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Conservation of flowering time genes in onion
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24	Title: Conservation of Arabidopsis thaliana photoperiodic flowering
25	time genes in onion (Allium cepa L.).
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30	Abbreviations:
31	AcCOL- Allium cepa CONSTANS-LIKE, AcCOP1- Allium cepa CONSTITUTIVE
32	PHOTOMORPHOGENIC 1, AcEF1α- Allium cepa ELONGATION FACTOR 1
33	ALPHA, AcGI- Allium cepa GIGANTEA, AcFKF1- Allium cepa FLAVIN-BINDING,
34	KELCH REPEAT, F-BOX 1, AcFTL- Allium cepa FLOWERING LOCUS T-LIKE,
35	AcPHYA- Allium cepa PHYTOCHROME A, AcZTL- Allium cepa ZEITLUPE, COL4-
36	CONSTANS-LIKE 4, CDF1- CYCLING DOF FACTOR 1, CO- CONSTANS, COP1-
37	CONSTITUTIVE PHOTOMORPHOGENIC 1, CRY- CRYPTOCHROME, EF1 $\alpha$ -
38	ELONGATION FACTOR 1 ALPHA, ELF3- EARLY FLOWERING 3, FKF1- FLAVIN
39	BINDING, KELCH REPEAT, F-BOX 1, FR- far-red, FT- FLOWERING LOCUS T,
40	GI- GIGANTEA, Hd1- Heading date 1, ID- intermediate day, LD- long day, LKP2-
41	LOV KELCH PROTEIN2, LOV- light, oxygen or voltage, NJ- neighbour-joining,
42	PPFD- photosynthetic photon flux density, PHY- PHYTOCHROME, PHYA-
43	PHYTOCHROME A, RACE- rapid amplification of cDNA ends, SD- short day,
44	TOC1- TIMING OF CAB EXPRESSION 1, ZT- zeitgeber time, ZTL- ZEITLUPE.
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#### Abstract

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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,

71 F-BOX 1 (FKF1), GIGANTEA (GI), onion, photoperiod.

#### Introduction

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crop production.

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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

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 GI has been shown to additionally regulate photoperiodic flowering 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

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an appreciable component of Far Red (FR) in the second half of the LD implying the involvement of phytochrome (Quail et al., 1995, Lercari, 1984). Perception of the LD signal takes place in the leaves (Sobeih and Wright, 1986, Brewster, 1990), as with flowering (Knott, 1934), while the response is in the meristem, requiring the transport of a signal within the plant. Other parallels between bulb initiation and floral initiation include a juvenile phase during which plants cannot respond to daylength and the involvement of a homeotic conversion of photosynthetic leaves to either floral organs or swollen bulb scales at the responsive meristem (Komeda, 2004, Lancaster et al., 1996, Massiah, 2007, Sobeih and Wright, 1986). Recent work has identified the *FLOWERING LOCUS T (FT)* protein and its homologue as a part of the mobile signal in *Arabidopsis* and rice (Corbesier et al., 2007, Jaeger and Wigge, 2007, Tamaki et al., 2007).

This study shows that genes controlling the daylength response are conserved between the model plant *Arabidopsis* and onion and supports the hypothesis that these genetic components regulate both bulbing and flowering end-processes.

#### Results

#### Conservation of flowering time genes

A clone of a putative onion *CO* homologue, identified in the *A. cepa* gene index, was assigned the name *Allium cepa CO*-like (*AcCOL*). Full-length sequence information was obtained which showed the gene to be more closely related to *Arabidopsis COL4* than *CO* (Table 1). A phylogenetic analysis based on B-box proteins (Supporting Information Fig. S1) showed that this gene is a group 1b CO-like 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.

