Functional Genomics of Photoperiodic Bulb Initiation in Onion (Allium cepa)

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DECLARATION

A small amount of the data presented in this thesis was used to produce a short paper for Acta Horticulturae (Appendix 7). This will be published in a book titled 'V International Symposium on Edible Alliaceae'.

SUMMARY

Bulb initiation is a process which is photoperiodically driven, drawing parallels with flowering. Photoperiodic flowering is well characterised at molecular and genetic levels and occurs when photoreceptors interact with the circadian clock, regulating the expression of *CONSTANS* (*CO*), which itself regulates the expression of floral pathway integrating genes such as *FLOWERING LOCUS T* (*FT*), leading to floral initiation. Two genes which regulate *CO* transcription are *FLAVIN-BINDING, KELCH REPEAT, F-BOX* (*FKF1*) and *GIGANTEA* (*GI*). The onion genome is very large with a high level of duplication, presenting challenges for any molecular-based study. The aim of this study was to test the hypothesis that genes controlling daylength response are conserved between the model plant *Arabidopsis* and onion and hence between the different end-processes bulbing and flowering.

Bulbing ratios were used to measure the response of onion plants to long day (LD) and short day (SD) conditions and the reversibility of the bulbing process. It was shown that bulbing is reversible, with a delay when plants are transferred from SDs to LDs, suggesting the accumulation of an inhibitor. Diurnal expression patterns of onion genes homologous to *Arabidopsis* flowering time genes were examined using quantitative RT-PCR. Phylogenetic analyses were conducted to validate the identity of the homologues. Molecular and phylogenetic data suggests that an onion *GIGANTEA* (*GI*) homologue was isolated. Peaks of expression of ZT10 in LDs and ZT7 in SDs mirror the expression of *Arabidopsis GI*. Homologues of *FKF1* and the circadian clock gene *ZEITLUPE* (*ZTL*) were also characterised. The putative *FKF1* homologue showed different expression patterns in varieties exhibiting different daylength responses. These differences may contribute to the different daylength responses. A *CO*-like gene, which is closely related to *Arabidopsis COL4*, and three *FT*-like genes were also characterised. It appears that many of the genes controlling daylength response are conserved in onion.

LIST OF ABBREVIATIONS

α	alpha
β	beta
<	less than
>	greater than
=	equals
°C	degrees Celsius
%	per cent
µg	micrograms
µl	microlitres
µM	micromolar
AcCOL	*Allium cepa CONSTANS*-like
AcFBox	*Allium cepa F-Box protein*
AcFKF1	*Allium cepa FLAVIN-BINDING, KELCH REPEAT, F-BOX 1*
AcFTL	*Allium cepa FLOWERING LOCUS T*-like
AcGI	*Allium cepa GIGANTEA*
AcPHYA	*Allium cepa PHYTOCHROME A*
AcZTL	*Allium cepa ZEITLUPE*
ANOVA	analysis of variance
AP1	*APETALA 1*
ACSO	S-alk(en)yl-L-cysteine sulphoxides
ASML2	*ACTIVATOR OF SPOMIN::LUC2*
B locus	bolting gene locus
BFT	*BROTHER OF FT*
bp	base pairs
CCA1	*CIRCADIAN CLOCK ASSOCIATED1*
CCT	*CO, CO*-like and *TOC1*
CDF1	*CYCLING DOF FACTOR 1*
CDF2	*CYCLING DOF FACTOR 2*
CDF5	*CYCLING DOF FACTOR 5*
cDNA	copy DNA
cfu	colony forming units

cm	centimetre
CO	*CONSTANS*
COL	*CONSTANS*-like
Col	Columbia
conz1	*CONSTANS OF ZEA MAYS 1*
COP1	*CONSTITUTIVE PHOTOMORPHOGENIC 1*
CRY1	*CRYPTOCHROME 1*
CRY2	*CRYPTOCHROME 2*
CSPD	Disodium 3-(4-methoxyspiro [1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan]-4-yl) phenyl phosphate
Ct	Cycle threshold
CTAB	cetyltrimethylammonium bromide
d.f.	degrees of freedom
DIG	digoxenin
DN	day-neutral
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EF1 α	*ELONGATION FACTOR 1 ALPHA*
ELF3	*EARLY FLOWERING 3*
EST	Expressed Sequence Tag
et al.	and others
FIO1	*FIONA1*
FKF1	*FLAVIN-BINDING, KELCH REPEAT F-BOX*
FLC	*FLOWERING LOCUS C*
FRI	*FRIGIDA*
FT	*FLOWERING LOCUS T*
g	relative centrifuge force
g	grams
GA	gibberellic acid
GFP	Green Fluorescent Protein
GI	*GIGANTEA*
HD1	*HEADING DATE 1*

HD3A	*HEADING DATE 3A*
hr	hours
IAA	3-indolylacetic acid
kb	kilobase pairs
kg	kilogram
l	litre
LB	Luria Broth
IAA	indolylacetic acid
ID	intermediate-day
LATE1	*PEA LATE BLOOMER 1*
LD	long-day
L*er*	Landsberg *erecta*
LFS	*LACHRYMATORY FACTOR SYNTHASE*
LFY	*LEAFY*
LHY	*LATE ELONGATED HYPOCOTYL*
LKP2	*LOV KELCH PROTEIN 2*
LOV	light, oxygen and voltage
M	Molar
MCSO	(+)-S-methyl-L-cysteine sulphoxide
mg	milligrams
MFT	*MOTHER OF FT*
$MgSO_4$	magnesium sulphate
min	minutes
miR172	MicroRNA172
ml	millilitre
ML	maximum likelihood
mm	millimetre
mM	milimolar
NaCl	sodium chloride
NaOH	sodium hydroxide
NASC	Nottingham *Arabidopsis* Stock Centre
NCBI	National Centre for Bioinformatic Information
ng	nanograms
NJ	neighbour-joining

OsCO3	*Oryza sativa CONSTANS LIKE 3*
OsFTL	*Oryza sativa FLOWERING LOCUS T*-like
p	probability (test statistic)
PAM	Percent Accepted Mutations
PAS	PER-ARNT-SIM
PCR	polymerase chain reaction
PCSO	(+)-S-propyl-L-cysteine sulpoxide
PEBP	phosphatidylethanolamine-binding protein
pg	pecograms
PGT2	*class II palatin gene*
PHYA	*PHYTOCHROME A*
PHYB	*PHYTOCHROME B*
PHYC	*PHYTOCHROME C*
PHYD	*PHYTOCHROME D*
PHYE	*PHYTOCHROME E*
PPD-H1	*PHOTOPERIOD H1*
PRR	*PSEUDO RESPONSE REGULATOR*
RACE	Rapid Amplification of cDNA Ends
RFT1	*RICE FLOWERING LOCUS T 1*
RKIP	Raf kinase inhibitor protein
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
RT-PCR	reverse transcription- polymerase chain reaction
SD	short-day
SDS	sodium dodecyl sulphate
sec	seconds
SED	standard error of the difference
SEM	standard error of the mean
SOC	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*
SPA1	*SUPRESSOR OF PHYA-105*
SSC	saline-sodium citrate

TEM	*TEMPRANILLO*
TFL1	*TERMINAL FLOWER 1*
TOC	*TIMING OF CAB EXPRESSION 1*
tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
tris-HCl	2-Amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride
TSF	*TWIN SISTER OF FT*
TUB	*TUBULIN*
UK	United Kingdom
UTR	untranslated region
UPL	ubiquitin-protein ligase
VRN2	*VERNALISATION 2*
v/v	volume by volume
w/v	weight by volume
ZIM	*ZINC FINGER PROTEIN EXPRESSED IN INFLORESCENCE MERISTEM*
ZT	zeitgeber time
ZTL	*ZEITLUPE*

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction to Onion

The onion (*Allium cepa* L.) is a monocotolydenous plant which belongs to the order Asparagales, the second most important monocot order (Stevens, 2001 onwards; Kuhl *et al*., 2004). Other important plants in the order Asparagales include asparagus, garlic, leek, Aloe, orchids and Narcissus. Onion belongs to the family *Aliaceae* and the genus *Allium* (Stevens, 2001 onwards). The genus *Allium* comprises over 700 species which can be found throughout the tropical, boreal, temperate and sub-temperate regions of the world (Fritsch and Friesen, 2002). Onions belong to the section *cepa*, a section which includes the Japanese bunching onion, *A. fistulosum* (Brewster, 2008). They are thought to have originated in central Asia and are not found in the wild, so may be an ancient hybrid or may simply have become extinct. Other wild species within the section *cepa* can be found in areas such as stony slopes and riverbanks. These species tend to have a long annual growth cycle and can take up to ten years to reach flowering maturity. There is evidence that onions have been cultivated for more than 4700 years. In fact, carvings of onions can be found on pyramid walls and in tombs in Egypt (Fritsch and Friesen, 2002). South-West Asia is regarded as the main area of domestication and variability. However, regions such as the Mediterranean are also considered as sites of great diversity.

