Changes in the expression of photoperiodic bulbing genes in response to increasing daylength in Long-day and Short-day onion varieties.

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ABSTRACT

Onion bulb initiation is photoperiod-dependent. Understanding this is crucial for adapting new varieties for growth at different latitudes as well as aiding germplasm screening for choice of current varieties. This study aims to gain further understanding of the molecular mechanisms involved in onion bulbing process based on the parallels with well characterised functional clock genes in the Arabidopsis flowering pathway. A comprehensive set of diurnal quantitative expression experiments was carried out to investigate the bulbing response in two different onion varieties, namely Renate, a long-day variety and Hojem, a short-day variety under increasing intermediate day-lengths. All onion homologous to Arabidopsis flowering time genes showed clear diurnal expression patterns peaking at different times of the day for both long/short-day onions, indicating their role in daylength dependent bulbing process at molecular level. Under intermediate daylengths, *AcFT1* expression level increased with daylengths in both varieties, while *AcFT4* was expressed in all daylengths. The two genes showed complementary expression with *AcFT4* peaking in the morning and *AcFT1* in the evening in longer days. The results indicate that *AcFT1* and AcFT4 are negatively co-regulated, but AcFT1 is the predominant regulator of bulb formation in response to daylength.

Keywords. Onion, bulbing, daylength, *FT* genes, *Alliums*, photoperiod.

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INTRODUCTION

The onion (*Allium cepa* L.) is a monocotyledonous flowering plant belonging to genus *Allium* (USDA Plant Database, 2019). The genus comprises over 700 species including garlic (*A. sativum* and *A. scordoprasum*), shallot (*A. ascalonicum*), leek (*A. porrum*), or chive (*A. schoenoprasum*). Onions belong to the section *cepa* (Brewster, 2008). Commercial common onion varieties are cultivated under a worldwide geographic range from temperate to tropical regions, with the production being a major source of income for rural families selling their produce in local, regional and international markets. According to the statistics from FAO (2019), present world production of dry onion is about 93.2 million tons of bulbs per annum, ranking the second important vegetable only exceeded by tomatoes in the light of global weight produced.

Onion is a biennial crop which only flowers after satisfying the vernalisation requirement. In the first-year spring after seed germinates, onion sends up leaves alternatively from a small flattened stem (base plate) from out to in, and photosynthesize to produce energy as the plant grows. Each leaf is composed of a photosynthetic leaf blade and a non-photosynthetic storage leaf base. During growth, base of leaves begins to swell, leaf scales thicken and form the character of bulb as central storage tissue. Bulb continues developing to its full expansion and being harvested for food in autumn (Lancaster et al., 1996).

The bulb initiation process in onion is a photoperiodic event along with other plant developmental changes such as flowering, tuberization, bud set, and many other responses that are triggered by the duration of illumination or pattern of light/dark cycles (Magruder and Allard, 1937; Heath, 1945; Kato, 1964; Lancaster et al., 1996). The critical daylength (CDL) is the point at which the photoperiod switches from being noninductive to inductive, which means initiation of a bulb can only be triggered once the CDL is reached or exceeded for the onion plant. Onion varieties are divided into 'short day' (SD, 10-12h/day), 'long day' (LD, >14h/day) and 'intermediate' (ID, 12-14h/day) by growers based on their CDL requirements.

At the molecular and genetic level, the photoperiodism of onion bulbing process has been compared with well characterized *Arabidopsis thaliana* flowering process. Arabidopsis is a facultative long-day plant that flowers only after being exposed to light periods longer than a CDL. In this model, leaf-to-apex communication initiates flowering in response to photoperiod (Navarro et al., 2011; Abelenda et al., 2014). The isolation of Arabidopsis mutants that had a compromised flowering response led to the

