Conservation of flowering time genes in onion

Author for correspondence: Andrew Taylor, Warwick HRI, University of Warwick, Wellesbourne, Warwick, CV35 9EF, UK. E-mail: andrew.taylor@warwick.ac.uk

Tel: +44 (0)24 7657 5030, Fax: +44(0)24 7657 4500

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Title: **Conservation of *Arabidopsis thaliana* photoperiodic flowering time genes in onion (*Allium cepa* L.).**

Andrew Taylor, Andrea Juliet Massiah and Brian Thomas

Warwick HRI, University of Warwick, Wellesbourne, Warwick, CV35 9EF, UK.

Abbreviations:

AcCOL- Allium cepa CONSTANS-LIKE, AcCOP1- Allium cepa CONSTITUTIVE PHOTOMORPHOGENIC 1, *AcEF1α- Allium cepa ELONGATION FACTOR 1 ALPHA, AcGI- Allium cepa GIGANTEA, AcFKF1- Allium cepa FLAVIN-BINDING, KELCH REPEAT, F-BOX 1*, *AcFTL- Allium cepa FLOWERING LOCUS T-LIKE, AcPHYA- Allium cepa PHYTOCHROME A, AcZTL- Allium cepa ZEITLUPE, COL4-CONSTANS-LIKE 4*, *CDF1- CYCLING DOF FACTOR 1*, *CO- CONSTANS, COP1-CONSTITUTIVE PHOTOMORPHOGENIC 1, CRY- CRYPTOCHROME, EF1α-ELONGATION FACTOR 1 ALPHA, ELF3- EARLY FLOWERING 3, FKF1- FLAVIN-BINDING, KELCH REPEAT, F-BOX 1*, FR- far-red, *FT- FLOWERING LOCUS T*, *GI- GIGANTEA*, *Hd1- Heading date 1*, ID- intermediate day, LD- long day, *LKP2-LOV KELCH PROTEIN2*, LOV- light, oxygen or voltage, NJ- neighbour-joining, PPFD- photosynthetic photon flux density, *PHY- PHYTOCHROME*, *PHYA-PHYTOCHROME A*, RACE- rapid amplification of cDNA ends, SD- short day, *TOC1- TIMING OF CAB EXPRESSION 1*, ZT- zeitgeber time, *ZTL- ZEITLUPE*.

Abstract

The genetics underlying onion development is poorly understood. Here the characterisation of onion homologues of *Arabidopsis* photoperiodic flowering pathway genes is reported with the end goal of accelerating onion breeding programmes by understanding the genetic basis of adaptation to different latitudes.

The expression of onion *GI*, *FKF1* and *ZTL* homologues under SD and LD conditions was examined using quantitative RT-PCR. The expression of *AcGI* and *AcFKF1* was examined in onion varieties which exhibit different daylength responses. Phylogenetic trees were constructed to confirm the identity of the homologues.

AcGI and *AcFKF1* showed diurnal expression patterns similar to their *Arabidopsis* counterparts while *AcZTL* was found to be constitutively expressed. *AcGI* showed similar expression patterns in varieties which exhibit different daylength responses whereas *AcFKF1* showed differences. It is proposed that these differences could contribute to the different daylength responses in these varieties. Phylogenetic analyses showed that all the genes isolated are very closely related to their proposed homologues.

The results presented here show that key genes controlling photoperiodic flowering in *Arabidopsis* are conserved in onion and a role for these genes in the photoperiodic control of bulb initiation is predicted. This theory is supported by expression and phylogenetic data.

Keywords: bulb initiation, daylength response, *FLAVIN-BINDING, KELCH REPEAT, F-BOX 1* (*FKF1*), *GIGANTEA* (*GI*), onion, photoperiod.

Introduction

The onion (*Allium cepa* L.) belongs to the order Asparagales, the second most important monocot order (Kuhl et al., 2004, Stevens, 2001 onwards). It is a diploid plant (2n=2x=16) with a very large genome (32pg/2n), about 36 and 107 times larger than rice and *Arabidopsis* genomes, respectively (McCallum et al., 2001; Kuhl et al., 2004). Onions are farmed worldwide and in 2007 68 million tonnes of onions were produced across the world (FAOSTAT, 2008). The onion is a biennial plant, the bulb being an overwintering stage of the life cycle (Lancaster et al., 1996). Flowering and seed production will occur following a period of vernalisation, provided the juvenile phase has been passed (Brewster, 1997). In terms of crop production, onions tend to be grown as annual crops.

The physiology of bulb initiation has been studied extensively. It is a process which is photoperiodically driven in temperate onions, drawing parallels with the photoperiodic control of flowering in other plant species (Mettananda and Fordham, 1997). Long days (LDs, $\geq$ 16 hours of light) will initiate bulbing in temperate onions. Commercially, onion cultivars are classified as long, short and intermediate daylength varieties (Brewster, 2008). The exact daylength required will vary between cultivars, but the broad classification gives an indication of which cultivar would be suited to growth at a particular latitude. Short-day (SD) onion varieties require a daylength of at least 12 hours to initiate bulbing. However, these varieties perform poorly in longer daylengths as they produce a bulb after only 1 or 2 leaves, leading to a very small final product (Brewster, 2008). Therefore a daylength of 12 hours is optimal for crop production.

Flowering has been well characterised at molecular and genetic levels. The flowering time genes in *Arabidopsis* mainly function in six different pathways: autonomous; vernalisation; gibberellin; temperature; light quality and photoperiod (Jack, 2004). In photoperiodic flowering, light interacts with the circadian clock (through *PHYTOCHROME (PHY)* and *CRYPTOCHROME (CRY)* genes) and the timing of the clock is controlled by feedback loops involving *TIMING OF CAB EXPRESSION 1* (*TOC1*). *CONSTANS (CO)* expression is high at the end of LDs and the CO protein is degraded at night (Valverde et al., 2004). CO regulates the expression of floral integrating genes such as *FLOWERING LOCUS T* (*FT*) leading to floral initiation (Jackson, 2009; Massiah, 2007). Flowering takes place when *CO* transcription and a blue or far-red light signal occur simultaneously. The *CO* gene is an integral part of this pathway and has been isolated from several species including both SD and LD plants (Griffiths et al., 2003).