Onions are grown in every part of the world where plants are farmed and can be grown from seeds, bulbs or sets (small bulbs). They show great variation in many characteristics such as size, colour, shape and pungency (Griffiths *et al*., 2002). Some Southern European varieties have been known to produce bulbs up to 1 kg in weight (Fritsch and Friesen, 2002). Cultivated onions (of the section *cepa*) fall into two major groups: the Common Onion Group and the Aggregatum Group. Varieties from the Common Onion Group form large single bulbs. The most economically important varieties are part of this group. Crops are either harvested at the mature bulb stage or at an immature stage as salad onions. Varieties from the Aggregatum Group are smaller and tend to form clusters of bulbs. The most economically important member of the Aggregatum group is the shallot. For the purpose of this

study, the common onion will be discussed. However, many of the same principles apply to members of the Aggregatum group.

1.2 Economic Importance

Onions are consumed throughout the world and are available in forms such as fresh, frozen, pickled and dehydrated. They are very widely used in Asian cuisine and are used as the base for most Indian dishes. In 2007, 68 million tonnes of onions were produced worldwide (Table 1.1). This highlights the economic importance of the crop throughout the world. The latest consumption figures show that 48 million tonnes of onions were consumed in 2003 (Table 1.2). In the UK the majority of onions produced are consumed within the country, with only 7000 tonnes being exported in 2005 (Table 1.3). More onions are consumed than are produced in the UK, the shortfall being accounted for by imports of around 300,000 tonnes a year.

Table 1.1: Production of onions (tonnes) in the UK and worldwide, 2003-2007 (FAOSTAT, 2008). (E)=estimated figures.

Year	UK	World
2003	390,000	57,246,783
2004	351,400	62,804,921
2005	404,500	64,223,568
2006	440,000 (E)	68,178,446
2007	375,000 (E)	68,063,462

Table 1.2: Consumption of onions (tonnes) in the UK and Worldwide, 1999-2003 (FAOSTAT, 2008)

Year	UK	World
1999	517,704	42,586,034
2000	509,012	44,315,017
2001	552,330	45,848,974
2002	491,495	46,863,762
2003	586,384	47,921,305

Table 1.3: Import/Export statistics for onions in the UK (tonnes), 2001-2005 (FAOSTAT, 2008).

Year	Import	Export
2001	240,203	4,318
2002	270,115	5,474
2003	279,050	12,454
2004	285,611	7,977
2005	315,636	6,927

1.3 Flavour, Colour and Potential Health Benefits of Onions

1.3.1 Biosynthesis of flavour precursors in onion and potential health benefits

An important feature associated with onions is the characteristic flavour and odour, leading to the culinary value of the crop. Plants are odourless until damaged, upon which they generate volatile sulphur-containing compounds (Jones *et al.*, 2004). These compounds come from the enzymatic hydrolysis of S-alk(en)yl-L-cysteine sulphoxides (ACSO's), with pyruvic acid and ammonia being produced as by-products (Mallor and Thomas, 2008). The reaction is catalysed by the enzyme alliinase. This enzyme is located in the vacuole and is released into the cytoplasm upon cell damage (Lancaster *et al.*, 1990). There are three ACSO's which are commonly found in onion (Griffiths *et al.*, 2002). The first is *trans*-(+)-*S*-(1-propenyl)-L-cysteine sulphoxide (PeCSO, isoalliin) which is responsible for the tear-producing effect. The other two are (+)-*S*-methyl-L-cysteine sulphoxide (MCSO, methiin) and (+)-*S*-propyl-L-cysteine sulpoxide (PCSO, propiin). The biosynthetic pathway producing these compounds is not yet fully understood, although recent work has focussed on improving the current knowledge of this pathway (Hughes *et al.*, 2005). This study focussed on *S*-allyl cysteine sulphoxide (alliin), a flavour precursor which is found in only trace amounts in onion but is abundant in garlic. Allyl cysteine was implicated as an intermediate in the synthesis of alliin in both garlic and onion, expanding the knowledge of ACSO synthesis.

The major site of synthesis of ACSO's in onion has been shown to be the leaves (Mallor and Thomas, 2008). It had been proposed that ACSO's are recycled in the plant, the leaf blades supplying ACSO's to the bulb scales during bulb

development and senescing bulb scales recycling their ACSO's to developing younger scales (Lancaster *et al.*, 1986). This was further tested by growing plants in inductive (in terms of bulb initiation) long-day (LD) and non-inductive short-day (SD) conditions (Mallor and Thomas, 2008). In the SD treatment, ACSO's accumulated in the foliage leaves. In the LD treatment, ACSO's initially accumulated in the foliage leaves and then moved to the bulb during bulb development. Plants which were grown in SDs accumulated more ACSO's than those in LDs, indicating that the length of the vegetative growth phase can drastically affect the level of flavour compounds in the plant. This work supports the theory of a recycling of ACSO's within the plant.

1.3.2 Flavonoid content of onions

Flavonoids are compounds which have been implicated as potential antioxidants and are present at high levels in onions (Griffiths *et al.*, 2002). There are two major groups of flavonoids in onions, the anthocyanins and the flavonols. The flavonols are often found in the skin, giving a yellow/brown colour to these onions. They can also be found in bulb scales, with the concentration increasing towards the outer scales. Sixteen different flavonols have been identified in onion including the glycosylated derivatives of quercetin (Price and Rhodes, 1997). The anthocyanins are responsible for the red colour of onions. Several anthocyanins have been isolated from onion, including cyanidin 3-glucoside, cyanidin 3-laminaribioside, cyanidin 3-malonylglucoside and cyanidin 3 malonyllaminaribioside (Donner *et al.*, 1997)

1.3.3 Potential health benefits of onions

For many years, the perceived health benefits of consuming onions have been investigated. Many potential health benefits have been proposed, including anti-carcinogenic and anti-bacterial properties. The ACSOs have been proposed to be responsible for the antibacterial and antifungal properties of onion (Al-Delaimy and Ali, 1970; Yin and Tsao, 1999). It has been proposed that flavonoids have antioxidant and anti-carcinogenic properties. This implies a possible health benefit of regular consumption. In addition, some of the downstream products of ACSO

biosynthesis are reported to be responsible for the anti-thrombotic, anti-platelet and anti-asthmatic activity of onion (Griffiths *et al.*, 2002). It has been shown that extracts of Welsh onion (*Allium fistulosum L.*) can lower blood pressure in rats through the inhibition of thromboxane production (Chen *et al*., 2000). Onion extracts have also been shown to have a hypoglycaemic effect in diabetic rats and rabbits (Srinivasan, 2005). This implies a potential use for onions (or onion extracts) in the treatment of diabetes mellitus.

The antioxidant properties of onions seem to be related to anthocyanin content (Griffiths *et al*., 2002). Anthocyanins are known to prevent plant cells being damaged by reactive oxygen species and free radicals. It is proposed that consuming foods containing such compounds can help reduce oxidative stress in humans. Studies into the anti-carcinogenic properties of onions show contrasting results. For example, a French study showed that the risk of breast cancer decreased when the intake of onion, garlic and fibre was increased (Challier *et al*., 1998). In contrast, a study in the Netherlands showed no link between increased onion consumption and breast cancer (Dorant *et al*., 1995).

A recent study investigated the antioxidant and free radical scavenging activities of phenols from onion (Prakash *et al*., 2007). It was found that the antioxidant activity was related to the total phenolic content. Antioxidants scavenge free radicals, leading to perceived anti-carcinogenic properties. They are also associated with reduced risk of cardiovascular disease. The onion varieties studied here showed a high phenol content, leading to possible health benefits. One particularly abundant compound was the flavonol quercetin, a compound which has been previously reported to benefit human health (Bingham *et al.*, 2003). The problem is that the highest proportion of quercetin is found in the outer, dry layers of the onion. These layers are usually discarded, negating some potential health benefits. However, this compound is found in the scale leaves of yellow onions.

There are many other perceived health benefits associated with onion consumption. Care must be taken as many compounds are either located in the outer layers of the bulb or are disrupted on cooking. Despite this, the numerous potential health benefits coupled with the widespread consumption of onions worldwide, further highlights the importance of the crop.

1.4 Physiology of the Onion Plant

1.4.1 The bulbing process

The onion is a biennial plant, the bulb being an overwintering stage of the life cycle (Lancaster *et al.*, 1996). Hence, the onion bulb is a storage organ. In terms of crop production, onions tend to be grown as annual crops. The plant itself is composed of leaves arising from a flattened stem (base plate), giving rise to older leaves on the outside and younger leaves on the inside. Leaves are composed of a photosynthetic leaf blade and a storage leaf base or scale. When bulb initiation occurs, the leaf sheaths swell and initiation of bladeless bulb scales occurs. The bulb scales form the storage tissue of the bulb. As the bulb matures, two or three foliage leaf initials are produced at the base (Brewster, 1990). These will elongate in the following season, producing bulb sprouts. The basic structure of an onion bulb is illustrated in Fig. 1.1.