identification of the *GIGANTEA* (*GI*), *FLAVIN KELCH F BOX 1* (*FKF1*), *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) genes, which all have a role in the photoperiod pathway. Plants are able to measure time by means of a circadian clock, an endogenous timekeeping mechanism controlled by various feedback loops (Jackson, 2009). In the leaf, light is perceived by different photoreceptors (cryptochromes in blue light and phytochromes in red/far-red light), which transmit light signals into the circadian clock. The clock drives the rhythmic expression of some of the key elements (*FKF1* and *GI*) (Fowler et al. 1999, Park et al., 1999, Nelson et al. 2000) and forms a *FKF1-GI* complex (Sawa et al., 2007). The complex regulates flower promoter *CO* (zinc-finger protein) transcription (Putterill et al., 1995) through the degradation of *CYCLING DOF FACTOR 1* (*CDF1*), a repressor of *CO* (Imaizumi et al. 2005, Sawa et al. 2007). Under long-days, the activated *CO* transcription will trigger the expression of floral integrating genes *FT (Flowering Locus T), SOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*) and *TSF* (*TWIN SISTER OF FT*). The RAF kinase inhibitor–like *FT* protein is afterwards translocated to the apical meristem and activates other genes which trigger the expression of *LEAFY* (*LFY),* finally leading to floral initiation in Arabidopsis (Massiah, 2007; Nakamichi, 2011; Golembeski and Imaizumi, 2015). However, if the plant is grown in the dark, *COP1* (*CONSTITUTIVE PHOTOMORPHOGENIC1*) and *SPA1* (*SUPPRESSOR OF PHYA-105*) proteins promote the degradation of the *CO* protein (Laubinger et al., 2006; Jang et al., 2008), leading to a delay or failure in flowering under short daylengths.

Although the mechanism underlying daylength-dependent bulbing in onion is not yet elucidated, there is good reason to use Arabidopsis flowering as a model. In both species, perception of daylength is in the leaves and the site of response is the apical meristem, which for Arabidopsis is in the shoot apex, whereas the meristem in onion is basal, which is where the bulb forms (Summerfield, 1991). Both processes therefore require a mobile signal to pass from the leaf to the apex. In Arabidopsis, this is FT, which is expressed under the control of CO in light at the latter part of the day. Studies have identified genes from Arabidopsis flowering pathway that are conserved in onion, including *AcFKF1* and *AcGI* (Taylor et al., 2010), six members of *AcFT* family (Lee et al., 2013), and three *CO-like* (*AcCOL*) genes (Rashid and Thomas, 2020). *AcGI* and *AcFKF1* were confirmed with the identity of their Arabidopsis homologs, with diurnal expression patterns similar to their Arabidopsis counterparts (Taylor et al., 2010); A new onion CO gene, *AcCOL2* was shown to be the only member of the AcCOL family that showed a diurnal pattern of expression similar to Arabidopsis *CO* (Rashid and Thomas, 2020). Two *AcFTs, AcFT1* and *AcFT4,* are bulbing regulators, acting as promoter and inhibitor respectively, and present different diurnal expression patterns peaking at different time of the day (Lee et al., 2013). Further studies also focused on the different daylength response in long-day and short-day onion

varieties. (Rashid and Thomas, 2020) compared diurnal expression in short-day onion type *Hojem* and long-day onion type *Renate* under 8h and 16h of daylengths and found that *AcFT1* was expressed in bulbing tissues at 16 h whereas *AcFT4* was more highly expressed in 8 h non – bulbing tissues. They also have shown that AcFT 1 and AcFT4 are expressed in the leaf but not in the bulbing tissues (Rashid et al. 2019) Although not conclusive, these studies indicate that a CO/FT mechanism based on daylength regulation of flowering in the model plant Arabidopsis, may be responsible for daylength regulation of onion bulbing with the added complexity of FT genes with opposite effects.