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Expression of AcFKF1 was examined in LD and SD grown Renate F<sub>1</sub> plants and a clear diurnal rhythm of AcFKF1 expression was observed under both growth conditions with a peak at ZT10 (Fig. 4 (a)). Whilst Arabidopsis FKF1 is similarly diurnally expressed, expression peaks at around ZT10 in LDs and ZT7 in SDs (Imaizumi et al., 2003). The expression of AcFKF1 was investigated using onion varieties with different daylength responses. Under LD conditions, AcFKF1 is seen to peak around ZT 7-8 in Agrifound Dark, the SD variety (Fig. 4 (b)) compared with ZT 10 in Renate F<sub>1</sub>, the LD variety. Expression peaks at an intermediate time (around ZT 9) in Candy F<sub>1</sub>, the ID variety. There is therefore a distinct difference between the timing of the peak of expression in varieties showing different daylength responses. Under SD conditions, the expression of AcFKF1 peaked around ZT 7-8 in both the SD and ID varieties (Fig. 4c) in contrast to the peak seen in the LD variety which was around ZT10 (Fig. 4a). Thus, as was observed for LD grown plants there is a difference in expression profiles between varieties with different daylength responses. The expression of AcZTL was examined in a SD onion variety (Agrifound Dark) and was shown to be constitutively expressed in both LD and SD grown plants (Fig. 5). There is no diurnal expression pattern and no obvious expression peaks, which is similar to Arabidopsis where ZTL does not show cyclic expression, although ZTL protein levels oscillate, with a peak at ZT10-13 (Kim et al., 2007). The expression profile, coupled with the phylogenetic data, is consistent with AcZTL being

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the onion ZTL homologue and a component of the photoperiod pathway.

#### Discussion

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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<sub>1</sub>) 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<sub>1</sub>) 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 AcFKFI 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

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

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

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

### **Materials and Methods**

### Gene isolation

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

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 EcoR 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<sub>1</sub> 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' 5'and 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 the gene-specific primer 5'-GGCACGAAGAAGAAGATCCGAGGCACTA-3' and 386 387 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 (5'poly dΤ primer

396	GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTT	
397	PCR was carried out using a primer designed to amplify both FKF1/ZTL homological	gues
398	(5'-ATGGTHTGTCARAAYGCDTGGGG-3') and a primer specific to the mod	ified
399	poly dT primer (5'-GCGAGCACAGAATTAATACGAC-3'). Sequence for the	e 5'
400	end of AcFKF1 and AcZTL genes were obtained using 5' RACE PCR (Invitro	gen,
401	Paisley, UK) using the gene-specific primer	5'-
402	CCACCCGCTTCCAAGTGGGCTGGTTTG-3' and the nested primer	5'-
403	CGAGGGTGAGTTCGCGGGAGAGT-3' for AcFKF1 and the gene-spe	cific
404	primer 5'-GCCCTTGCCTACCACATCCACCAAAT-3' and nested primer	5'-
405	CCTGCTGCTGGCATGGTTTCTAACGC-3' for AcZTL. Primers were designed	d to
406	the UTR's of both genes to obtain full-length clones	(5'-
407	TCCAAATCCCAAACCAATTACAGC-3' and	5'-
408	GCATGAAAACGAGCACAATCAGA-3' for AcFKF1,	5'-
409	CACAACCACACTGATTTTCACA-3' and	5'-
410	CTCGTTCCTCCAATCGATCA-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  $52^{\circ}12^{\circ}$ ). 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  $\mu$ mol m<sup>-2</sup> sec<sup>-1</sup> 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

sections, flash frozen in liquid nitrogen and stored at -80°C until required for analysis.

Leaf material was harvested from three separate plants and pooled. Plants were selected for harvest using a random number generator.