GIGANTEA (*GI)* and *FLAVIN-BINDING, KELCH REPEAT, F-BOX 1* (*FKF1)* have been shown to regulate *CO* expression. Both are circadian regulated (Fowler et al., 1999, Nelson et al., 2000, Park et al., 1999) and control flowering by regulating *CO* transcription through the degradation of *CYCLING DOF FACTOR 1* (*CDF1*), a repressor of *CO* (Imaizumi et al., 2005; Sawa et al., 2007). Recent work has shown that additional *CDF* genes (*CDF2*, *CDF3* and *CDF5*) act redundantly with *CDF1* to repress *CO* (Fornara et al., 2009). GI shows no homology with any gene of known function (Fowler et al., 1999). The FKF1 protein has three characteristic domains: the light, oxygen and voltage (LOV)-sensing domain, the F-box and the Kelch repeat (Nelson et al., 2000). FKF1 has been shown to regulate the precise timing of CO expression, a function which is light dependent, leading to flowering only in LDs (Imaizumi et al., 2003). Studies have shown that GI and FKF1 form a

protein complex in blue light (Sawa et al., 2007) dependent on blue light absorption within the LOV domain of FKF1 (Imaizumi et al., 2003). The complex binds to CDF1 and forms on the *CO* promoter regulating *CO* expression. This occurs in the late afternoon in LDs, leading to CO activation of *FT* expression and subsequently flowering. GI has been shown to additionally regulate photoperiodic flowering through a mechanism independent of *CO*, which involves regulation of miR172 abundance and hence its targets, leading to activation of *FT* (Jung et al., 2007). *GI* is also involved in the maintenance of circadian rhythms (Mizoguchi et al., 2005). This is mediated through an interaction with ZEITLUPE (ZTL) under blue light conditions (Kim et al., 2007). ZTL is a circadian clock-associated protein which is very closely related to FKF1. It is involved in the control of proteosome-dependent degradation of TIMING OF CAB EXPRESSION 1 (TOC1) (Mas et al., 2003). GI is required to establish and maintain the oscillation of the ZTL protein through protein-protein interactions which are enhanced by blue light acting through the LOV domain. It has also been shown that ZTL and another member of the same gene family, LOV KELCH PROTEIN2 (LKP2), can act redundantly with FKF1 in the degradation of CDF proteins (Fornara et al., 2009).

Two further genes involved in photoperiodic flowering are *CONSTITUTIVE PHOTOMORPHOGENIC 1* (*COP1*) and *EARLY FLOWERING 3* (*ELF3*). COP1 and ELF3 have been shown to control flowering by regulating GI stability (Yu et al., 2008). COP1 is also involved in the degradation of CO protein at night (Jang et al., 2008).

At the physiological level, bulb initiation in LD onions is regulated in a similar way to the photoperiodic regulation of flowering in LD plants such as *Arabidopsis* (Thomas and Vince-Prue, 1997). Bulb initiation requires the perception of light with

147 an appreciable component of Far Red (FR) in the second half of the LD implying the
148 involvement of phytochrome (Quail et al., 1995, Lercari, 1984). Perception of the LD
149 signal takes place in the leaves (Sobeih and Wright, 1986, Brewster, 1990), as with
150 flowering (Knott, 1934), while the response is in the meristem, requiring the transport
151 of a signal within the plant. Other parallels between bulb initiation and floral initiation
152 include a juvenile phase during which plants cannot respond to daylength and the
153 involvement of a homeotic conversion of photosynthetic leaves to either floral organs
154 or swollen bulb scales at the responsive meristem (Komeda, 2004, Lancaster et al.,
155 1996, Massiah, 2007, Sobeih and Wright, 1986). Recent work has identified the
156 *FLOWERING LOCUS T* (*FT*) protein and its homologue as a part of the mobile signal
157 in *Arabidopsis* and rice (Corbesier et al., 2007, Jaeger and Wigge, 2007, Tamaki et
158 al., 2007).
159 This study shows that genes controlling the daylength response are conserved
160 between the model plant *Arabidopsis* and onion and supports the hypothesis that these
161 genetic components regulate both bulbing and flowering end-processes.
162
163 **Results**
164
165 Conservation of flowering time genes
166 A clone of a putative onion *CO* homologue, identified in the *A. cepa* gene
167 index, was assigned the name *Allium cepa CO*-like (*AcCOL*). Full-length sequence
168 information was obtained which showed the gene to be more closely related to
169 *Arabidopsis COL4* than *CO* (Table 1). A phylogenetic analysis based on B-box
170 proteins (Supporting Information Fig. S1) showed that this gene is a group 1b CO-like
171 gene (according to the groupings described by Griffiths et al., 2003). This gene did

not exhibit a discernable diurnal expression pattern (Supporting Information Fig. S2), as shown by both *Arabidopsis CO* and the rice homologue *Hd1* (Izawa et al., 2002, Suárez-López et al., 2001). Attempts to clone other *CO*-like sequences using degenerate primers or screening a normalised cDNA library consistently resulted in isolation of the same sequence as *AcCOL*.

Partial sequence information was obtained for an onion putative *PHYA* homologue. This gene was assigned the name *AcPHYA* and was shown to share a high level of nucleotide and amino acid identity with *Arabidopsis* and rice *PHYA* (Table 1). A Phylogenetic analysis (Supporting Information Fig. S3) supported its identity as the onion *PHYA* homologue. In onion, FR is essential for bulb initiation (Lercari, 1982) whilst in *Arabidopsis*, *PHYA* mediates the response of seedlings to FR, which is consistent with a role for *AcPHYA* in mediating bulb initiation in response to FR (Thomas, 2006). Further analysis of *AcPHYA* is required to elucidate the exact function of this gene in onion bulbing.

A full – length cDNA clone bearing homology to *GI* was identified following a normalised library screen and 5' RACE – PCR. The gene represented by this clone was assigned the name *Allium cepa GIGANTEA* (*AcGI*) and was shown to share very high nucleotide and amino acid identities with both *Arabidopsis* and rice *GI* (Table 1). This gene was further analysed using expression and phylogenetic analyses.