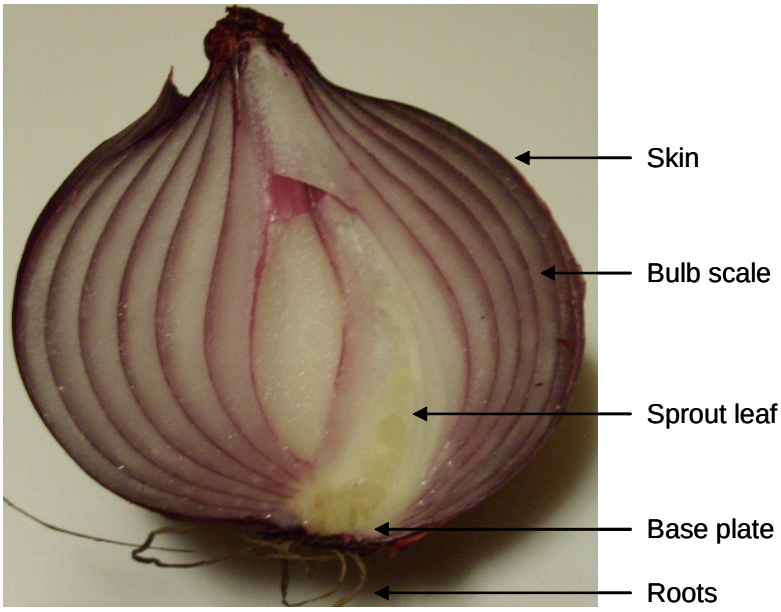


Fig. 1.1: Cross-section of an onion bulb.

Bulb initiation can be quantified using the 'bulbing ratio' (maximum bulb diameter/minimum neck diameter, Fig. 1.2). When this value increases to a value greater than two, bulbing is considered to have been initiated (Clark and Heath, 1962). The bulb diameter is usually measured in two different planes to allow for the

fact that not all bulbs are round. Different methods of quantifying bulb initiation have been described, but the bulbing ratio is the preferred method as it can be measured non-destructively.

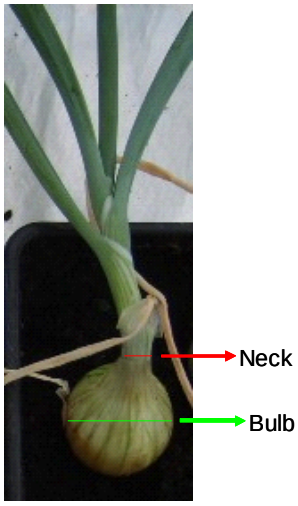


Fig. 1.2: Measurements to be taken for the calculation of bulbing ratios

1.4.2 Photoperiodic nature of the bulbing process

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 (16 or more 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. Care must be taken when selecting a cultivar as long-day (LD) cultivars will not produce a bulb at latitudes around the equator whereas short-day (SD) cultivars will produce a very small bulb almost as soon as the leaves have emerged in LD conditions. Generally, each country or area will have its traditional varieties which have been bred to produce their crop most effectively in that region.

Table 1.4: The photoperiod response of different onion cultivars (adapted from Brewster, 2008).

Cultivar	Daylength at which bulbing is initiated
Long-day	>16 hours
Intermediate day	13-14 hours
Short day	12 hours

1.4.3 Other factors affecting bulb initiation (summarised in Fig. 1.3)

There are factors other than daylength that affect bulb initiation. One such factor is plant age. Bulbs will grow more rapidly in older plants while young plants will not initiate bulbing unless they have at least 4 leaves (Sobeih and Wright, 1986). This implies the presence of a juvenile phase during which plants will not initiate bulbing, even under inductive conditions. In addition, onion leaves must be constantly exposed to an inductive photoperiod in order to initiate and complete bulbing (Brewster, 1997). Bulbing can be reversed if plants are transferred to a non-inductive photoperiod. These observations draw comparisons with the photoperiodic control of flowering which is discussed in detail in section 1.5.

Temperature is another important factor affecting bulb initiation. Elevated day or night temperatures have been shown to accelerate bulbing (Steer, 1980). Temperature has been suggested as the major factor controlling bulbing in short-day onions. It has been shown that bulb initiation requires a combination of a minimum thermal time and a minimum daylength (Lancaster *et al*., 1996). This suggests a major role for temperature in bulb initiation.

Light quality has a major affect on bulb initiation (Austin, 1972). It has been shown that phytochrome has a role in the photoperiodic control of bulbing (Lercari, 1984; Sobeih and Wright, 1987). Therefore, the rate of bulbing in a particular photoperiod is dependent on light quality, where high levels of far-red light accelerate bulbing. Thus, it is the ratio of red:far-red light which controls bulb initiation (Mondal *et al*., 1986). Bulbing will not be initiated under high red:far-red ratios. Blue light, to a lesser extent, also controls bulb initiation (Terabun, 1965).

Light quantity also affects bulb initiation. It has been shown that high irradiance can accelerate bulbing in LDs (Wright and Sobeih, 1986). However, it seems that light quality has a more pronounced effect on initiation. Once initiated, it

has also been shown that elevated carbon dioxide levels can accelerate bulbing (Daymond *et al.*, 1997). However, the major factor controlling bulb initiation in LD onions is daylength and this will usually be the limiting factor. This allows for a seasonal response, bulbing being initiated when the days get longer (in the spring in the UK). This then allows for rapid bulb development under favourable environmental conditions (e.g. warm temperatures and high irradiance during early to mid summer).

Gaining a greater knowledge of the daylength response in onion is important for adapting new varieties for growth at different latitudes, as well as aiding germplasm screening for choice of current varieties. Although much is known about the physiological aspects of onion development and initiation of bulbing, little work has been done on the genetics. This is despite the obvious economic value of the crop (section 1.2). Conversely, the photoperiodic control of flowering (especially in model species) has been studied thoroughly. This allows for parallel genetic studies on the photoperiodic control of bulbing to be undertaken. The genetic pathway controlling photoperiodic flowering is discussed in section 1.5.

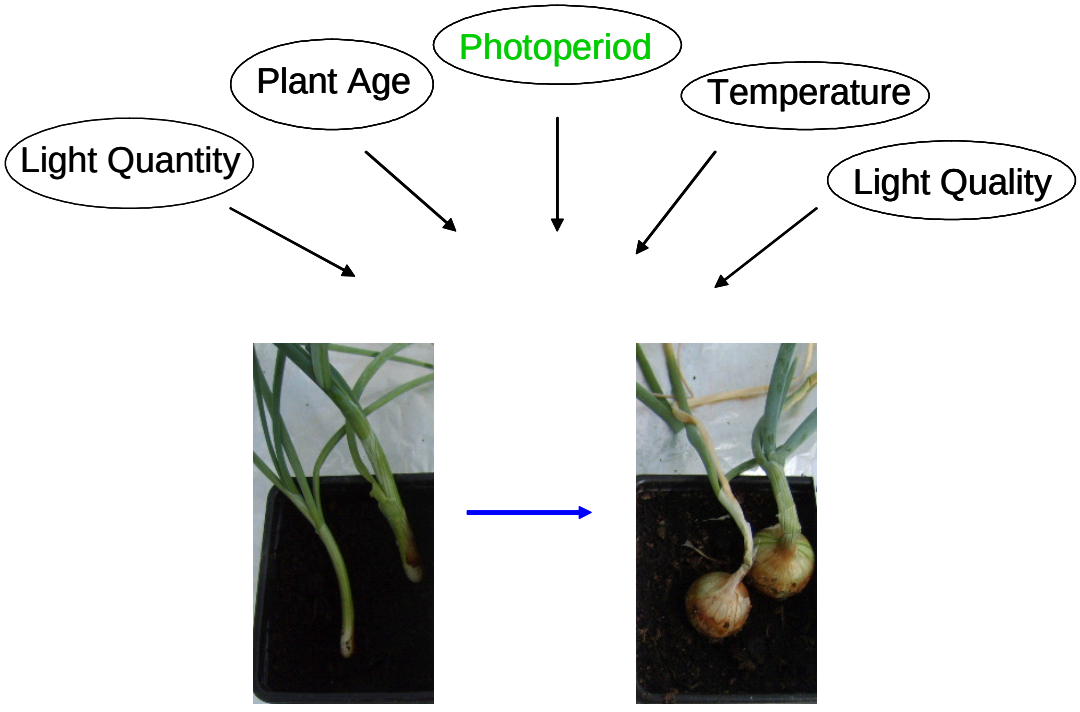


Fig. 1.3: The major factors controlling bulb initiation. Photoperiod is considered the most important factor in temperate onions.

1.4.4 The hormonal control of bulbing

Many attempts have been made to isolate a hormone which is singly responsible for bulbing. There have been many theories about different hormones which have a certain role in bulbing, none of which have been particularly conclusive. However, auxin, cytokinin, gibberellins and ethylene have all been shown to have some role (Brewster, 2008). Ethylene has been shown to stimulate bulb initiation, but ethylene antagonists do not repress bulb initiation in inductive photoperiods (Lercari, 1983). This suggests that ethylene is not involved in the endogenous control of bulbing. It has also been shown that the level of 3-indolylacetic acid (IAA) increases when plants are exposed to an inductive photoperiod (Clark and Heath, 1962). This then decreases rapidly after 5-7 days and eventually reaches a level lower than that of non-induced plants. IAA has been shown to induce bulbing in non-inductive photoperiods. However, this has only been observed at one particular concentration and in plants which have had their roots removed. The exact role of IAA in bulb initiation remains unclear.

