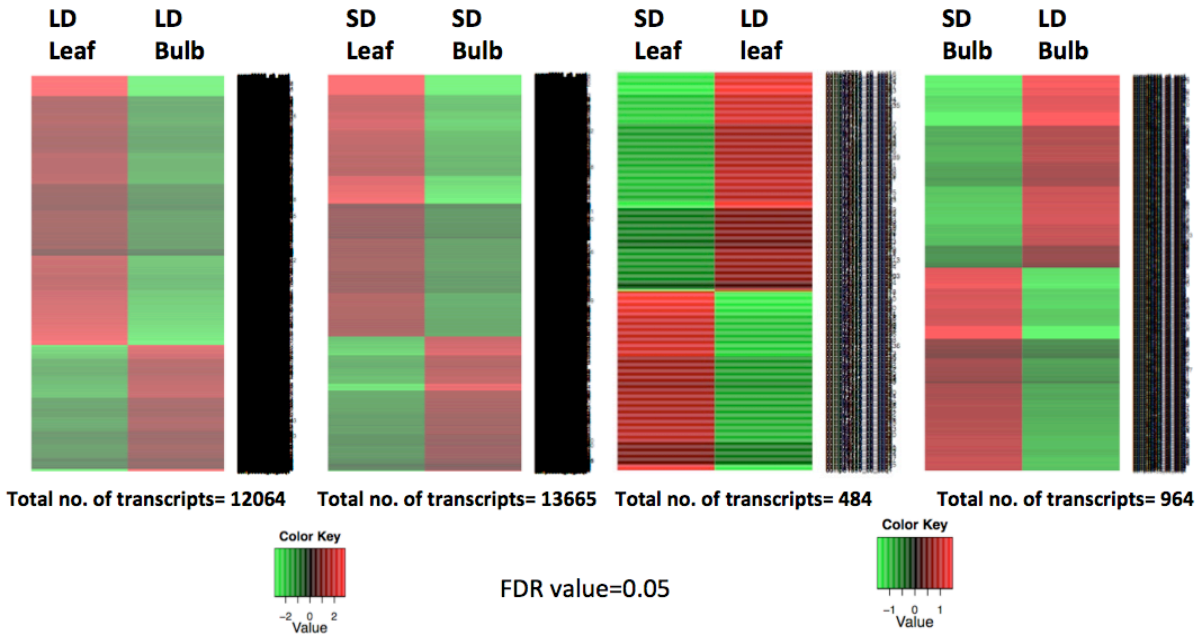


Figure 2. Heat Map showing differential expressed transcripts in *Renate F1* grown under different daylengths.