Full – length cDNA clones bearing homology to *FKF1* and *ZTL* were identified using degenerate PCR and 5' RACE – PCR. Genes representing these cDNAs were assigned the names *Allium cepa FKF1* (*AcFKF1*) and *Allium cepa ZTL* (*AcZTL*). Both genes showed very high nucleotide and amino acid identities with *Arabidopsis* and rice *FKF1* and *ZTL* respectively (Table 1) and were further characterised through expression and phylogenetic analyses.

The *Allium cepa* Gene Index also contains EST sequences with sequence similarity to *Arabidopsis FT* and *COP1*. These genes were assigned the names *Allium cepa FT-like* (*AcFTL*) and *AcCOP1*. *AcCOP1* showed very high nucleotide and amino acid identity with *Arabidopsis* and rice *COP1*, suggesting a potentially similar role for this gene in onion. *AcFTL* appeared to be more closely related to a group of *FT*-like genes than *FT*/*Hd3a* (Supporting Information Fig. S4). This gene showed no clear diurnal expression pattern although its expression level was much higher in LDs than in SD's (Supporting Information Fig. S5).

Phylogenetic analysis of *GI* homologues

A phylogenetic analysis was carried out in order to establish the relationship between AcGI and other GI proteins. AcGI clustered with other monocot GI homologues, with the support of high bootstrap values (Fig. 1). As there are no other genes known to show homology with *GI* in *Arabidopsis* or other species (Fowler et al., 1999) we conclude that *AcGI* is the onion *GI* homologue.

Expression of *AcGI*

The expression of *AcGI* over a 24 h period under both LD and SD conditions was determined using quantitative RT-PCR. In a LD onion variety (Renate F_1) the expression of *AcGI* was seen to peak around ZT10 in LDs compared with ZT7 in SDs (Fig. 2a). This expression pattern is very similar to that shown by *Arabidopsis GI*, which peaks at ZT10 in LDs and ZT8 in SDs (Fowler et al., 1999). *AcGI* has a diurnal expression pattern, characteristic of genes involved in the photoperiod response (Jackson, 2008) implying it is circadian regulated, although expression in constant light conditions would be required to confirm this.

The expression of *AcGI* was also examined in Candy F_1 and Agrifound Dark, ID and SD onion varieties, respectively. When grown under LD and SD conditions patterns of *GI* expression were similar in both varieties to those observed for Renate F1 the LD variety when grown under similar conditions, peaking at around ZT10 and ZT7-8, respectively (Fig. 2).

Analysis of AcFKF1 and AcZTL proteins

The predicted protein sequences of AcZTL and AcFKF1 were compared with *Arabidopsis* ZTL and FKF1. Both AcZTL and AcFKF1 contain an F-box, six kelch repeats and a LOV domain, as observed for the homologous *Arabidopsis* genes (Supporting Information Fig. S6).

A phylogenetic analysis of FKF1 and ZTL family proteins was carried out. A NJ tree was constructed and rooted through a clade containing F-Box genes which lack the other domains essential for FKF1 and ZTL function (Fig. 3). The FKF1 and ZTL homologues form a large clade which is clearly divided into two smaller clades, with strong support from high bootstrap values. AcFKF1 is present in the clade containing the FKF1 homologues, providing further evidence that this gene is the onion FKF1 homologue. AcZTL is present in the clade which contains the ZTL homologues, suggesting that it is the onion ZTL homologue. Both the FKF1 and ZTL clades show a clear split between monocot and dicot sequences. In both cases, the onion putative homologue clusters with monocot gene sequences. The high level of sequence conservation, mirrored by the relationships seen in the phylogenetic tree, may also suggest a level of conservation of function.

247 Expression of *AcFKF1* and *AcZTL*

248 Expression of *AcFKF1* was examined in LD and SD grown Renate F_1 plants
249 and a clear diurnal rhythm of *AcFKF1* expression was observed under both growth
250 conditions with a peak at ZT10 (Fig. 4 (a)). Whilst *Arabidopsis FKF1* is similarly
251 diurnally expressed, expression peaks at around ZT10 in LDs and ZT7 in SDs
252 (Imaizumi et al., 2003).

253 The expression of *AcFKF1* was investigated using onion varieties with
254 different daylength responses. Under LD conditions, *AcFKF1* is seen to peak around
255 ZT 7-8 in Agrifound Dark, the SD variety (Fig. 4 (b)) compared with ZT 10 in Renate
256 F_1, the LD variety. Expression peaks at an intermediate time (around ZT 9) in Candy
257 F_1, the ID variety. There is therefore a distinct difference between the timing of the
258 peak of expression in varieties showing different daylength responses.

259 Under SD conditions, the expression of *AcFKF1* peaked around ZT 7-8 in
260 both the SD and ID varieties (Fig. 4c) in contrast to the peak seen in the LD variety
261 which was around ZT10 (Fig. 4a). Thus, as was observed for LD grown plants there
262 is a difference in expression profiles between varieties with different daylength
263 responses.

264 The expression of *AcZTL* was examined in a SD onion variety (Agrifound
265 Dark) and was shown to be constitutively expressed in both LD and SD grown plants
266 (Fig. 5). There is no diurnal expression pattern and no obvious expression peaks,
267 which is similar to *Arabidopsis* where *ZTL* does not show cyclic expression, although
268 ZTL protein levels oscillate, with a peak at ZT10-13 (Kim et al., 2007). The
269 expression profile, coupled with the phylogenetic data, is consistent with *AcZTL* being
270 the onion *ZTL* homologue and a component of the photoperiod pathway.

271

Discussion

The genetic network controlling photoperiodic flowering in *Arabidopsis* is proposed to be broadly conserved across plant species, including legumes, *Brassicas*, rice, potato and wheat (Hecht et al., 2005, Robert et al., 1998, Kojima et al., 2002, Martínez-García et al., 2002, Miller et al., 2008). In the case of potato, the photoperiod genes control a different end process, namely tuberisation (Martínez-García et al., 2002). Our working hypothesis is that the same genes also control daylength – dependent bulb initiation in onion. This is supported by the results obtained with the onion homologues of *GI*, *FKF1* and *ZTL* presented in this paper. It is possible that expressed paralogues of the onion *Arabidopsis* flowering time gene homologues discussed may exist. However, their spatial or temporal expression may be such that they were not represented in the cDNA library synthesised and screened.