Onions are characterised by diversity in their CDL, depending on their geographical distribution. This raises the question of whether the CDL, as defined by the response of onion to increasing daylength, is determined by an increase in the promoter (AcFT1) or decrease in the inhibitor (AcFT4). The Rashid and Thomas (2020) paper compared expression in 16 h and 8 h treatments. However, these treatments would give the same bulbing responses in both varieties (bulbing under 16h and non-bulbing under 8h). To address the question, the experiments in this study were carried out over a range of intermediate daylengths (10-14h) where differential bulbing responses between the varieties are observed (*Renate* bulbing at ≥14h, *Hojem* bulbing at >10-12h). By looking at the differences between diurnal expression profiles of *AcFT1* and *AcFT4* in increasing daylengths, the relative contributions of promoter and inhibitor can be compared.

MATERIAL AND METHODS

The investigation was carried out at the School of Life Sciences and Phytobiology Facility (PBF), the University of Warwick, UK, during the period from May 2016 to November 2017.

Plant material

Two onion (*Allium cepa* L.) varieties with different daylength responses were used: a long-day onion variety "*Renate*", seeds obtained from the seed company (Elsoms Seeds Ltd., Spalding, UK); and a short-day variety "*Hojem*", seeds obtained from VeGIN, UK (Vegetable Genetic Improvement Network Project Diversity Set).

Experimental treatments

All plants were grown under the operation of a central monitoring system, which monitors and logs temperature and humidity in rooms and cabinets. Carbon dioxide content and light were also controlled during plant growth (Table 2-1).

Table 0-1 General conditions for growth compartment

Conditions	PBF Grodome Compartment	PBF Sanyo 2279 Cabinets
Humidity Range	70%	60%
Day/Night temperature (°C)	20/18	22/18
Light	Daylight plus supplementary 400 W SONT lamps	Fluorescent + Tungsten lamps
CO_2 Injection	None / ambient	Maintained at ambient
Irrigation	Manual	Manual

During the seedling stage, *Renate* plants were grown in a glasshouse of PBF Grodome Compartment with natural daylength condition during the period from 18th March to 25th May 2016 when the daylight was 12h 3min to 16h 12min, respectively. At 69d from sowing when bulb initiation was expected, the plants were separated randomly (using Completely Randomised Design) into three groups and transferred to three SANYO 2297 controlled environmental cabinets with the daylength treatments of 10h, 12h and 14h, respectively, with fluorescent lamps supplemented by incandescent lamps. The beginning of photoperiod was set at 8:00am (ZT0) for all cabinets, and lasted for 10h, 12h and 14h for each cabinet.

Sampling was conducted 14 days after transfer to the differential daylength treatment, to allow plants to become acclimatized to the experimental environment. Harvesting of a 1 cm section from the mid-point of the second newly expanded leaf was scheduled at 3h intervals starting from 8:00am (ZT0), and covered two consecutive days. Six timepoints were set for sampling from ZT0 to ZT15 for each day, and three plants were selected randomly from each cabinet (using Completely Randomised Design) as biological replicates.

Table 0-2 Sampling timepoints for Renate plants. Clear cells without shades indicate illuminated parts of a day, Lt Trellis shades indicate dark period. Six samplings each day for consecutive two days, from ZT0 to ZT15 at 3 hours intervals.

Sampling time	Sampling timepoints for the 1st day	Sampling timepoints for the 2nd day

		8:00	11:00	14:00	17:00	20:00	23:00	8:00	11:00	14:00	17:00	20:00	23:00
ZT time		ZT0	ZT3	ZT6	ZT9	ZT12	ZT15	ZT24	ZT27	ZT30	ZT33	ZT36	ZT39
Number of plants harvested & pooled	**10h**	3	3	3	3	3	3	3	3	3	3	3	3
	12h	3	3	3	3	3	3	3	3	3	3	3	3
	14h	3	3	3	3	3	3	3	3	3	3	3	3

For the SD variety *Hojem*, a similar experimental design was employed. After germination, *Hojem* seedlings were kept in PBF Grodome Compartment with natural daylength conditions from 22/06/2017 to 03/08/2017 when daylengths were 16h 48min to 15h 24min, respectively. At 69 days from sowing when bulbing had been initiated, plants were separated and transferred into three controlled SANYO cabinets with the same settings as used for the *Renate* experiment, providing 10h, 12h and 14h of intermediate daylengths.