Several studies also indicate that gibberellins have a role in bulb initiation (Brewster, 2008). It has been shown that an inhibitor of gibberellin biosynthesis can initiate bulbing in non-inductive photoperiods (Mita and Shibaoka, 1984). This suggests an inhibitory role for gibberellins in bulb initiation. Gibberellin has been shown to promote flowering in *Arabidopsis* (Blazquez *et al*., 1998). This promotion is achieved through activation of the promoter of the floral integrator gene *LEAFY* (*LFY*). Gibberellin has also been shown to inhibit flowering in some species, for example strawberry (Thompson and Guttridge, 1959). It has been suggested that gibberellin is involved in onion flowering as high activity is observed during bulb storage, with a peak shortly before the growth of flower initials (Rabinowitch, 1990). It is possible that gibberellin inhibits bulbing but promotes flowering in onion.

Cytokinin has also been proposed to have a role in bulb initiation. The level of cytokinins is seen to increase upon transfer to an inductive photoperiod, reaching a maximum around 29 days after transfer (Lercari and Micheli, 1981). The precise role of the plant hormones discussed here in bulb initiation is unclear. However, it is clear that there is a role for these hormones in either bulb initiation or bulb development.

1.4.5 Onion flowering

An interesting complication to consider is the fact that, in addition to bulbing, onions produce flowers in order to set seed. Flowering will normally occur in the second growing season, but can occur in the first growing season under favourable conditions (Rabinowitch, 1990). When onions are grown for the harvesting of mature bulbs, flowering is undesirable as it results in a very poor quality crop. However, if plants are grown for seed then yield depends entirely on the flowering response.

Flowering will occur following a period of vernalisation, provided the juvenile phase has been passed (Brewster, 1997). The optimum temperature for vernalisation of Japanese and Northern European varieties is reported to be 9-12 ºC. The number of days required to induce the vernalisation response is dependent on cultivar (approximately 20-40 days). Long photoperiods have been shown to shorten the period of time required to vernalise plants (Brewster, 1983). After vernalisation, the rate of floral development increases with photoperiod and temperature (in the range of 6-12 ºC).

The stages in the life cycle of onions can be divided into four phases: the juvenile phase, the thermophase, the competition phase and the completion phase. During the juvenile phase, floral initiation will not occur as plants must reach a critical size. During the thermophase, plants can be induced to flower by vernalisation. However, inflorescences are destroyed during the competition phase if conditions favour bulb development. Therefore, it is bulb initiation which is the photoperiod response in onion and the photoperiod response genes, characterised in *Arabidopsis*, are hypothesized to be involved in bulb initiation. This appears to be the case in potato where the *Arabidopsis CONSTANS* (*CO*) gene, when overexpressed in potato, impairs tuberisation (Martínez-García *et al.*, 2002). Tuberisation is the photoperiod response in potato, a response which is initiated by SDs. This is discussed in more detail in section 1.8.1.

Another interesting complication in the physiology of onion development is the formation of small bulbs (referred to as bulbils) in place of normal flowers and seed capsules (Brewster, 2008). If bulbs are stored at high temperatures, growing points in the inflorescence can revert to vegetative growth. If a flower has already started to develop at the start of the heat treatment, this may cause the meristem to

develop bulbils. The degree of bulbil development depends on which stage the inflorescence is at when the heat treatment is applied. There is no evidence for bulbil development in response to photoperiod. It is bulb initiation which is considered to be the photoperiod response in onion.

1.5 The Photoperiodic Control of Flowering in *Arabidopsis*

1.5.1 Overview

Flowering has been well characterised at molecular and genetic levels (Fig. 1.4). The flowering time genes in *Arabidopsis* mainly function in six different pathways: autonomous; vernalisation; gibberellin (GA); temperature; light quality and photoperiod (Jack, 2004). The photoperiod pathway is of particular interest for this study. In photoperiodic flowering (Fig. 1.5), light interacts with the circadian clock (through *PHYTOCHROME* and *CRYPTOCHROME* genes) and the timing of the clock is controlled by feedback loops involving *TIMING OF CAB EXPRESSION 1* (*TOC1*, discussed in section 1.5.2). *CO* expression is high at the end of long-days and the CO protein is degraded at night. *CO* regulates the expression of the floral integrating genes *FT* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)* leading to floral initiation. 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 (discussed in more detail in section 4.1).

Two of the genes which have been shown to regulate *CO* expression are *GIGANTEA* (*GI)* and *FLAVIN-BINDING, KELCH REPEAT, F-BOX 1* (*FKF1)*. These genes regulate *CO* transcription by repressing *CYCLING DOF FACTOR 1* (*CDF1*), a repressor of *CO* (Sawa *et al.*, 2007). A recent study showed that *FKF* and *GI* form a complex which directly regulates *CDF1* stability (Sawa *et al.*, 2007). The roles of individual flowering time genes are discussed separately in sections 4.1, 5.1, 6.1 and 7.1.

A final complication to consider is the involvement of MicroRNA's. A recent study showed that MicroRNA172 (miR172) regulates photoperiodic flowering through *FT*, independently of *CO* (Jung *et al*., 2007). *GI* has been shown to regulate

miR172, shedding light on a new genetic pathway controlling photoperiodic flowering. It appears that as more genes involved in the photoperiod response are discovered, the genetic pathway becomes more complex. There may well be other genes, which are yet to be characterised, that have a significant role in the photoperiod response.

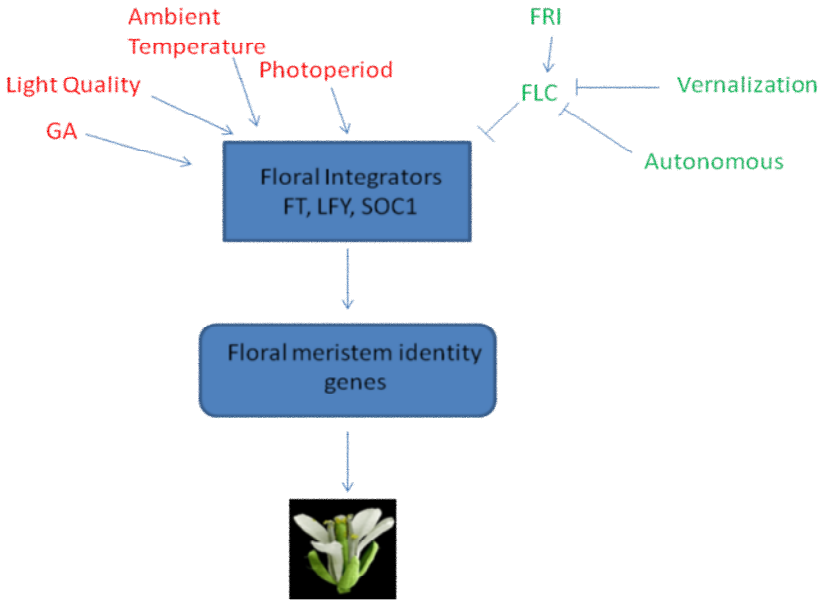


Fig. 1.4: The different floral initiation pathways in *Arabidopsis*. *FRI= FRIGIDA*, *FLC=FLOWERING LOCUS C* (Adapted from Jack, 2004). *Arabidopsis* flower picture copyright Juergen Berger, Max Planck Institute for Developmental Biology.

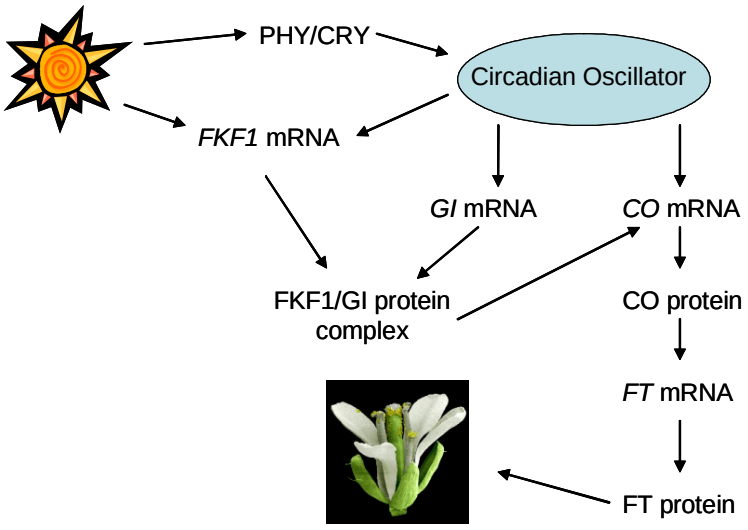


Fig. 1.5: Photoperiodic control of flowering in *Arabidopsis* (adapted from Massiah, 2007). *Arabidopsis* flower picture copyright Juergen Berger, Max Planck Institute for Developmental Biology.

1.5.2 The circadian clock

The circadian clock is an endogenous timekeeping mechanism which is controlled by various feedback loops (Jackson, 2008). This allows the clock to continue to cycle under constant conditions. Many responses are mediated by the clock including shade avoidance, stomatal opening/closing, photosynthesis and notably floral initiation (Hotta *et al*., 2007). The basic feedback loop involves the positive regulation of *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) by *TOC1* (Alabadi *et al*., 2001). *LHY* and *CCA1* proteins then negatively regulate *TOC1* by binding to a motif called the EVENING ELEMENT in the *TOC1* promoter. At the end of a day, the levels of CCA1/LHY are low enough for *TOC* expression to occur. In addition, *TOC1* induces *LHY/CCA1* expression in an indirect manner. *ZEITLUPE* (*ZTL)* also regulates *TOC1* by targeting the protein for degradation via the 26S proteosome (Mas *et al*., 2003). An additional consideration is the role of Clock-Associated Pseudo-Response Regulators (*PRR9*, *PRR7* and *PRR5*). It has been suggested that these genes act antagonistically with *CCA1*/*LHY* and activate *CO* by repressing *CDF1* (Nakamichi *et al*., 2007). A final component to consider is a recently characterised gene termed *FIONA1* (*FIO1*). This gene is proposed to regulate period length in the clock (Kim *et al*., 2008a).