In *Arabidopsis*, the timing of expression of *GI* is an essential component of the photoperiodic control of flowering (Fowler et al., 1999). This gene shows a later expression peak in LDs compared to SDs, leading to increased expression of *CO* and induction of flowering when daylength increases. Similarly, the expression of *AcGI* in the LD onion variety (Renate F_1) peaked later under inductive (LD) conditions than in non-inductive (SD) conditions (Fig. 2a). This is consistent with a role for *AcGI* in the photoperiodic control of bulb initiation. In addition, *AcGI* shows the same expression pattern in ID and SD onion varieties. This suggests that a key circadian rhythm component of the photoperiod pathway is active in LD, ID and SD onion varieties and that if a difference in daylength response is associated with a change in the photoperiod pathway it should be downstream of *AcGI*.

In contrast, the expression of *AcFKF1* was seen to vary in varieties showing different daylength responses. A consequence of this variation is that in SDs, the peaks of expression of *AcFKF1* in the SD and ID varieties occur during the light period, whereas in the LD variety, the peak occurs in the dark period. It is possible that the differential expression of this gene contributes towards the different daylength responses seen in the three varieties tested. The earliest LD expression peak was seen in the SD variety, a variety which quickly initiate bulbing under LDs (Brewster, 2008). This precocious bulbing response could be partly due to a build up of AcFKF1 protein. Later peaks were seen in the ID and LD varieties, which will initiate bulbing in LDs, but only after a certain number of leaves have been produced.

In *Arabidopsis*, GI forms a complex with FKF1, leading to the degradation of CDF1, a repressor of CO, and eventually floral initiation (Sawa et al., 2007). The GI/FKF1 complex appears to directly regulate CDF1 stability in the afternoon. In onion, *AcFKF1* and *AcGI* show very high percentage identities with *Arabidopsis FKF1* and *GI* respectively (Table 1). Therefore it is feasible that the same interactions occur in onion. In the LD onion variety (Renate F_1) it was shown that the expression profiles of *AcGI* and *AcFKF1* are very similar in LD conditions, peaking around ZT10 (Figs. 2a & 4a). This would allow the two gene products to form a complex to control the expression of onion *CO* (or an equivalent), allowing bulb initiation in LDs. Under SD conditions, there is a difference in the timing of the expression peaks. The expression of *AcGI* is seen to peak around ZT 7.5, compared with ZT 10 for *AcFKF1*. Moreover, the expression of *AcFKF1* is seen to peak in the dark period. This would mean that an FKF1/GI protein complex would only be formed into the dark period and *CO* expression would be repressed by CDF1 during the day. However, the GI protein is degraded at night in *Arabidopsis* so it is possible

that the GI/FKF1 complex is not formed at all in SDs (David et al., 2006). This could explain, at least partly, why this variety does not initiate bulbing under SD conditions.

The expression patterns of *AcGI* and *AcFKF1* were very similar in an ID onion variety (Figs 2 & 4). This variety (Candy F_1) was seen to produce bulbs in both LD and SD conditions and is described by seed companies as 'day neutral'. This would allow for a daytime complex to form, leading to *CO* transcription and hence bulb initiation.

In a SD onion variety (Agrifound Dark), the expression profiles of *AcFKF1* and *AcGI* were very similar under SD conditions, peaking at ZT 7.5 (Figs. 2c & 4c). This would allow for a daytime complex to form and bulbing to be initiated in SD, i.e. the phenotype which was observed in this variety. Under LD conditions, the expression patterns of *AcFKF1* and *AcGI* are seen to differ (Figs. 2b & 4b). A peak in *AcFKF1* expression is seen around ZT7-8 compared to ZT10 for *AcGI*. This may lead to an accumulation of the FKF1/GI protein complex to occur at an earlier time of day than is seen for LD and ID varieties. This could then explain the precocious bulbing response seen in this variety.

In *Arabidopsis*, GI also forms a protein complex with ZTL (Kim et al., 2007). The formation of this complex is enhanced in blue light. ZTL is involved in controlling circadian rhythm through TOC1. It has been shown that GI is required to maintain the oscillation of the ZTL protein. *AcZTL* shows a high level of sequence identity with *Arabidopsis ZTL*, suggesting a conservation of function. Therefore, it is hypothesised that AcGI also forms a complex with AcZTL in order to control circadian rhythm. It was shown that *AcZTL* is constitutively expressed. The formation of the complex in *Arabidopsis* is predicted to allow the rapid deployment of ZTL during the light period (Kim et al., 2007). In all the varieties tested (and in all

346 daylengths), the expression of *AcGI* was seen to peak in the light period. This would
347 allow a peak in AcZTL protein levels to occur in the light period and hence circadian
348 rhythm to be controlled through the onion equivalent of *TOC1*.

349 Onion plants will also flower, usually following a period of vernalisation
350 (Brewster, 1997), raising the question of which genes are involved in flowering and
351 which are involved in bulbing. It is clear that bulb initiation is photoperiodically
352 controlled (Lancaster et al., 1996). Under inductive daylength conditions, onions will
353 initiate bulbing and flowering will be inhibited (Brewster, 2008). Gaining a greater
354 knowledge of the genetic control of flowering in onion would be useful for
355 controlling and stopping flowering during bulb production.

356 The rationale behind this study is that knowledge of the daylength response in
357 onion is important for adapting new varieties for growth at different latitudes.
358 Molecular genetic studies with onion are difficult because of its very large genome
359 size and biennial habit. The results in this paper indicate that expression patterns of
360 genes involved in the daylength response in *Arabidopsis* are also seen in onion.
361 Furthermore, variations in *AcFKF1* expression in onions with different daylength
362 responses are consistent with a role for those genes in establishing daylength
363 sensitivities. If confirmed in a wider range of germplasm, this information may be
364 useful in accelerating onion breeding programmes.