Hojem plants were also kept for 14 days in the cabinets for adapting to the provided daylengths before harvesting for molecular analysis. Similar sampling timepoints of that of *Renate* were conducted except for one additional timepoint at ZT21 for each day (Table 2-3).

Table 0-3 Sampling timepoints for Hojem plants. Red shades indicate illuminated parts of a day, grey shades indicate dark period. Seven samplings each day for consecutive two days, from ZT21 to ZT15 at 3 hours intervals.

Sampling time		Sampling timepoints for the 1st day							Sampling timepoints for the 2nd day						
		5:00	8:00	11:00	14:00	17:00	20:00	23:00	5:00	8:00	11:00	14:00	17:00	20:00	23:00
ZT time		ZT21	ZT0	ZT3	ZT6	ZT9	ZT12	ZT15	ZT21	ZT24	ZT27	ZT30	ZT33	ZT36	ZT39
Number of plants harvested & pooled	**10h**	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	12h	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	14h	3	3	3	3	3	3	3	3	3	3	3	3	3	3

RNA extraction, DNase treatment and first strand cDNA synthesis

Total RNA was extracted from onion leaf material from specific growth conditions using the Z6 buffer method, following the manufacturer's (Roche manufacturing Ltd., Republic of Ireland) guidelines. Approximately 100 mg of frozen plant tissue was homogenised using a pestle and mortar. Followed by further grinding using a Dremel drill in liquid nitrogen. In this step, Z6 buffer reagent and *β*-

Mercaptoethanol were added in order to remove RNase. Two extra reagents, 3M sodium acetate (NaOAC) and 7.5M lithium chloride, which removes carbohydrates and polysaccharides, respectively, were included to obtain high quality RNA. After isolation, the quality and quantity of total RNA was measured with the Thermo Scientific NanoDrop™ 1000 Spectrophotometer (NanoDrop Technologies, Inc., USA).

Samples were then DNase treated using TURBO DNA-free™ (Ambion Inc, Cat. No. AM1907) in order to eliminate the genomic DNA contamination, procedures following the manufacturer's guidelines. A PCR was set up to check for genomic DNA contamination using primers for *ALLIINASE* gene and visualized on RNA gel electrophoresis.

First-strand cDNA was synthesised using 2µg total RNA with ThermoScript™ Reverse transcription polymerase chain reaction System (Invitrogen by Life Technologies, Cat. No. 11146-016) following the manufacturer's guidelines. The primer used for this procedure was oligo$^{(dT)}$. All samples were treated with RNase H.

qRT-PCR

After the first strand cDNA was obtained, the expression of reference and candidate genes was analysed by Real-time PCR quantification using the CFX384 Touch™ Real-time PCR machine from BioRad (Bio-Rad Laboratories Ltd., UK). All primer pairs used were initially tested to ascertain the optimum primer and cDNA concentrations. Each reaction contained 5 µl GoTaq® qPCR Master Mix, 0.5 µl of cDNA, either 0.2 or 0.3 µM of each primer, 0.2 µL fluorescein dye and SDW to make up 10 µl reaction volume. Each sample was run in triplicate and the average CT value calculated. The optimisation and selection of dilution series for making standard curves were conducted. Eight reference genes were examined (*PP2A1*, *PP2AA3*, *TIP41*, *AcTUA*, *AcTUB*, *UBQ1*, *UBC9* and *UBL*). The top three with the best stability and performance would be selected and further analysed together with the target genes. After each run, the quality and other running information of PCR was checked on the software screen. The acceptable PCR efficiency (standard curve, slopes on log amplification curves) is between 90-110%, R^2 higher than 0.985 and the melt curve should show a unique peak indicating only one single type of expected amplicon is present (Eurogentec qPCR guide).