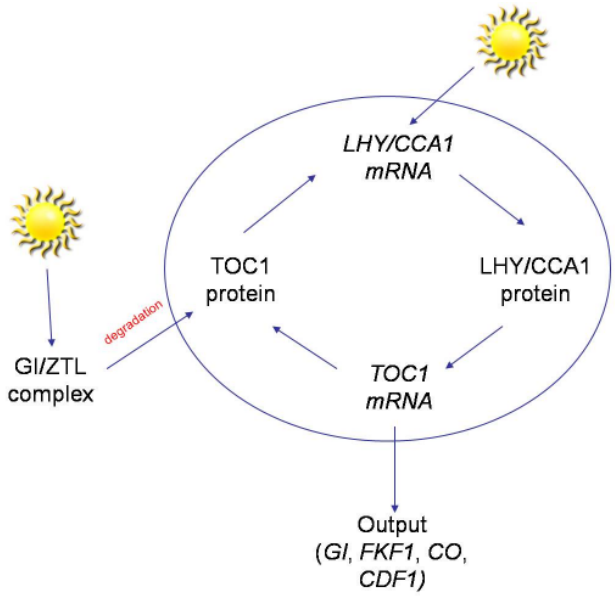


Fig. 1.6: Schematic representation of the function of the circadian clock in the photoperiodic flowering pathway (adapted from Alabadi *et al*., 2001 and Mas *et al*., 2003).

1.5.3 The role of light

Light plays a critical role in the photoperiod response in *Arabidopsis*, through both blue and red light photoreceptors. As mentioned in section 1.5.2, light interacts with the circadian clock as part of the photoperiodic flowering pathway. Light entrains the clock to 24-hour cycles (Michael *et al*., 2003). This entrainment of the clock is achieved through both cryptochromes in blue light and phytochromes in red light (Somers *et al*., 1998). *PHYB* is the major photoreceptor for circadian control in high intensity red light and *PHYA* is important in low intensity red light. *CRY1* mediates high intensity blue light signals to control the period of the clock. In addition, *CRY1* and *PHYA* transmit low fluence blue light to the clock. The role of CRY2 in transmitting light to the clock is discussed in section 7.1.2.

Light is also important in promoting the formation of a complex between *GI* and *FKF1*. This complex is specifically formed under blue light conditions (Sawa *et al*., 2007). Light is also important in controlling the stability of the CO protein. In the dark, *CONSTITUTIVE PHOTOMORPHOGENIC 1* (*COP1*) promotes the degradation of the CO protein (Jang *et al*., 2008). This leads to a delay in flowering in SDs. CO protein is also degraded in the morning. This is mediated by *PHYB* (Valverde *et al*., 2004).

1.6 Conservation of the Photoperiodic Flowering Pathway

The genetic network controlling photoperiodic flowering in *Arabidopsis* is proposed to be conserved across plant species. Homologues of the major flowering time genes *CO*, *FT* and *GI* have been characterised from many species (Table 1.5). Work has been carried out on the conservation of *Arabidopsis* flowering time genes in legumes (Hecht *et al*., 2005). Homologues of *CO*, *FT*, *GI* and other flowering time genes have been isolated and characterised in the model species *Medicago truncatula* (barrel medic) and the economically important garden pea (Hecht *et al*., 2005). Homologues of flowering time genes have also been isolated from many other economically important species including wheat, maize and *Brassica* species (Robert *et al*., 1998; Xiang *et al*., 2005; Miller *et al*., 2008).

Table 1.5: Conservation of the *Arabidopsis* flowering time genes *CO, FT* and *GI* across plant species. This table focuses on genes which have been functionally characterised.

Species	Genes	Key References
Rice	*CO, FT, GI*	(Yano *et al.*, 2000; Kojima *et al.*, 2002; Hayama *et al.*, 2003)
Wheat	*CO, FT, GI*	(Nemoto *et al.*, 2003; Xiang *et al.*, 2005; Li and Dubcovsky, 2008)
Barley	*FT, GI*	(Dunford *et al.*, 2005; Faure *et al.*, 2007)
Pea	*CO, FT, GI*	(Hecht *et al.*, 2005; Hecht *et al.*, 2007)
Maize	*CO, FT*	(Danilevskaya *et al.*, 2008; Miller *et al.*, 2008)
Perrenial ryegrass	*CO*	(Martin *et al.*, 2004)
Darnel ryegrass	*FT*	(King *et al.*, 2006)
Potato	*CO*	(Martínez-García *et al.*, 2002)
Oilseed rape (and other Brassicas)	*CO*	(Robert *et al.*, 1998)
Japanese morning glory	*CO, FT*	(Liu *et al.*, 2001; Hayama *et al.*, 2007)
Sugar beet	*CO*	(Chia *et al.*, 2008)
Radish	*GI*	(Curtis *et al.*, 2002)
Barrel medic	*CO, FT, GI*	(Hecht *et al.*, 2005; Paltiel *et al.*, 2006)
Tomato	*FT*	(Lifschitz *et al.*, 2006)
Norway spruce	*FT*	(Gyllenstrand *et al.*, 2007)
Squash	*FT*	(Lin *et al.*, 2007)
Moss *(Physcomitrella patens)*	*CO*	(Zobell *et al.*, 2005)
Lombardy poplar	*FT*	(Igasaki *et al.*, 2008)
Red goosefoot	*FT*	(Cháb *et al.*, 2008)

The photoperiod pathway has been studied in detail in rice, a SD plant. There is a very strong conservation of the genes within this pathway, despite a flowering response being induced in SDs and not LDs. It has been shown that *Hd3a* (the rice *FT* orthologue) promotes flowering, specifically under SD conditions (Kojima *et al.*, 2002). In contrast to *Arabidopsis*, *Hd3a* expression is inhibited by *Hd1* (the rice *CO* orthologue). Thus, flowering is inhibited in LDs in a similar way to which it is promoted in LDs in *Arabidopsis* (Hayama *et al.*, 2003). A *CO* orthologue has also been characterised in the SD plant *Pharbitis nil* (Liu *et al.*, 2001). This suggests the genes involved in the photoperiod response may be conserved in many SD plants.

In barley, the major component of photoperiod flowering (in LDs) is the pseudo-response regulator, *PHOTOPERIOD-H1* or *Ppd-H1* (Decousset *et al*., 2000). A reduced photoperiod response is seen in the *ppd-h1* mutant (Turner *et al*., 2005). This is explained by an altered circadian expression of *CO* and has reduced expression of *FT*. Reduced photoperiod response is highly desirable in barley, so this gene is of particular interest. This case highlights the fact that some differences exist in the genetic control of photoperiod response. However, it seems that many other photoperiod response genes are still conserved in barley. This includes *GI*, which is proposed to play an important role in the photoperiod response in barley (Dunford *et al*., 2005).

Another interesting case is floral transition in sugar beet (*Beta vulgaris*). Bolting is associated with rapid stem elongation and is undesirable in crop production. Sugar beet is an obligate LD plant where bolting is strongly linked to the early bolting (*B*) gene locus (Chia *et al*., 2008). A sugar beet gene homologous to *Arabidopsis CO* has been shown to functionally complement *Arabidopsis co* mutant plants (Chia *et al*., 2008). However, this gene maps to a separate location from the *B* locus. In addition, it shows a different expression pattern to *Arabidopsis CO*. This suggests a slightly different regulation of photoperiod response in sugar beet.

1.7 Comparing Photoperiodic Bulb Initiation with Photoperiodic Floral Initiation

On a physiological level, bulb initiation in LD onions is regulated in a way that is similar to the regulation of flowering in *Arabidopsis*. The two processes can be compared in terms of photoperiodic control and the involvement of phytochrome. The role of phytochrome in the photoperiodic control of bulb initiation is discussed in section 1.4. The role of phytochrome in the photoperiodic control of flowering is long established and the roles of individual phytochrome genes (*PHY A-E*) in *Arabidopsis* are well characterised (Jackson, 2008). In addition, blue light is important in the photoperiodic control of flowering. Blue light acts through the photoreceptor genes *CRY1* and *CRY2*, regulating the expression of *CO* (Jackson, 2008). *CRY2* is thought to be the major photoreceptor, as *cry2* mutants are late flowering (El-Assal *et al.*, 2003). This is discussed in section 7.1.2. Recent work

showed that *FKF1* also acts as a blue light photoreceptor, regulating *CO* expression (Imaizumi *et al.*, 2003). Blue light has also been shown to have a stimulatory effect on bulb initiation (Terabun, 1965). The *A.cepa* gene index contains expressed sequence tags (ESTs) showing sequence similarity with *Arabidopsis PHYA*, *CRY2* and *FKF1* (DFCI, 2008).