365

366 **Materials and Methods**

367

368 Gene isolation

369 Renate F_1 onions, a LD variety from the UK which requires a daylength of
370 approximately 16 hours or more to initiate bulbing (Elsoms Seeds Ltd., Spalding,

371 UK), grown in the glasshouse in Spring and Summer were used as the starting
372 material for gene isolation. Initially, the *A.cepa* Gene Index was screened for genes
373 which showed sequence similarity with *Arabidopsis* photoperiod pathway genes.
374 Clones homologous to *CO* and *PHYA* (*AcCO* and *AcPHYA*) were obtained (cloned
375 into the *Eco*R V site of a pCMV.SPORT 6 vector, Invitrogen, Paisley, UK) and
376 sequenced.

377 The onion *GI* homologue (*AcGI*) was obtained by screening a normalised
378 cDNA library (produced by vertis Biotechnologie AG, Freising, Germany) produced
379 using RNA extracted from onion Renate F_1 leaf and bulb tissue harvested every three
380 hours over a 24-h period. The probe was generated from cDNA by PCR using the
381 primers 5'-CAGGCCGAGAAGGATTTACAAC-3' and 5'-
382 CAAAACTCCGGTTCTGACAGTG-3' at an annealing temperature of 61°C and a
383 digoxigenin (DIG) non-radioactive nucleic acid labelling kit (Roche, Welwyn Garden
384 City, UK) following the manufacturer's guidelines. RACE PCR was used to obtain
385 sequence for the 5' end of the gene (Invitrogen, Gene Racer Kit, Paisley, UK) using
386 the gene-specific primer 5'-GGCACGAAGAAGAAGATCCGAGGCACTA-3' and
387 the nested primer 5'-CAACATCACAAAGCGCATCCACTACCT-3' following the
388 manufacturer's instructions. Full length clones were generated using primers
389 designed to the UTR's (5'-GCCTTCTTCACGAAAAATCGCAGTG-3' and 5'-
390 CCAAGACGATTACAAGGATGATAGA-3').

391 Members of the *FKF1*/*ZTL* gene family were obtained using degenerate PCR
392 using a protocol adapted from the 3' RACE PCR method (Borson et al., 1992),
393 communicated by Dr. Ken Manning, University of Warwick, UK. Superscript™ II
394 Reverse Transcriptase (Invitrogen, Paisley, UK) was used to synthesise cDNA using a
395 modified poly dT primer (5'-

GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTVN-3'). PCR was carried out using a primer designed to amplify both *FKF1*/*ZTL* homologues (5'-ATGGTHTGTCARAAYGCDTGGGG-3') and a primer specific to the modified poly dT primer (5'-GCGAGCACAGAATTAATACGAC-3'). Sequence for the 5' end of *AcFKF1 and AcZTL* genes were obtained using 5' RACE PCR (Invitrogen, Paisley, UK) using the gene-specific primer 5'-CCACCCGCTTCCAAGTGGGCTGGTTTG-3' and the nested primer 5'-CGAGGGTGGTGAGTTCGCGGGAGAGT-3' for *AcFKF1* and the gene-specific primer 5'-GCCCTTGCCTACCACATCCACCAAAT-3' and nested primer 5'-CCTGCTGCTGGCATGGTTTCTAACGC-3' for *AcZTL*. Primers were designed to the UTR's of both genes to obtain full-length clones (5'-TCCAAATCCCAAACCAATTACAGC-3' and 5'-GCATGAAAACGAGCACAATCAGA-3' for *AcFKF1*, 5'-CACAACCACACACTGATTTTCACA-3' and 5'-CTCGTTCCTTCTCCAATCGATCA-3' for *AcZTL*).

Growth and harvesting of plants for expression analyses

Renate F_1 onions were grown from May to August 2006 at Wellesbourne (latitude 52º12'). All plants received 8 h of natural daylight within a glasshouse. Plants in LDs were subjected to a daylength extension of 8 h using low-level incandescent light within a photoperiod chamber with an average photosynthetic photon flux density (PPFD) of 5 µmol m^{-2} sec^{-1} whilst plants in SDs were subjected to 16 h of darkness in a photoperiod chamber. At bulb initiation in LD grown plants (99 days after sowing), harvests were carried out at set times over a 48-h period. Middle sections of the youngest fully expanded leaves were harvested, chopped into small

421 sections, flash frozen in liquid nitrogen and stored at -80°C until required for analysis.
422 Leaf material was harvested from three separate plants and pooled. Plants were
423 selected for harvest using a random number generator.

424 Agrifound Dark, a SD onion variety from India which requires a daylength of
425 approximately 12 hours for optimal bulb formation and Candy $F_{1,}$ an intermediate day
426 (ID) variety from USA which requires a daylength of approximately 14 hours or more
427 to initiate bulbing were grown from April to September 2007 at Wellesbourne
428 (latitude 52º12'). Seed was sourced from the Warwick HRI Genetic Resources Unit,
429 Wellesbourne, UK. Plants were subjected to 8 h of natural daylight plus a daylength
430 extension of 8 h (LD) or 4 h (SD) using low-level incandescent light within a
431 photoperiod chamber with a mean PPFD of 5 µmol m^{-2} sec^{-1}. Plants were placed in
432 specific locations using a Latin square design (Mead et al., 1993). Leaf material was
433 harvested at set times over a 48-hr period as described for the LD onion expression
434 experiment. Harvests were carried out when bulbing had been initiated under
435 inductive daylengths (62 days after sowing).

436

437 Quantitative expression analyses

438 Total RNA was extracted from 100 mg leaf material harvested at each time-
439 point using Trizol® reagent (Invitrogen, Paisley, UK) following the manufacturer's
440 guidelines. Samples were DNase treated using TURBO DNA-free™ (Ambion,
441 Huntingdon, UK) and first-strand cDNA synthesised from 2 µg total RNA using
442 Superscript™ II Reverse Transcriptase (Invitrogen, Paisley, UK) following the
443 manufacturers' guidelines. Quantitative real-time PCR was carried out using an I-
444 Cycler (Bio-Rad Laboratories, iCycler Thermal Cycler). To assess the expression of
445 *AcGI* in a LD onion variety (Renate F_1), reaction volumes of 25 µl were used,

containing 1 µl of cDNA, 1x PCR Mastermix containing SYBR green (Eurogentec, Southampton, UK) and 0.4 µM of each primer (5'-CACAGATGGATTGCTTGTTGATG-3' and 5'-ATTGGCTACGAGATGAACTGCTC-3'). Cycling was carried out as described in the manufacturer's guidelines with an annealing temperature of 61°C. All samples were run in triplicate and data normalised to the expression of *Elongation Factor 1 Alpha* (*AcEF1α*, accession number CF437531) using the primers 5'-. TGGCATCCAACTCTAAGGACGAT-3' and 5'-AATGTGAGATGTGTGGCAATCCA-3'.