The Real-time data obtained were then analysed using Biogazelle qBase+ software (www.biogazelle.com) after completion of each PCR run. Three reference housekeeping genes (*PP2AA3*, *PP2A1* and *TIP41*) were achieved by using qBase+ software, on the basis of 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. Normalisation was achieved by dividing the expression of the gene of interest at a specific time-point by the expression of the mean of three reference genes at that same timepoint.

RESULTS

Expression of clock genes AcGI and AcFKF1

Average relative mRNA expression was measured for both *Renate* and *Hojem* over two light/dark cycles of daylengths of 10h, 12h and 14h using RT-PCR (Figures 1 and 2). The expression of *AcGI* in both varieties showed obvious diurnal pattern which peaks at the latter part of the day at around ZT 9 and 12. The peak time moved from ZT12 to ZT9 with longer daylengths for Renate and from ZT9 to 12 in Hojem.

For *AcFKF1*, both LD and SD onion varieties showed obvious diurnal expression patterns under all intermediate daylengths with transcripts peaking in the evening. For *Renate*, the expression profile showed peaks moving forward from ZT12 to ZT9 with the increase of the daylengths. For *Hojem*, the peak time remained at ZT9 in all daylengths.

Expression of AcCOL2

AcCOL2 showed a similar diurnal expression pattern under all intermediate daylengths in both *Renate* and *Hojem* (Figure 3). A clear pattern of increasing to the highest point at the end of a day can be observed. Both varieties showed low *AcCOL2* expression level during the day from ZT0 to ZT9, and began to increase around ZT9-ZT12. Under 10h and 12h of daylight, *AcCOL2* expression peaks in the darkness, whereas under14h peaks towards the end of the day, for both varieties.

Expression of AcFT1and AcFT4

Previous study Rashid (2020) found *AcFT1* had no expression under 8h but a distinct diurnal pattern if under 16h for both *Renate* and *Hojem*. This experiment confirmed and extended these results (Figure 4). The daylength response of *AcFT1*was similar in both varieties, showing higher expression levels with increased daylengths. Both showed very low expression of *AcFT1* under 10h of daylight, with a visible trend of increasing in the dark at the end of the day (ZT12). As the daylength extended, the level of transcripts increased, leading to a clear diurnal pattern, peaking at ZT12, for both varieties.

In contrast to *AcFT1*, *AcFT4* showed expression under all three daylengths, with the diurnal pattern becoming clearer with longer daylengths (Figure 5). For *Renate*, *AcFT4* had a low expression pattern under 10h, but showed clear diurnal expression, peaking in the early part of the daylight at ZT3 under 12h and 14h. For *Hojem*, the gene showed broader expression under 10h and 12h with expression in the morning and the evening. When the daylight reached 14h, a similar diurnal pattern to *Renate* with a definite morning peak was seen. Furthermore, *AcFT4* showed a reverse diurnal expression pattern to *AcFT1* under 14h, with *AcFT1* peaking at ZT12 in the evening, and *AcFT4* peaking at ZT3, shortly after dawn.

Expression of AcFT5 and AcFT6

Generally, the gene *AcFT5* was expressed in all samples with no consistent diurnal pattern (Figure S1). The broad expression level was not affected by different daylengths. However, *AcFT5* began to show a distinct and repeatable diurnal pattern under 14h for both varieties, which was not seen in 10h or 12h. When daylength reached 14h, *AcFT5* appeared to have clear diurnal patterns, repeatable over both cycles, for both LD and SD varieties, though the peak time was different between varieties (ZT9 in *Renate* and ZT3 in *Hojem*).