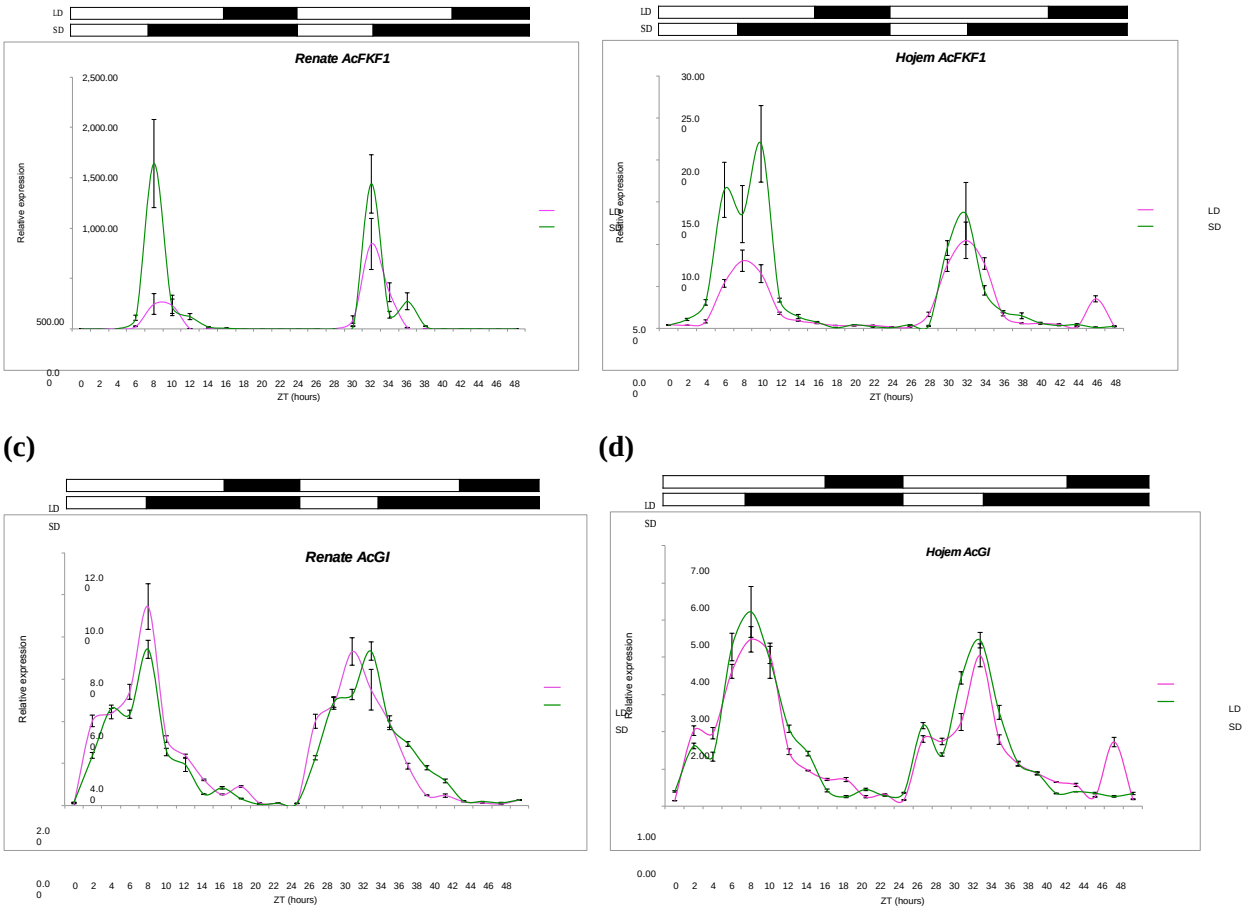


Figure 3. Expression of *AcFKF1* and *AcGI* genes in lon-day (*cv. Renate F1*) and short-day (*cv. Hojem*) varieties of onion over a 48-hour period using qRT-PCR. White and black bars denote light/dark cycles. Error bars represent the SEM. (a) *AcFKF1* in *Renate F1*. (b) *AcFKF1* in *Hojem*. (c) *AcGI* in *Renate F1*. (d) *AcGI* in *Hojem*.