For all other qRT-PCR analyses, a MESA GREEN qPCR MasterMix for SYBR® green with fluorescein (Eurogentec, Southampton, UK) was used, following the manufacturer's guidelines. Reactions were carried out in 15 µl volumes, containing 0.5 µl of cDNA. All samples were run in triplicate and all data normalised to *Acß–Tubulin* (accession number AA451549) using the primers 5'-GTCTTCAGAGGCAAGATGAGCAC-3' and 5'-TCAGTCCAGTAGGAGGAATGTCG-3'. For analysis of *AcFKF1*, the primers 5'CCGGTGCAGTTGTTTATGTTGGAT-3' and 5'-TCCCACCCACCACACAGGTACTAT-3' with an annealing temperature of 65°C were used. For *AcZTL*, the primers 5'-GTTTGGTGGTCTGGCTAAGAGTG-3' and 5'-CTCCAGGCATACTGCTACCTGTT-3' with an annealing temperature of 65°C were used. Data from both cycles of the 48 h time course were combined to calculate average expression over a 24 h period.

Phylogenetic analyses

Phylogenetic analyses of *GI* and *FKF1*/*ZTL* homologues were conducted in the same way. Published sequences were collated, converted to predicted amino acid sequences using the EditSeq package of DNAStar and alignments carried out using Clustal X (Thompson et al., 1997). Neighbour-joining (NJ) trees were constructed using Clustal X and bootstrap values calculated using 1000 replicates. Phylogenetic trees were viewed and edited using NJPlot (Perrière and Gouy, 1996).

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621

622 **Tables**

623

624 **Table 1**: Conservation of *Arabidopsis* flowering time genes in onion.
625 The percentage of the coding region covered was estimated based on *Arabidopsis* and
626 rice gene sequences. *Arabidopsis* accessions: *FKF1*- NM_105475, *GI*- NM_102124,
627 *ZTL*- NM_125119, *CO*- X94937, *COL4*- NM_122402, *FT*- NM_105222, *PHYA*-
628 NM_001123784, *COP1*- NM_128855.3. Rice accessions: *FKF1*- NM_001074600,
629 *GI*- NM_001048755, *ZTL*- NM_001064973, *Hd1*- AB041840, *Hd3a*- Os01g06320,
630 *OsFT5*- Os0239064, *PHYA*- AB109891, *COP1*- AB040053.

631
632

Arabidopsis/rice gene	Annotation	Accession number	Percentage amino acid identity (*Arabidopsis*)	Percentage amino acid identity (rice)	Percentage of coding region covered
FKF1	*AcFKF1*	GQ232754	63.4	69.7	100
GI	*AcGI*	GQ232756	69.0	75.8	100
ZTL	*AcZTL*	GQ232755	72.1	75.5	100
CO/*Hd1*	*AcCOL*	GQ232751	34.9 (48.1 for *COL4*)	40.6	100
FT/*Hd3a*	*AcFTL*	CF438000	52.9	56.2 (56.9 for *OsFT5*)	86
PHYA	*AcPHYA*	GQ232753	55.8	56.3	33
COP1	*AcCOP1*	CF451443	79.4	81.9	35

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635

Figures

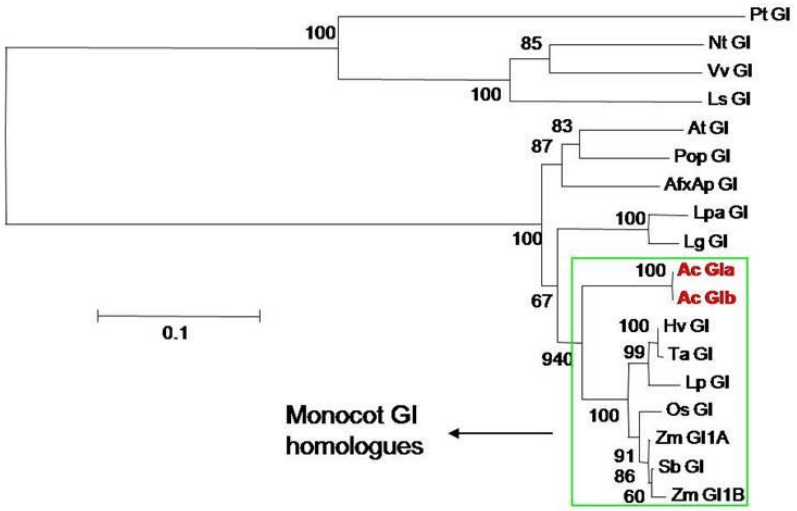
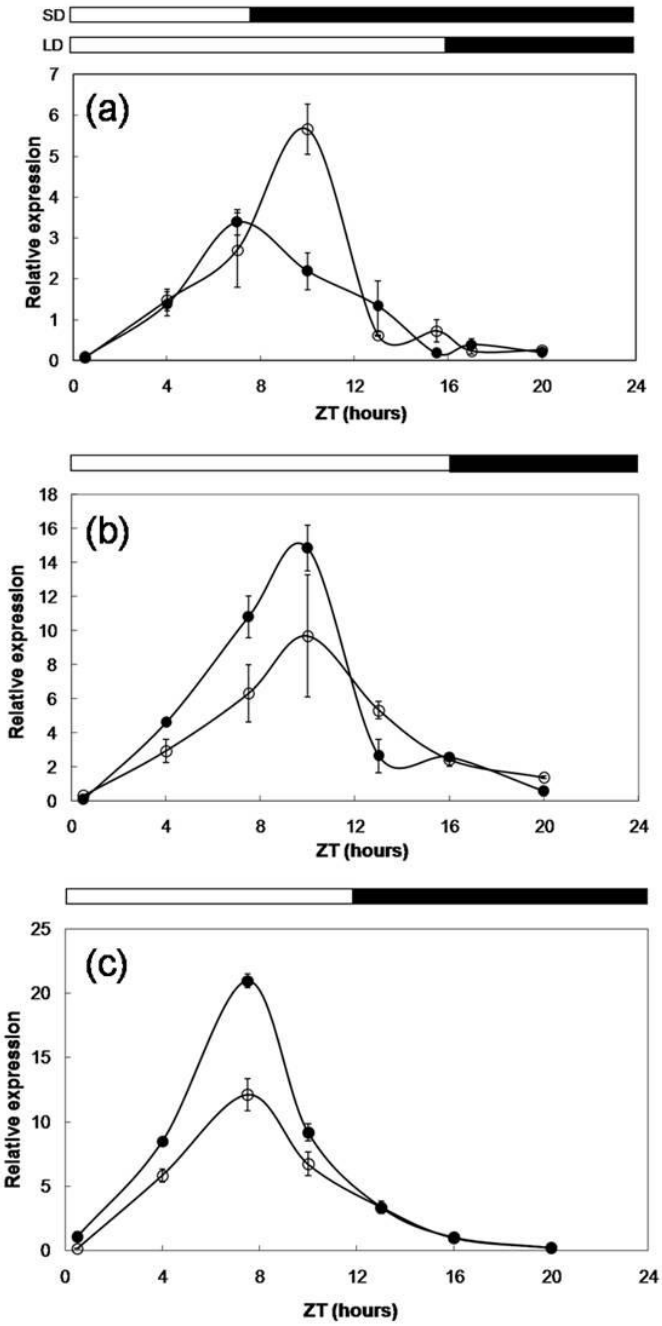