Unlike *AcFT5* which showed no difference in expression level with different daylengths, *AcFT6* was seen to have more transcripts with longer days (Figure S2), for both varieties. For *Renate*, *AcFT6* showed very low expression under 10h with no obvious trend, but a peak at ZT3 can be seen in 12h. When the daylength reached 14h, a clear repeatable rhythm expression with an additional peak time at

ZT9 appeared. For *Hojem*, there was no expression of *AcFT6* in 10h plant. Under 14h, the gene showed rhythmic expression peaking at ZT6, and a lower peak at ZT12. The plants in 12h showed non-repeatable pattern but a similar peak time with 14h in the second day.

DISCUSSION

This work details the diurnal time-course expression of genes in onion that are linked to circadian regulation in Arabidopsis. All candidates, *AcGI, AcFKF1, AcCOL2, AcFT1* and *AcFT4,* presented clear diurnal expression patterns in both long-day and short-day varieties of onion consistent with the hypothesis that the photoperiodism genes under circadian regulation in Arabidopsis would also be under circadian regulation in onion.

The experimental design involved following quantitative gene expression over two diurnal cycles. The sampling pattern was limited by the capacity of the cabinets, the need for biological replication of samples and the need to sample over two cycles to confirm whether patterns were truly diurnal. Based on statistical advice, pooling samples with three technical replicates and assaying over two cycles as biological replicates was the approach we adopted. It was judged that it was more informative to have more frequent sampling points during the day than at night. Consequently, there is a longer gap during the dark period between the last sampling point of the first cycle and the first point of the second cycle. A further limitation of this study is that samples are all taken from the same 1 cm mid-section of the second recently expanded leaves. This tissue was chosen as it is likely to be exporting photosynthate to the basal bulbing tissues, which has been shown to be the route of transmission for leaf-generated FT proteins, the mobile flowering signal in Arabidopsis (Corbesier *et al*. 2007. It may not, however, be the site of maximum expression for all the genes assayed and thus quantitative comparisons of expression levels bet wen different genes should be only be made with caution. Rashid and Thomas have looked at the spatial expression of several of these genes along the leaf (Rashid et al.2019)

Genes linked to daylength perception

Photoperiodic responses can be separated into daylength perception and the consequent response of the organism. The working hypothesis is that the response to long days involves the interaction of light with CO protein, which is made when Co mRNA is expressed late in the day under the control of the circadian clock. Thus the timing perception is determined by the expression patterns of the onion homologues of *AcGI, AcFKF1* and *CO*. In Arabidopsis, *FKF1* peaks at around ZT10 in LD and ZT7

in SD (Imaizumi et al., 2003); *GI* mRNA peak levels occur around 8-10 hours after dawn (Fowler et al., 1999). In onion under intermediate daylengths, *AcFKF1* presented clear diurnal expression patterns for both *Renate* and *Hojem*. The peak time moves forward from ZT12 to ZT9 with increased daylengths in *Renate*, but in *Hojem*, was consistent at about ZT9. As these two genes work as a complex for internal controls, the shift of *AcFKF1* timing in *Renate* may be decisive in the timing of their action, causing insufficient *AcFKF1-GI* complex formed to activate *AcCOL2* transcription at the shorter daylengths. In Hojem, the expression of both genes peak at ZT9, at 10 and 12 hour daylengths, which may lead to earlier AcFT1 activation.

The Arabidopsis *FKF1-GI* complex then binds to a repressor of *CO* (*CDF1*) and forms on the *CO* promoter, regulating *CO* expression. The regulation occurs in the late afternoon in LDs, leading to *CO* protein expression and eventually flowering in Arabidopsis (Sawa et al., 2007). The onion homolog *AcCOL2* showed a similar and constant diurnal expression pattern in both *Renate* and *Hojem*, peaking towards the end of the long day and slightly later into darkness in shorter daylengths. This is supportive evidence that *AcCOL2* is a candidate for being a homologue of Arabidopsis *CO* (Rashid and Thomas , 2020). There was no obvious relationship between the timing of *AcCOL2* mRNA expression and the differential response to daylengths at the intermediate daylengths. This could be because *AcCOL2* is not responsible for AcFT1 activation, either because an alternative mechanism is involved or another, as yet undiscovered, AcCOL gene is involved. However, it should be noted that *CO* has been shown to be subject to post transcriptional regulation in Arabidopsis and other species and daylength sensitivity could be established at that level.