Bulb initiation and floral initiation can also be compared in terms of the presence of a juvenile phase, as mentioned in section 1.4.3 (Brewster, 1997). During both processes, initiation will not occur regardless of environmental conditions until this phase is passed (Massiah, 2007). The juvenile phase has been shortened in certain tree species by overexpression of the *FLOWERING LOCUS T* (*FT*) gene (Hsu et al., 2006). This implies that *FT* is important in controlling the transition from juvenile to adult development or is produced as a consequence of the transition. There is an EST in the *A.cepa* gene index which shows sequence similarity with *Arabidopsis FT* (DFCI, 2008).

The reversible nature of bulbing is comparable with flowering in certain species (e.g. soyabean and wild Vigna species) where the process can be reversed even after floral buds are well developed (Summerfield et al., 1991). The classic case indicating the reversibility of flowering comes from *Impatiens balsamina.* This plant has been studied in detail and shows that reversion from reproductive to vegetative growth can serve a function in the perenniality of a plant (Tooke *et al.*, 2005). Bulbing has been shown to be reversible, even when plants are at an advanced stage of bulbing (Sobeih and Wright, 1986). Foliar leaf production was seen to be resumed despite the fact that many bulb scales had been formed.

A major comparison between the two processes is that both involve homeotic conversions. In *Arabidopsis,* the shoot apical meristem produces leaf primordia until environmental and internal signals cause a change of fate and an inflorescence meristem is produced (Komeda, 2004). A homeotic conversion refers to the conversion of leaves to sepals, petals, stamen and carpels. This can be compared to bulb initiation where the leaf sheaths swell and production of bladeless bulb scales occurs upon initiation (Lancaster *et al.*, 1996). The homeotic conversion here is from a leaf to a bulb scale.

Another clear comparison between photoperiodic bulbing and flowering relates to the perception of the signal. Several studies have shown that the leaf blade is the receptor for the photoperiodic control of bulb initiation (Brewster, 1990). It

has been shown that removal of parts of the leaf blade leads to an increase in the number of leaves formed before bulbing occurs (Kato, 1964). It then follows that the signal is transported to the bulb. The mechanism by which this occurs is currently unknown. This is clearly comparable with photoperiodic flowering where perception of daylength occurs in the leaves and the signal is transported to the apex (Knott, 1934). The flowering signal has been referred to as 'florigen' and a lot of research has been carried out trying to identify the biochemical nature of 'florigen'. Recent developments indicate an involvement of the *FLOWERING LOCUS T* (*FT*) protein in the mobile signal in *Arabidopsis* and rice (Corbesier et al., 2007). This is discussed in section 7.1.1.

1.8 Other Plant Responses to Photoperiod

1.8.1 Tuberisation in potato

The production of tubers in potato (*Solanum tuberosum*) is essential for the success of the crop. It has been proposed that at least two independent pathways are involved in the control of tuberisation, a photoperiod dependent pathway and a gibberellin-dependent pathway (Martínez-García *et al.*, 2001). It is a SD photoperiod which induces tuberisation in potato (Jackson, 1999). Plants require a daylength of 12 hours or less in order to initiate tuberisation. It has been shown that *PHYB* is involved in the inhibition of tuberisation in LDs, but not the induction of tuberisation in SDs (Jackson, 1999). Grafting experiments suggested that common factors may be involved in photoperiod flowering and tuberisation (Jackson, 1999). It was shown that when tobacco leaves from a plant which had been induced to flower were grafted onto potato plants kept in non-inductive conditions, tuberisation occurred. This suggests that the genetic control of tuberisation is similar to the genetic control of photoperiodic flowering. This is due to the fact that the FT protein has been shown to be (at least part of) the mobile signal previously referred to as 'florigen' (Corbesier *et al.*, 2007)

The genetic control of tuberisation in potato has not been well characterised. However, a recent study revealed that *CO* has a role in this process (Martínez-García *et al.*, 2002). It was shown that when *Arabidopsis CO* is overexpressed in potato,

tuberisation is inhibited in SD conditions. Grafting experiments showed that the site of this inhibition is the leaves. It is therefore proposed that the photoperiod pathway genes are conserved in potato and function in the photoperiodic control of tuberisation. Three *CO* homologues have been identified in potato, one of which has a predicted role in tuberisation (Rodríguez-Falcón *et al.*, 2006). Characterisation of these *CO* homologues is currently in progress. In addition, two potato genes which show a high level of homology to *Arabidopsis FT* have been identified (Rodríguez-Falcón *et al.*, 2006). At least one of these genes is predicted to have a function which is orthologous to *FT*.

Studies on potato tuberisation show a conservation of gene function between different photoperiod responses. This leads to the hypothesis that the same genes are involved in the photoperiodic control of bulb initiation in onion.

1.8.2 Additional responses to photoperiod

Trees and other perennial plants show adaptations in their growth rhythm in response to changes in the environment (Gyllenstrand *et al.*, 2007). These plants respond to SDs and lower temperatures with a cessation of growth and initiation of bud set (Partanen and Beuker, 1999). This is essential for the survival of these plants over the cold winter period and subsequent growth the following spring (through the opening of buds which are set in response to shortening days). In the Norway Spruce (*Picea abies L.*) tree species, an *FT* homologue has been implicated in the control of growth rhythm (Gyllenstrand *et al.*, 2007). This shows that a gene which is involved in the photoperiodic control of flowering is also involved in control of growth rhythm in response to photoperiod.

In strawberries (*Frageria* species) flowering and runner production are both controlled by daylength (Serçe and Hancock, 2005). In terms of the flowering response, LD, SD and day-neutral (DN) varieties exist. The production of runners in strawberries has been shown to be stimulated by LDs in all the different flowering classes (Durner *et al.*, 1984). The genetic control of flowering and runnering has not been studied so the molecular mechanisms controlling the processes remain unknown. However, a full-length clone of a strawberry *CO* homologue can be found in a publicly available database (NCBI, 2008). There is currently no information available on the function of this gene.

1.9 The Onion Genome

Onion is a diploid plant (2n=2x=16) with a very large genome (32pg/2n), about 36 and 107 times larger than rice and *Arabidopsis* respectively (McCallum *et al*., 2001; Kuhl *et al*., 2004). The level of duplication in the onion genome is reported to be greater than for other diploids such as *Arabidopsis*, barley, tomato and rice (King *et al*., 1998). However, it is reported to be less than the duplication in maize, soybean and some *Brassica* species (King *et al*., 1998). The relatively high level of duplication may suggest that the onion is of polyploid origin. However, there is no evidence for this and it has been proposed that duplication is due to tandem (through infidelity or meiotic recombination), transpositional (including the movement of DNA fragments) or RNA-mediated retropositional duplication of specific regions of chromosomes. It is possible that chromosomal rearrangement followed this. Duplication within the onion genome provides a challenge for molecular-based studies such as this one. However, it has been reported that genes encoding non-abundant proteins are rarely repeated (Pichersky, 1990). This may be advantageous for the isolation of onion homologues of non-abundant genes such as *CO* and *FKF1*.

An onion EST database has been created where sequencing was carried out in order to assess the genomic differences between the Asparagales (of which onion is a member) and the Poales (e.g. rice, barley), the two most economically important monocot orders (Kuhl *et al*., 2004). This is available online and is known as the *A.cepa* Gene Index (DFCI, 2008). The material used for the construction of a normalised cDNA library originated from RNA isolated from leaf, bulb, root and callus tissue. It was shown that there are significant similarities between single positions of the onion and rice genomes, but little colinearity. It was shown that the GC content in onion is similar to *Arabidopsis* and quite different from rice (see table 1.6). This correlates to the similar frequencies of codon usage observed for *Arabidopsis* and rice. It was also shown that onion has a similar GC content across the coding region. This is similar to the eudicots and in contrast to other Poales, which show a high GC content at the 5’ end of the coding region. This suggests that the genomic information available on the Poales may not provide an accurate model for the Asparagales (Martin *et al*., 2005). Therefore, extensive genomic research into the Asparagales would be advantageous.

Table 1.6: Comparison of percentage GC content in onion, *Arabidopsis* and rice (Kuhl *et al*., 2004)

Species	Number of EST's	GC content (%)
Onion	11,008	41.9
Arabidopsis	30,542	42.7
Rice	32,400	51.1

Despite the value of the crop, little research has been carried out on onion genomics. A recent study details the pilot sequencing of onion genomic DNA (Jakse *et al*., 2008). Among the findings were that onion has a very low gene density, reported to be only 1 gene per 168 kb. This is among the lowest reported of any species to date. There was also no evidence for gene rich regions. This suggests that the large genome size in onion is not due to a larger number of genes, but due to large areas of non-coding DNA and high levels of duplication.

There are several EST's, found in the *A.cepa* gene index, which show significant sequence similarity with *Arabidopsis* flowering time genes. This includes EST's which show similarity with *CO*, *FKF1* and *GI*. This was used as a starting point for the molecular and genetic characterisation of the photoperiodic control of bulb initiation in onion.