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

# Quantitative expression analyses

Total RNA was extracted from 100 mg leaf material harvested at each time-point using Trizol® reagent (Invitrogen, Paisley, UK) following the manufacturer's guidelines. Samples were DNase treated using TURBO DNA-free<sup>TM</sup> (Ambion, Huntingdon, UK) and first-strand cDNA synthesised from 2 μg total RNA using Superscript<sup>TM</sup> II Reverse Transcriptase (Invitrogen, Paisley, UK) following the manufacturers' guidelines. Quantitative real-time PCR was carried out using an I-Cycler (Bio-Rad Laboratories, iCycler Thermal Cycler). To assess the expression of *AcGI* in a LD onion variety (Renate F<sub>1</sub>), reaction volumes of 25 μl were used,

446	containing 1 µl of cDNA, 1x PCR Mastermix containing SYBR green (Eurogentec,
447	Southampton, UK) and $0.4~\mu M$ of each primer (5'-
448	CACAGATGGATTGCTTGATG-3' and 5'-
449	ATTGGCTACGAGATGAACTGCTC-3'). Cycling was carried out as described in
450	the manufacturer's guidelines with an annealing temperature of 61°C. All samples
451	were run in triplicate and data normalised to the expression of Elongation Factor 1
452	Alpha (AcEF1α, accession number CF437531) using the primers 5'
453	TGGCATCCAACTCTAAGGACGAT-3' and 5'-
454	AATGTGAGATGTGGCAATCCA-3'.
455	For all other qRT-PCR analyses, a MESA GREEN qPCR MasterMix for
456	SYBR® green with fluorescein (Eurogentec, Southampton, UK) was used, following
457	the manufacturer's guidelines. Reactions were carried out in 15 $\mu$ l volumes,
458	containing 0.5 $\mu$ l of cDNA. All samples were run in triplicate and all data normalised
459	to $Ac\beta$ -Tubulin (accession number AA451549) using the primers 5'-
460	GTCTTCAGAGGCAAGATGAGCAC-3' and 5'-
461	TCAGTCCAGTAGGAGGAATGTCG-3'. For analysis of AcFKF1, the primers
462	5'CCGGTGCAGTTGTTTATGTTGGAT-3' and
463	5'-TCCCACCCACCACACAGGTACTAT-3' with an annealing temperature of 65°C
464	were used. For AcZTL, the primers 5'-GTTTGGTGGTCTGGCTAAGAGTG-3' and
465	5'-CTCCAGGCATACTGCTACCTGTT-3' with an annealing temperature of 65°C
466	were used. Data from both cycles of the 48 h time course were combined to calculate
467	average expression over a 24 h period.
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## 471 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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# 622 **Tables**

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**Table 1**: Conservation of *Arabidopsis* flowering time genes in onion.

The percentage of the coding region covered was estimated based on Arabidopsis and

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.

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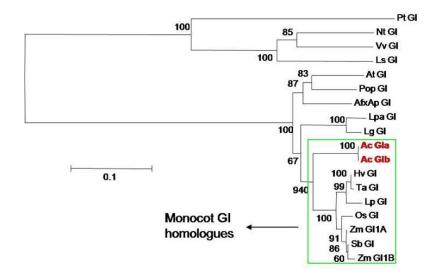
Arabidopsis/rice	Annotation	Accession	Percentage	Percentage	Percentage
gene		number	amino acid	amino acid	of coding
			identity	identity	region
			(Arabidopsis)	(rice)	covered
FKF1	AcFKF1	GQ232754	63.4	69.7	100
GI	AcGI	GQ232756	69.0	75.8	100
ZTL	AcZTL	GQ232755	72.1	75.5	100
CO/Hd1	AcCOL	GQ232751	34.9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