Fig. 1: Section of a neighbour-joining (NJ) tree showing the evolutionary relationships (based on amino acid sequences) among *GI* homologues. Full-length and partial gene sequences were included in this analysis and numbers represent bootstrap values from 1000 replicates. The tree was constructed using Clustal X (Thompson et al., 1997) and the bar indicates 0.1 substitutions per site. Accession numbers: PtGI (*Pinus taeda*): TC105492; NtGI (*Nicotiana tabacum*): DV162002; VvGI (*Vitis vinifera*): TC75660; LsGI (*Lactuca sativa*): TC27622; AtGI (*Arabidopsis thaliana*): NM_102124; Pop GI (*Populus*): TC124305; AfxAp GI (*Aquilegia formosa x pubescens*): TC20877; Lpa GI (*Lemna Paucicostata*): AB210843; Lg GI (*Lemna gibba*): AB210848; Ac GIa (*Allium cepa*): GC232756; Ac GIb: GC232757; Hv GI (*Hordeum vulgare*): AY740523; Ta GI (*Triticum aestivum*): AF543844; Lp (*Lolium Perenne*): DQ534010; Os GI (*Oryza sativa*): NM_001048755; Zm GI1A (*Zea mays*): BK006299; Zm GI1B: BK006298; Sb GI (*Sorghum bicolor*): TC113678. TC numbers refer to unigenes made up of EST's held by the Dana-Farber Cancer Institute (http://compbio.dfci.harvard.edu/tgi/).



655

656 **Fig. 2**: Average expression of *AcGI* over 24 hours in onion leaves. (a) LD (open
657 circles) and SD (closed circles) expression in Renate F_1 (LD variety), relative to
658 *EF1α.* (b) LD expression in Agrifound Dark (SD variety, open circles) and Candy F_1
659 (ID variety, closed circles), relative to ß-*tubulin.* (c) SD expression in Agrifound
660 Dark (open circles) and Candy F_1 (closed circles), relative to ß-*tubulin*. Error bars
661 represent SEM of six replicates. White and black bars represent light/dark cycles.