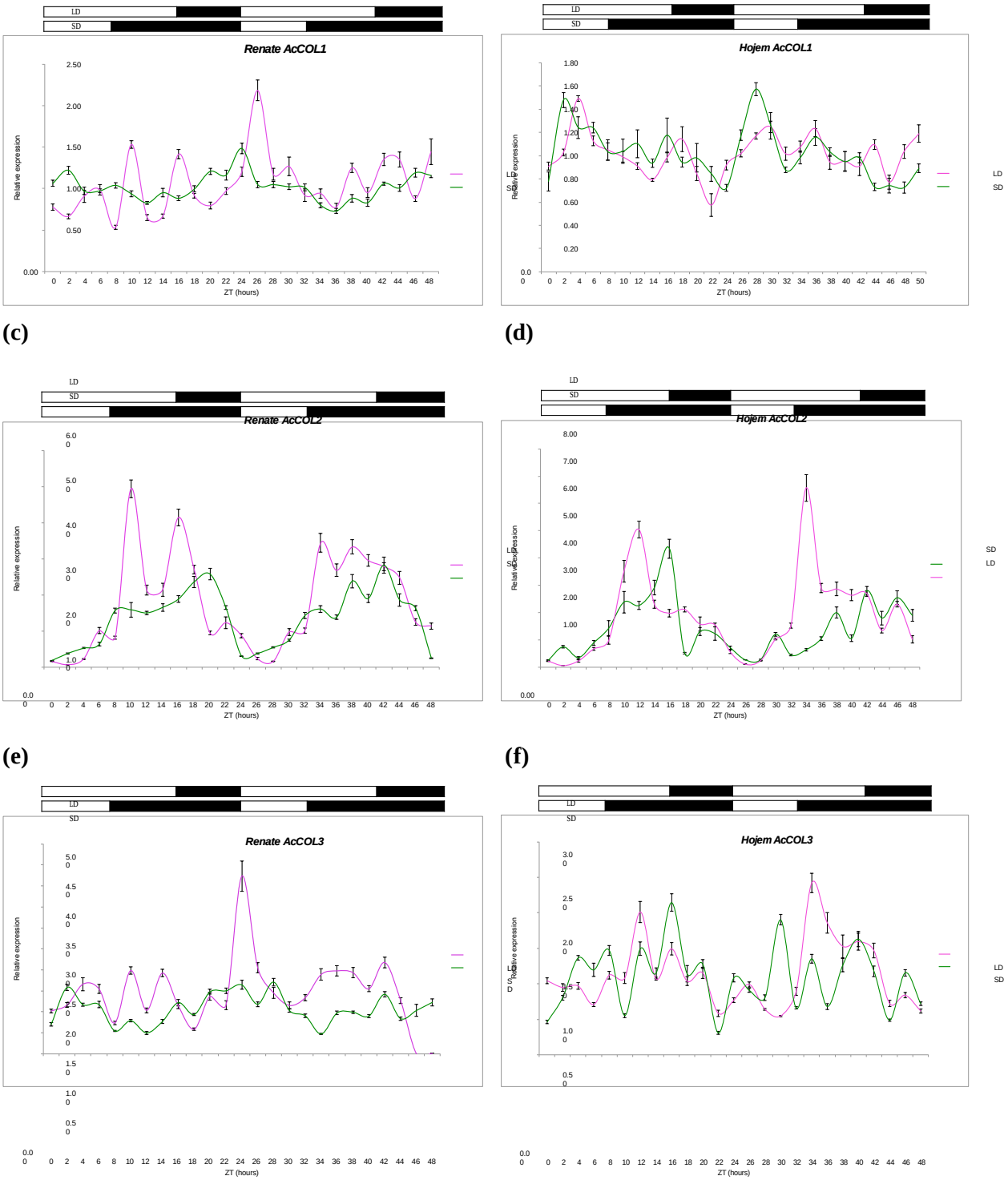
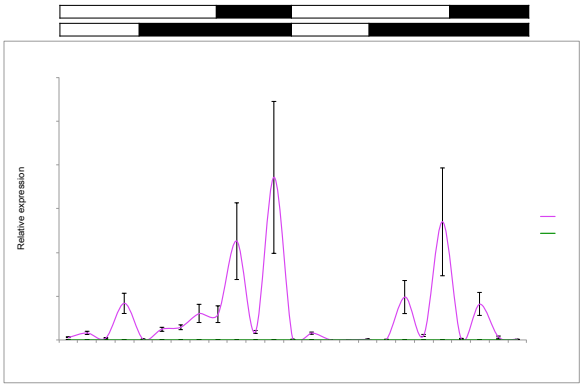
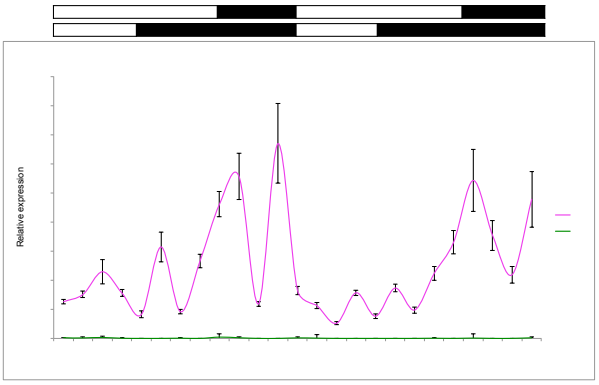