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### 636 Figures

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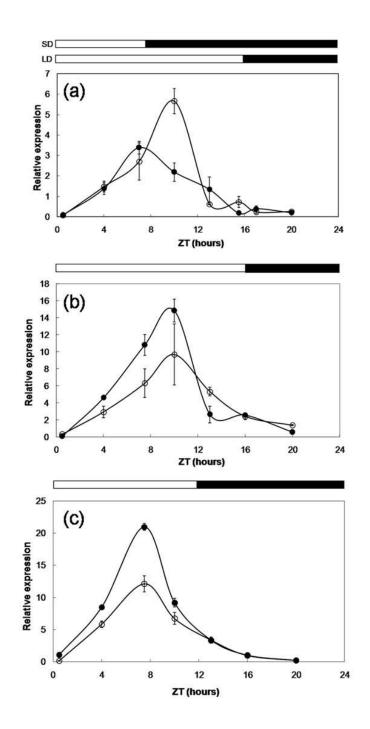
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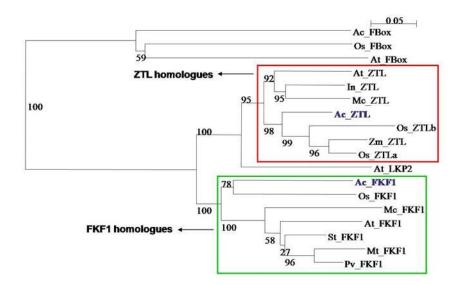
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Fig. 1: Section of a neighbour-joining (NJ) tree showing the evolutionary relationships (based on amino acid sequences) among GI homologues. Full-length and partial gene sequences were included in this analysis and numbers represent bootstrap values 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/).



**Fig. 2**: Average expression of AcGI over 24 hours in onion leaves. (a) LD (open circles) and SD (closed circles) expression in Renate  $F_1$  (LD variety), relative to  $EF1\alpha$ . (b) LD expression in Agrifound Dark (SD variety, open circles) and Candy  $F_1$  (ID variety, closed circles), relative to β-tubulin. (c) SD expression in Agrifound Dark (open circles) and Candy  $F_1$  (closed circles), relative to β-tubulin. Error bars represent SEM of six replicates. White and black bars represent light/dark cycles.



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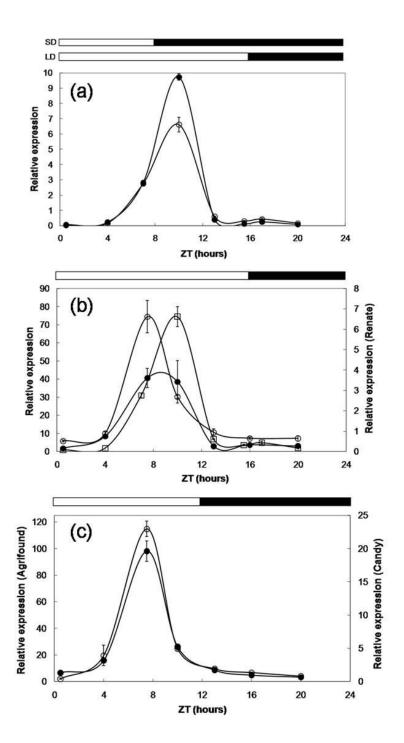
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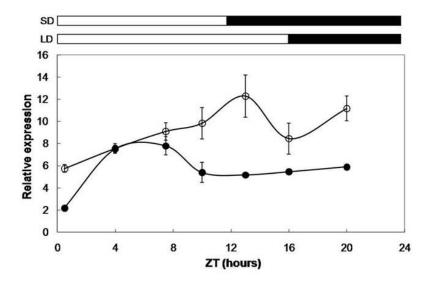
Fig. 3: Neighbour-joining (NJ) tree showing the evolutionary relationships (based on amino acid sequences) between members of the FKF1/ZTL gene family. The tree was constructed using Clustal X (Thompson et al., 1997) and is rooted through a clade containing other F-box genes. Numbers represent bootstrap 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 DQ309278; Mc\_ZTL (Mesembryanthemum crystallinum): (Impomoea nil): Os ZTLa: AY371291; Ac ZTL: GQ232755; NM 001064973; Os ZTLb: AK111850, Zm\_ZTL (Zea mays): AY104996; At\_LKP2: NM\_179652; Ac\_FKF1: GO232754; 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/).

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