Genes linked to response.

In Arabidopsis, *FT* is an early target of *CO*, where its expression is directly regulated by *CO* protein (Kobayashi et al., 1999). In Arabidopsis, LD-specific *FT* induction occurs in leaf phloem companion cells with expression around dusk in long days (Mouradov et al., 2002; Song at al., 2013). In onion, six AcFT genes (*FT1-6*) were identified by Lee et al. (2013), and bulb initiation was proposed to be regulated by upregulation of *AcFT1* along with downregulation of *AcFT4*, which were proposed as bulbing promoters and inhibitors respectively

The previous study by Rashid et al. (2020) suggested *AcFT1* showed a diurnal pattern, being expressed in the later part of the day under 16h of long-daylength while showing no expression under 8h short-daylengths. The results presented in the current paper show *AcFT1* with a similar diurnal pattern, and also describes dynamic transcripts changes in long- and short-day varieties with increasing

intermediate daylengths between 10 h and 14 h. In both *Renate* and *Hojem*, *AcFT1* transcript levels increase with daylengths. There are limited transcripts at 10 h and a clear diurnal pattern can be seen only in longer daylengths. *Hojem* had higher expression and a stronger diurnal pattern than *Renate* at 12 h, particularly in the first cycle. This result is supportive of *AcFT1* being responsible for the correlation of bulbing with LD conditions (Lee et al., 2013), and consistent with *Hojem'*s better bulbing response under 12h. However, as the results represent only relative levels of expression against the particular reference genes selected, it is not possible to know what level of expression is sufficient to promote bulbing. A study conducted in tropical SD conditions (Lyngkhoi et al., 2019) also suggested that the *AcFT1* expression short-day onion increased more rapidly compared with long-day varieties.

In contrast, *AcFT4* was expressed in all daylengths, suggesting it may have a less dominate role than *AcFT1*in establishing the critical daylength. With increasing daylengths, a repeatable diurnal pattern occurred earlier in *Renate* but at 14 h, both varieties showed clear rhythmic expression of *AcFT4*, peaking at dawn and with a lower peak around dusk. The complementary daily expression pattern of *AcFT1*, which peaks in the evening, and *AcFT4*, which peaks in the morning, may indicate that their expression is negatively correlated. Nevertheless, the expression of *AcFT4* in all the intermediate daylengths may suggest it has a less important role in onion bulb initiation than *AcFT1*, which showed increased expression with intermediate daylengths in both varieties. Again, some caution is needed when considering what level of relative expression is required for inhibition by *AcFT4*.

Lee et al. (2013) identified AcFT5 and AcFT6 in onion but in limited studies did not identify roles for these genes. In this study, for *AcFT5* and *AcFT6*, a clearer and repeatable diurnal pattern only appears with longer daylengths for both varieties. *AcFT5* showed measurable transcripts under all daylengths, and shifted peak times in different daylengths. On contrast, *AcFT6* showed higher expression level with longer daylengths, and the peak times remained the same within variety. In addition, *AcFT6* peaked at 14h in *Renate* and earlier in the day at 12-14h in *Hojem*. Those daylengths match the varieties' daylength requirement for bulb initiation therefore *AcFT6* may have a role in determine the onion's ability to bulb under a particular daylength. Rashid *et al*. (2019) showed that AcFT5 and AcFT 6 had a wider tissue distribution in onion leaves, being expressed in bulbing and intermediate tissue. It may be that multiple AcFT genes with different daylength specificities have a role in daylength dependent bulbing. However, any such roles for AcFT5 and AcFT6 would be required to be confirmed in functional studies.