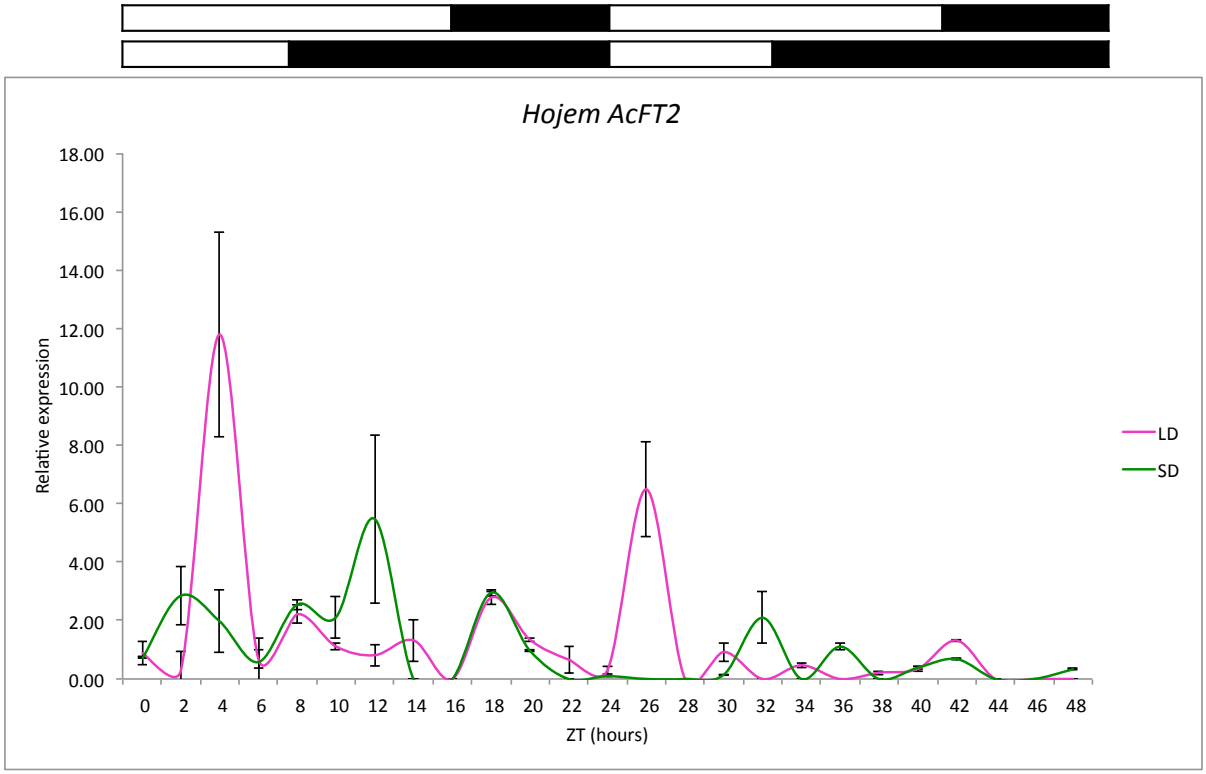
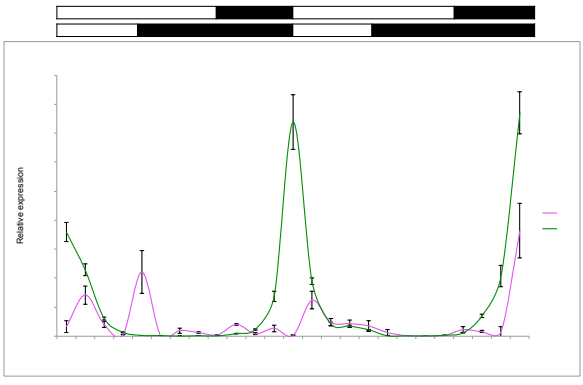