1.10 Genetic Transformation of Onion

Two main methods can be used for the genetic transformation of onion. The first, microprojectile bombardment, was developed more than 20 years ago (Klein *et al*., 1987). It was demonstrated here that onion epidermal cells could be transformed. The second method, *Agrobacterium*-mediated transformation, was developed shortly afterwards (Dommisse *et al*., 1990). This was the first report of onion being a host for *Agrobacterium tumefaciens.* Since then, there have been several reports of successful onion transformation protocols. However, the first report of transformation followed by the regeneration of transgenic plants did not appear until the new millennium (Eady *et al*., 2000). The technique used on this occasion was the transformation of immature embryos with *Agrobacterium* containing a binary vector encoding Green Florescent Protein (GFP) expression. A further report details

transformations carried out using callus induced from mature embryos and also reports the successful regeneration of transgenic plants (Zheng *et al*., 2001). In both studies, a vector conferring resistance to one or more antibiotics was used to select positive transformants. In both studies, it was reported to take around six months to generate transgenic plants. This is a limitation of any onion transformation system as it then takes a further two growing seasons to generate seed from any transgenic lines.

Agrobacterium-mediated transformation was shown to be successful in the generation of herbicide resistant plants (Eady *et al*., 2003a; Eady *et al*., 2003b). Plants showing resistance to glyphosate and phosphinothricin were produced, highlighting the commercial potential of this technology. A recent study reported the use of phosphomannose isomerase as a selective marker, eliminating the need for an antibiotic resistance marker (Aswath *et al.*, 2006). This is advantageous, as the presence of antibiotic resistance markers is perceived as hazard to the environment and to human health. Despite recent advances, transgenic onions have yet to be grown on a commercial scale.

The preferred transformation technique for down-regulating genes at the current time is RNA interference (RNAi). This is a powerful natural mechanism which regulates gene expression and has been used to silence plant genes (Hammond *et al*., 2000). Recently, RNAi has been applied to onions in order to produce a tearless onion (Eady *et al*., 2008). This was achieved by silencing the lachrymatory factor synthase (LFS) gene. The LFS enzyme normally catalyses the production of lachrymatory factor, the major compound which causes eye irritation when onions are chopped. There is potential for the commercialisation of this technology as a tearless onion would be seen as desirable. At the current time, genetically modified onions are not grown commercially in any part of the world. However, this recent technological advancement shows the potential of RNAi technology for onion improvement.

1.11 Project Aims

Bulb initiation is similar to floral initiation on a physiological level. The similarities between the photoperiodic control of bulb initiation and the photoperiodic control of flowering were discussed in detail in section 1.7 and are summarised in Fig. 1.7. The overall aim of this project was to test the hypothesis that: **the genes controlling daylength response are conserved between the model plant *Arabidopsis* and onion and hence between the different end-processes bulbing and flowering.**

Specific aims:

- Isolate and clone onion *CO* and *CO*-like genes
- Determine the expression patterns and function of *CO*/*CO*-like genes in LD and SD grown plants
- Characterise the physiological aspects bulb development and establish a system for studying the expression of putative photoperiod response genes
- Isolate and characterise other candidate genes involved in the photoperiodic flowering pathway which may function in the photoperiodic regulation of bulbing in onion (e.g. *GI* and *FKF1*)

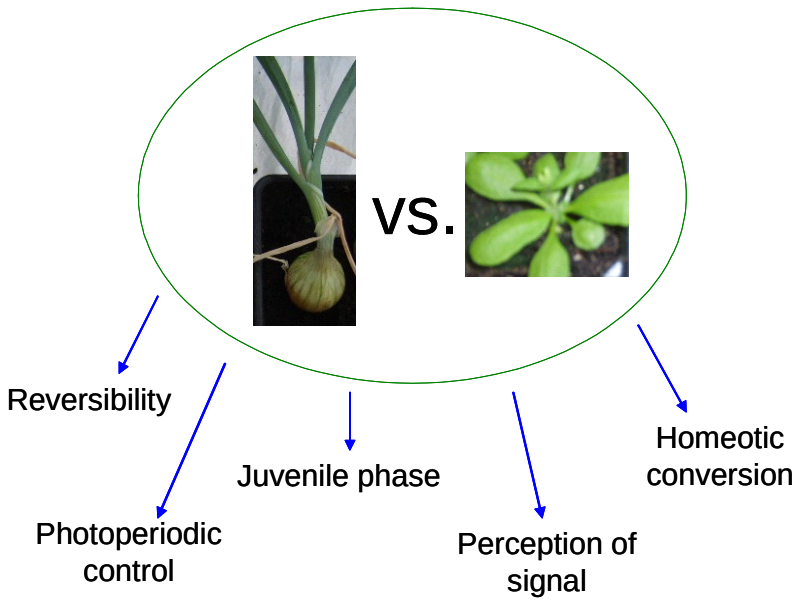


Fig. 1.7: Comparing bulb initiation with floral initiation.

CHAPTER 2: STANDARD MATERIALS AND METHODS

This chapter details the plant and biological materials which were used in this study. Unless otherwise stated, the methods and protocols set out in this chapter were used throughout the project.

2.1 Standard Materials

2.1.1 Plant materials

The onion (*Allium cepa L.*) variety used throughout this project was 'Renate F_1' (Elsoms Seeds Ltd., Spalding, UK). Varieties with different daylength responses were grown for the experiment described in section 2.3.4. The SD variety Agrifound Dark, the intermediate day variety Candy F_1 and the LD variety Ailsa Craig were selected. All seeds were sourced from the Warwick HRI Genetic Resources Unit. Onion plants which had been induced to bolt (unknown variety) were provided by Dr. Paul Hand/Dr. Dave Pink (Warwick HRI). *Arabidopsis thaliana co-2* (Landsberg background) and *fkf1-1* (Columbia background) mutant as well as Landsberg *erecta* (L*er*) and Columbia (Col-0) wild-type seed was provided by Dr. Karl Morris (Warwick HRI). *Arabidopsis gi-3* mutant seed (L*er* background) was provided by Ioannis Matsoukas (Warwick HRI). The original source of all *Arabidopsis* seed was the Nottingham *Arabidopsis* Stock Centre (NASC).

2.1.2 Other materials

Onion EST clones, cloned into the *Eco*R V site of a pCMV.SPORT 6 vector (Invitrogen Ltd., Fig. 2.1), were provided by Prof. Mike Havey (USDA, University of Wisconsin). Maize (*Zea mays*) genomic DNA was provided by Dr. Jose-Marcos Gutierrez (Warwick HRI). The pB2GW7 vector used for transformations was provided by Dr. Sharon Hall (Warwick HRI). Onion double haploid genomic DNA was provided by Dr John McCallum (Crop & Food Research, New Zealand). Plasmid DNA extracted from a clone containing the maize *conz1* gene (in the pGEM-T vector, Promega Corporation) was provided by Dr. Theresa Miller (Marquette University, Milwaukee).

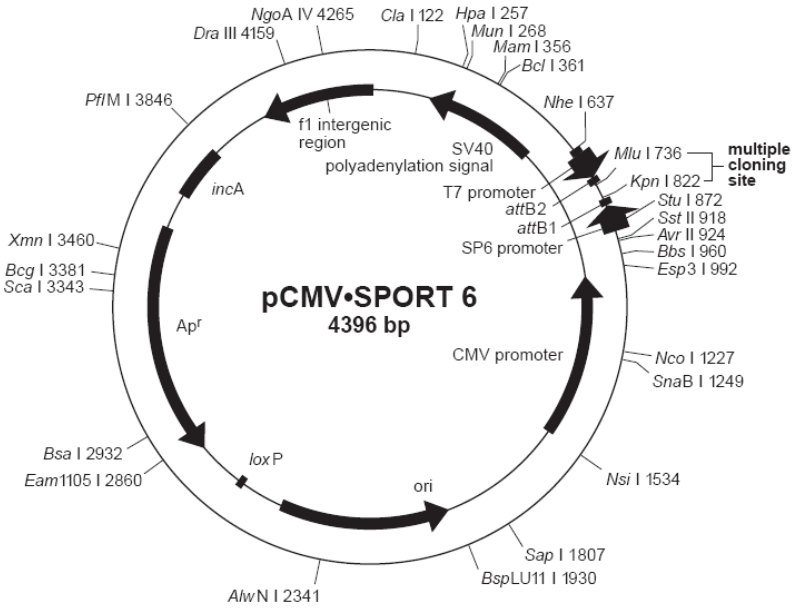


Fig. 2.1: Map of the pCMV.SPORT 6 vector

2.2 Standard Methods

2.2.1 PCR & gel electrophoresis

PCR reactions were set up in 20 µl volumes containing 0.4 units KOD Hot Start DNA Polymerase (Merck Bioscience, Cat No. 71086-3), 1 µl of template, 1 µM of each primer, 0.2 mM each dNTP and 2 mM $MgSO_4$. Reactions were carried out with an initial denaturation step of 94 °C for 2 min followed by cycles of denaturation at 94 °C for 15 seconds, annealing at a temperature deemed appropriate for the primer pair for 30 seconds and extension at 72 °C for 1 min per kb of product. In the case of colony PCR, an extra 1µl of water was added to each reaction and a pipette tip containing a single colony placed in the mix.