CONCLUSIONS

This work details the diurnal time-course expression of genes in different onion types that are linked to circadian regulation in Arabidopsis. All candidates presented clear diurnal expression patterns in both LD and SD variety of onion, consistent with a role in daylength-dependent bulbing process at the molecular level, and confirming the hypothesis that the clock genes in Arabidopsis would be circadian regulated in onion. Both *AcFKF1* and *AcGI* showed distinct diurnal expression with similar patterns to that of Arabidopsis. *AcCOL2* showed a consistent diurnal pattern in both varieties under all daylengths, peaking towards the end of the long day and slightly later into darkness in shorter daylengths. There was no obvious relationship between the timing of *AcCOL2* mRNA expression and the differential response to daylengths at the intermediate daylengths. Further research is required to confirm a role for AcCOL2 in daylength-dependent bulbing. The AcFTs all showed different diurnal expression patterns, peaking at different times of the day. *AcFT5* and *AcFT6*, showed peaks at different time between varieties. A clearer and repeatable diurnal pattern only appears with longer daylengths for both varieties, suggesting they might be the active components present for circadian or diurnal regulation under LD conditions. The expression pattern of *AcFT6* suggested a possible role in responding to daylength but that would need to be tested in further studies. With regard to the original question of the relative roles of promotion and inhibition in the response to increasing daylengths, it was found that *AcFT1* expression level increased with daylength in both LD and SD onion. In contrast *AcFT4* was expressed in all daylengths, suggesting it has a less important role in daylength dependent bulbing process. The results indicate that *AcFT1* and AcFT4 are negatively co-regulated, but AcFT1 is the predominant regulator of bulb formation in response to daylength.

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FIGURE LEGENDS

Figure 1: Expression of *AcGI* in long-day (*Renate*) and short-day (*Hojem*) varieties of onion over a 48h period using qRT-PCR, relative to *PP2AA3, PP2A1* and *TIP41*. Light and dark shades denote light/dark cycles. Error bars represent the SEM.

Figure 2: Expression of *AcFKF1* in long-day (*Renate*) and short-day (*Hojem*) varieties of onion over a 48h period using qRT-PCR, relative to *PP2AA3, PP2A1* and *TIP41*. Light and dark shades denote light/dark cycles. Error bars represent the SEM.

Figure 0: Expression of *AcCOL2* in long-day (*Renate*) and short-day (*Hojem*) varieties of onion over a 48h period using qRT-PCR, relative to *PP2AA3, PP2A1* and *TIP41*. Light and dark shades denote light/dark cycles. Error bars represent the SEM.

Figure 4: Expression of *AcFT1* in long-day (*Renate*) and short-day (*Hojem*) varieties of onion over a 48h period using qRT-PCR, relative to *PP2AA3, PP2A1* and *TIP41*. Light and dark shades denote light/dark cycles. Error bars represent the SEM.

Figure 5: Expression of *AcFT4* in long-day (*Renate*) and short-day (*Hojem*) varieties of onion over a 48h period using qRT-PCR, relative to *PP2AA3, PP2A1* and *TIP41*. Light and dark shades denote light/dark cycles. Error bars represent the SEM.

SUPPLEMENTARY FIGURES

Figure S1: Expression of *AcFT5* in long-day (*Renate*) and short-day (*Hojem*) varieties of onion over a 48h period using qRT-PCR, relative to *PP2AA3, PP2A1* and *TIP41*. Light and dark shades denote light/dark cycles. Error bars represent the SEM.

Figure S2: Expression of *AcFT6* in long-day (*Renate*) and short-day (*Hojem*) varieties of onion over a 48h period using qRT-PCR, relative to *PP2AA3, PP2A1* and *TIP41*. Light and dark shades denote light/dark cycles. Error bars represent the SEM.