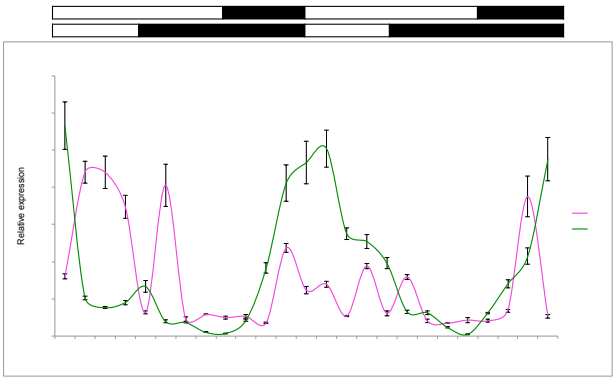
Figure 4. Expression of *CONSTANS LIKE (COL)* genes in long-day (*cv. Renate F1*) and short-day (*cv. Hojem*) varieties of onion over a 48-hour period using qRT-PCR. White and black bars denote light/ dark cycles. Error bars represent the SEM. (a) *AcCOL1* in *Renate F1*. (b) *AcCOL1* in *Hojem*. (c) *AcCOL2* in *Renate F1*. (d) *AcCOL2* in Hojem. (e) *AcCOL3* in *Renate F1*. (f) *AcCOL3* in *Hojem*.