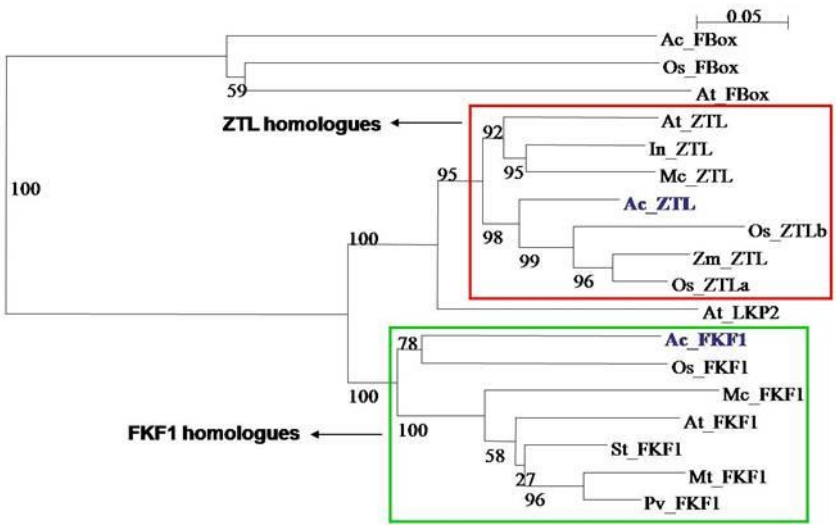
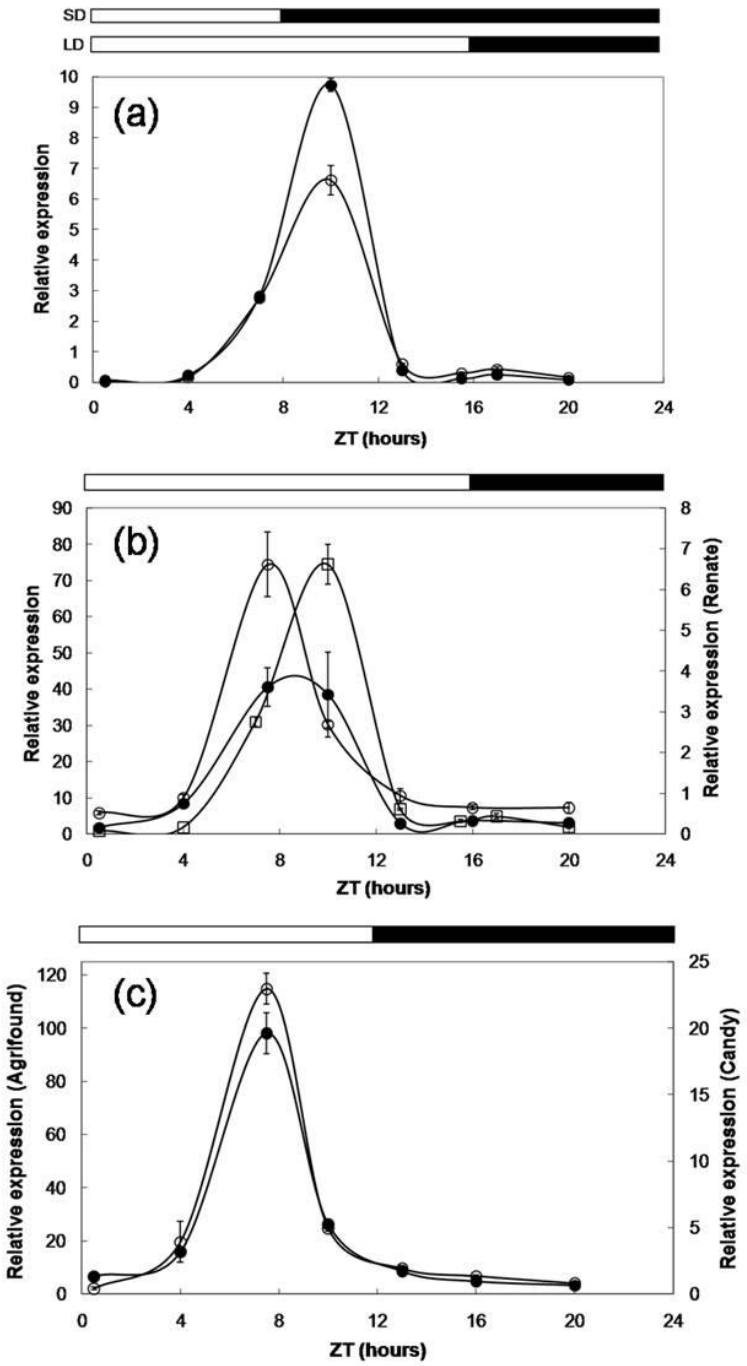


Fig. 3: Neighbour-joining (NJ) tree showing the evolutionary relationships (based on amino acid sequences) between members of the FKF1/ZTL gene family. The tree was constructed using Clustal X (Thompson et al., 1997) and is rooted through a clade containing other F-box genes. Numbers represent bootstrap values from 1000 replicates and the bar indicates 0.05 substitutions per site. Accession numbers: Ac_FBox (*Allium cepa*): GQ232752, Os_FBox (*Oryza sativa*): NM_001067833; At_FBox (*Arabidopsis thaliana*): NM_104033; At_ZTL: NM_105475; In_ZTL (*Impomoea nil*): DQ309278; Mc_ZTL (*Mesembryanthemum crystallinum*): AY371291; Ac_ZTL: GQ232755; Os_ZTLa: NM_001064973; Os_ZTLb: AK111850, Zm_ZTL (*Zea mays*): AY104996; At_LKP2: NM_179652; Ac_FKF1: GQ232754; Os_FKF1- NM_001074600; *Mc_FKF1*: AY371291; At_FKF1: NM_105475; St_FKF1 (*Solanum tuberosum*): DR751881; Mt_FKF1 (*Medicago truncatula*): TC130448; Pv_FKF1 (*Phaseolus vulgaris*): EF643234. TC numbers refer to unigenes made up of EST's held by the Dana-Farber Cancer Institute (http://compbio.dfci.harvard.edu/tgi/).



681

682 **Fig. 4**: Average expression of *AcFKF1* over 24 hours in onion leaves, relative to ß-
683 *tubulin*. (a) LD (open circles) and SD (closed circles) expression in Renate F_1 (LD
684 variety). (b) LD expression in Agrifound Dark (SD variety, open circles) and Candy
685 F_1 (ID variety, closed circles) compared with Renate F_1 (open squares) (c) SD
686 expression in Agrifound Dark (open circles) and Candy F_1 (closed circles). Error bars
687 represent SEM of six replicates. White and black bars represent light/dark cycles.

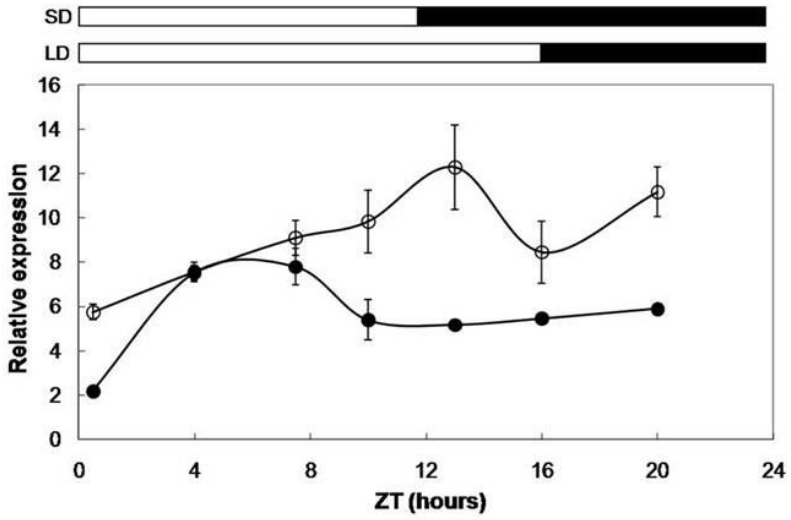


Fig. 5: Average twenty-hour expression of *AcZTL* in the leaves of Agrifound Dark (SD variety) in LD (open circles) and SD (closed circles) conditions relative to ß-*tubulin*. Error bars represent the SEM of six replicates. White and black bars denote light/dark cycles.