Relative expression

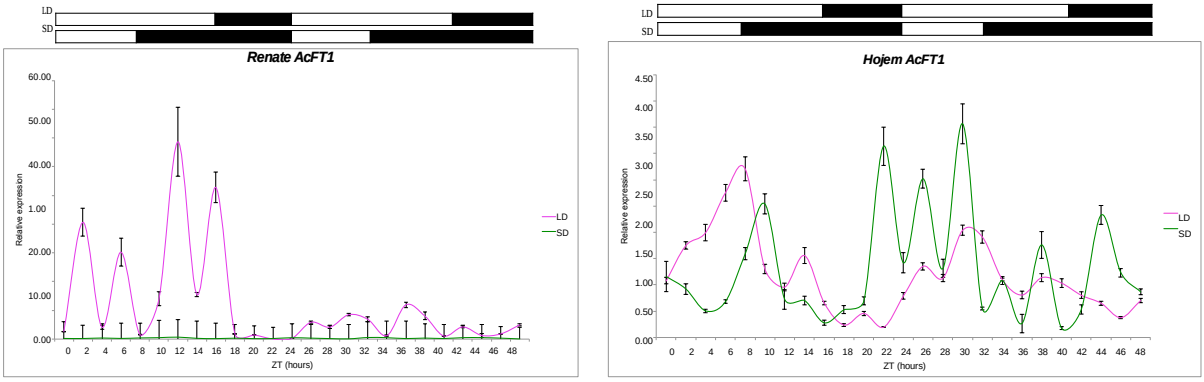

Relative expression


Hojem AcFT2
Relative expression
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48
ZT (hours)
LD
SD

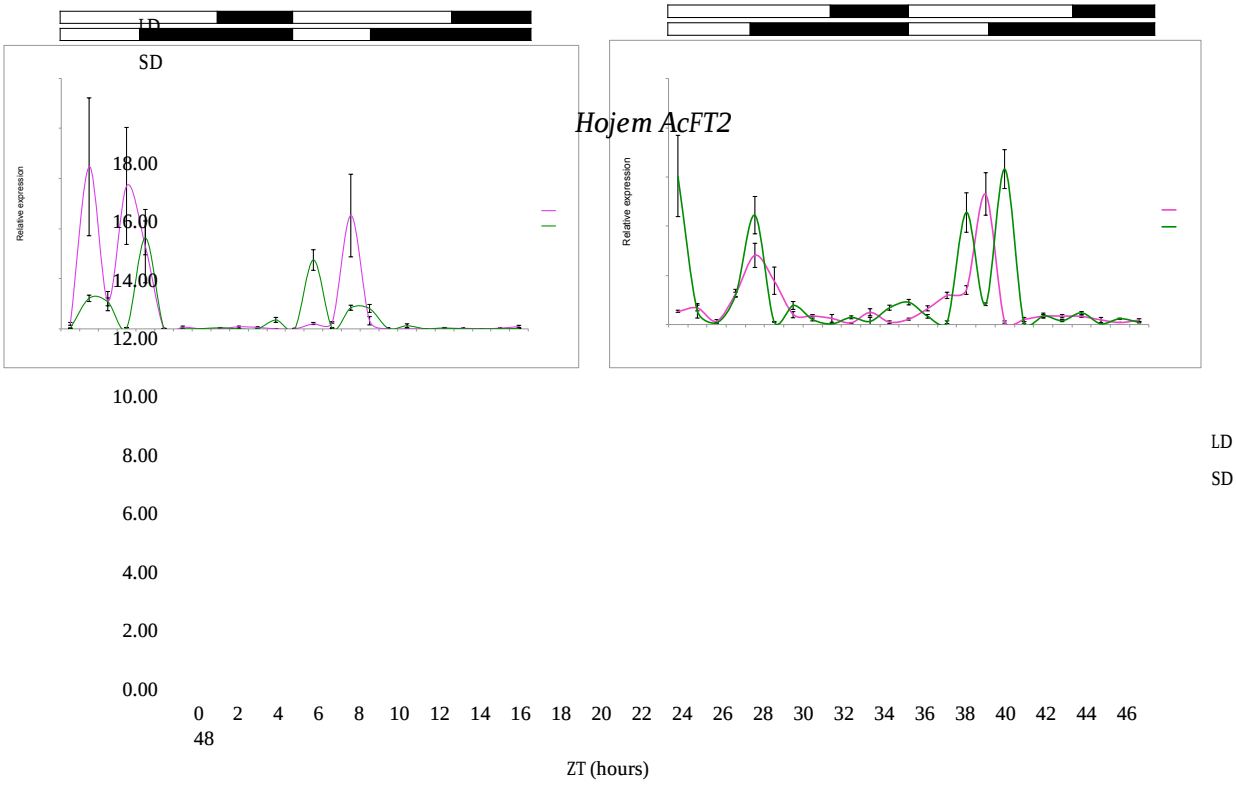

Relative expression


Relative expression

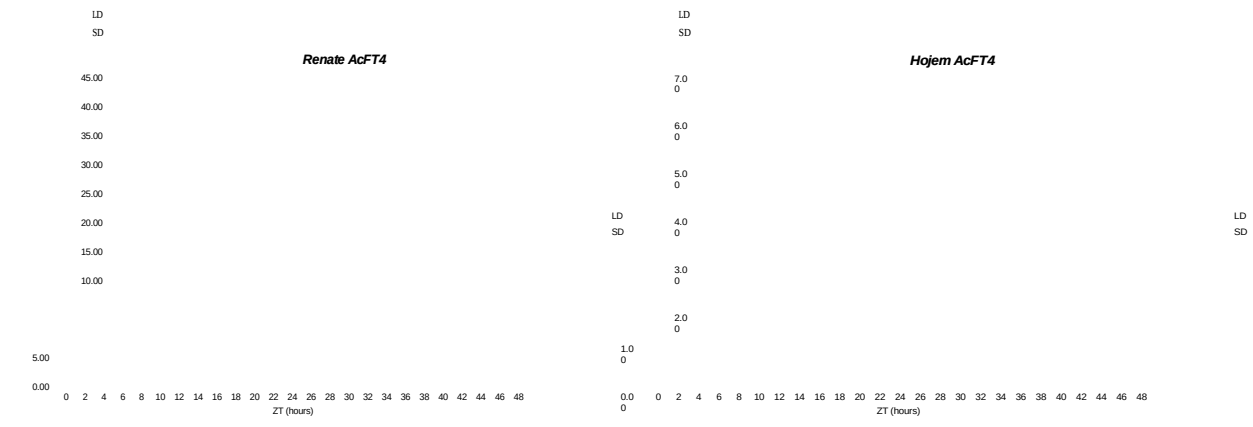
578 **(a)** **(b)**



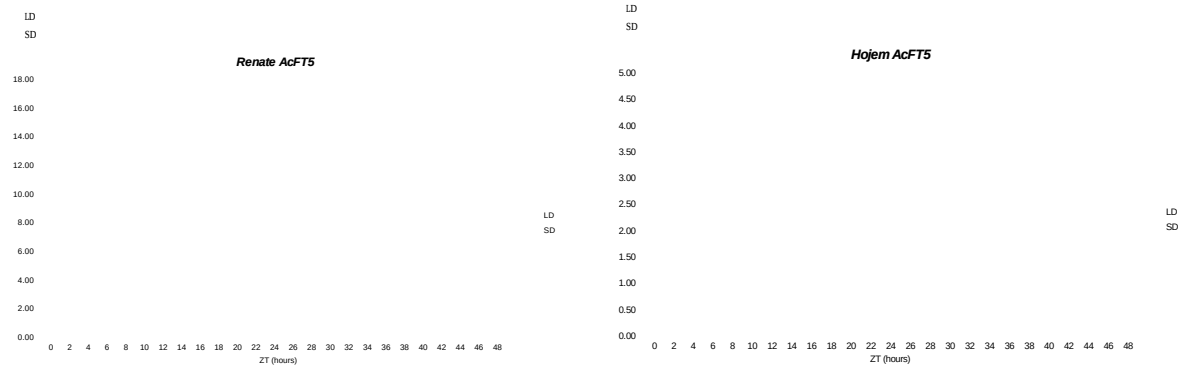
579 **(c)**



580 **(d)** **(e)**



(f) **(g)**



(h) **(i)**

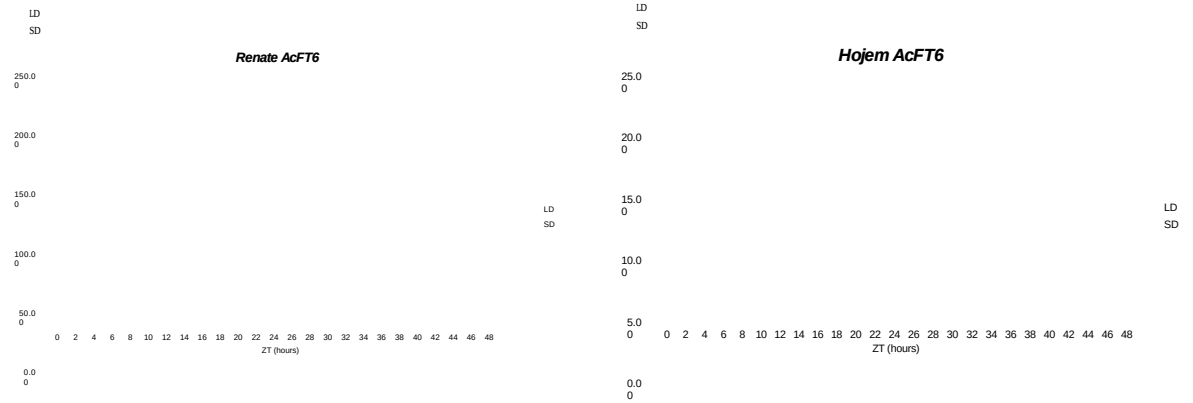


Figure 5. Expression of *AcFT1* and *AcFT4* genes in long-day (*cv. Renate F1*) and short-day (*cv. Hojem*) varieties of onion over a 48-hour period using qRT-PCR. White and black bars denote light/ dark cycles. Error bars represent the SEM. (a) *AcFT1* in *Renate F1*. (b) *AcFT1* in *Hojem*. (c) *AcFT2* in *Hojem*. (d) *AcFT4* in *Renate F1* (e) *AcFT4* in *Renate F1*. (f) *AcFT5* in *Renate F1*. (g) *AcFT5* in *Hojem*. (h) *AcFT6* in *Renate F1*. (i) *AcFT6* in *Hojem*.