Following PCR, agarose gel electrophoresis was carried out. Orange G ($C_{16}H_{10}N_2O_7S_2Na_2$, Sigma-Aldrich®, Cat. No. O3756) loading dye was added to each sample. For a standard 20 µl reaction, 3 µl of Orange G was added prior to loading. The size of products was estimated by also loading 5 µl of 1 kb Plus DNA ladder (Invitrogen Ltd., Cat. No. 10787). Gels were made up using 0.5x TAE buffer (VWR International, Cat. No. 44125D) containing either 1 or 2 % Agarose

(Invitrogen Ltd., Cat. No. 15510). Gels were run in tanks containing 0.5x TAE buffer at approximately 100 mA for 30-90 min depending on the size of the gel. Photographs were taken using a G:BOX gel documentation system (Syngene).

2.2.2 *RNA extraction, DNase treatment and first strand cDNA synthesis*

Total RNA was extracted using Trizol® reagent (Invitrogen Ltd., Cat. No. 15596-026), following the manufacturer's guidelines. Approximately 100 mg of frozen plant tissue was homogenised using a pestle and mortar. Samples were DNase treated using TURBO DNA-free™ (Ambion Inc, Cat. No. AM1907) and first-strand cDNA synthesised using Superscript™ II Reverse Transcriptase (Invitrogen Ltd., Cat. No. 18064-14) following the manufacturer's guidelines. The primer used for this procedure was oligo(dT). All samples were treated with RNase H (Invitrogen Ltd., Cat. No. 18021-14), following the manufacturer's guidelines.

2.2.3 Genomic DNA extraction (from onion)

Genomic DNA extractions were carried out using a modified CTAB method (Doyle and Doyle, 1987; Porebski *et al.*, 1997). Leaf tissue was placed in a microfuge tube and homogenised with a microfuge pestle. Pre-warmed CTAB buffer was then added (500 µl) and the material homogenised further. The reaction was then incubated at 65 ºC for 10 minutes. Dichloromethane:isoamyl alcohol (24:1 v/v) was then added (500 µl) and samples were centrifuged at 16,500 x g for 2 min (in a microfuge). The top phase was then transferred to a fresh tube and 300 µl of isopropanol added. Samples were than centrifuged at 16,500 x g for 2 min and the supernatant removed. Wash buffer was added (500 µl) and samples were left at room temperature for 2 min then centrifuged at 16,500 x g for 3 min and the supernatant removed. The pellet was re-suspended in 200 µl of sterile distilled water. This protocol was provided by Linda Brown (Warwick HRI).

2.2.4 Rapid Arabidopsis DNA extraction method

A single leaf was homogenised for 15 sec using a Dremel drill with an attachment designed to fit a microfuge tube. The next step was to add 400 µl of extraction buffer (200 mM tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % w/v SDS) and homogenise further. Samples were then centrifuged at full speed in a microfuge and 300 µl of the supernatant transferred to a fresh tube. Isopropanol was added (300 µl) and reactions mixed and left for 2 min. Samples were then centrifuged at 16,500 x g for 5 min, the supernatant removed and the pellet air-dried. The pellet was re-dissolved in 50 µl of 10 mM Tris buffer (pH 7.5). All steps were carried out at room temperature.

2.2.5 Plasmid DNA extraction

Liquid bacterial cultures were set up by seeding centrifuge tubes containing approximately 3 ml of liquid LB media (VWR International, Cat. No. 1.10285) and the appropriate antibiotic. Cultures were grown at 37 ºC, 250 rpm overnight. Bacteria were harvested by centrifuging at 6000 x g for 15-20 minutes. Plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (QIAGEN, Cat. No. 27106) following the manufacturer's guidelines. DNA was eluted in 50 µl of sterile distilled water.

2.2.6 Purification of PCR products

PCR products were purified following gel electrophoresis. Bands were cut out of the gel under UV light with a wavelength of 302nm (Bio-Rad UV Transilluminator 2000) using a scalpel blade and purification was carried out using a QIAquick Gel Extraction Kit (QIAGEN, Cat. No. 28704), following the protocol provided with the kit. Samples were eluted in 30-50 µl of sterile water.

2.2.7 Quantification of samples

DNA and RNA samples for all experiments were quantified using a NanoDrop™ ND-1000 spectrophotometer (Thermo Scientific). A volume of 1.2 µl was loaded onto the spectrophotometer.

2.2.8 Sequencing

Sequencing was carried out using a Big Dye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Cat No. 4337456). Reactions were set up using 2 µl of Big Dye, a primer concentration of 0.42 µM and an appropriate concentration of template, as stated in the manufacturer's protocol. Reaction volumes of 10 µl were used. Cycling was set up with 1 cycle of 96 ºC for 1 min followed by 25 cycles of 96 ºC for 10 sec, 50 ºC for 5 sec and 60 ºC for 4 min. Sequencing was carried out by the Warwick HRI Genomics Resource Centre. Sequence files were viewed and edited using the EditSeq package of DNAStar (DNAStar Inc.). Chromatograms were viewed using the BioEdit software package (Hall, 2007)

2.2.9 Cloning

Cloning of PCR products was carried out using the cloning kit specified in the appropriate section. The standard method of transformation used was electroporation. Overnight ligations were precipitated and re-suspended in 10 µl of sterile distilled water. A single microlitre was then added to 20 µl of Electrocompetant EC100 cells (Cambio Ltd., Cat. No. EC10005). This mixture was added to a 1 mm electroporation cuvette (Geneflow Ltd., Cat. No. E6-0050) and electoporated using the EC1 setting of a Bio-Rad laboratories Micropulser. SOC medium (1 ml, recipe in Appendix 1) was then added to the cuvette and the contents mixed. The contents of the cuvette were then transferred to a microfuge tube and agitated at 37 ºC, 250 rpm for 1 hr. Following this, 10, 50 and 100 µl were plated on LB media (VWR International) containing the appropriate antibiotic.

2.3 Experiments Relating to Several Chapters

2.3.1 Screening the A.cepa Gene Index (the onion EST database)

The onion EST database (DFCI, 2008) was screened for genes showing sequence similarity with *Arabidopsis* photoperiod pathway genes. Keyword searches were carried out and sequences of *Arabidopsis* flowering time genes BLASTED against the EST database. EST clones were received in bacterial culture. Information on the onion EST clones is displayed in Table 2.1. Clones were grown on plates containing LB (VWR International) and 100 µg ml^{-1} ampicillin (Melford). Plasmid DNA was isolated from the onion EST clones and sequencing carried out using gene-specific primers as well as T7, SP6 and M13 forward and reverse primers, as described in sections 2.2.5 and 2.2.8. Primers specific to each EST were designed to complete the double stranded sequence of BR20 and AV44 (see Appendix 2 for sequences).

Table 2.1: Onion EST's clones studied; all except EE96 and DQ45 were obtained in bacterial culture.

EST I.D.	**Accession Number**	**Sequence Similarity with *Arabidopsis* gene**
BR20	CF435233	*CO*
AV44	CF447089	*FKF1*
GK28	CF441736	*PHYA*
DQ45	CF451504	*GI*
BP76	CF434985	*LHY*
CY05	CF436612	*LHY*
EE96	CF438000	*FT*

2.3.2 Preliminary gene expression experiment

Onion Renate F_1 plants were grown under artificial lights in a growth chamber (Sanyo MLR Plant Growth Chamber). Plants were grown in LDs (16 hours of light, 22 °C). Leaf material was harvested at ZT8, 11.5 and 15 and 'bulb' and root material harvested at ZT15 only. ZT (zeitgeber time) is the number of hours after subjective dawn. RNA was extracted, DNase treated and first-strand cDNA

synthesised as described in section 2.2.2. Approximately 0.5 µg of RNA was used to synthesise cDNA for each sample. PCR was then set up to assess the expression of BR20 and AV44 at different time-points and in different tissues (discussed in sections 4.2.1.2 and 6.2.1.1).

2.3.3 Time-course experiment to study diurnal gene expression patterns

Plants were grown from May to August 2006 at Wellesbourne (latitude 52º12'). Initially, onion Renate F_1 seed was sown in modular trays (8th May 2006) and kept under natural conditions within a glasshouse. After 4 weeks, plants were potted up into 9 cm pots containing Levington M2 compost and left in SDs (8 hr of light). Two plants were placed in each pot. A week later, half the plants (150) were transferred to LDs (16 hr of light) and half remained in SDs. Plants in LDs were subjected to a daylength extension of 8 hrs using low-level incandescent light within a photoperiod chamber. Plants in SDs were subjected to 16 hr of darkness in a photoperiod chamber. At the first sign of bulb initiation in LD grown plants, harvests were carried out at ZT 0.5, 4, 7, 10, 13, 15.5, 17 and 20 over a 48-hr 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. SD and LD grown plants were harvested at every time-point and every harvest was carried out in duplicate. Plants were selected for harvest using a random number generator (Haahr, 2006).

Total RNA was extracted from harvested material, DNase treated and first strand cDNA synthesised from 2 µg of total RNA following the protocol in section 2.2.2. Quantitative real-time PCR was carried out using an I-Cycler (Bio-Rad Laboratories, iCycler Thermal Cycler). Reaction volumes of 25 µl were used, containing 1 µl of cDNA, 1x PCR Mastermix containing SYBR green (Eurogentec Ltd., Cat. No. RT-QP2X-03-15+) and an appropriate concentration of each primer. An initial step of 55 ˚C for 2 min was carried out to activate uracil-*N*-glycosylase. Denaturation was carried out with an initial cycle of 95 ˚C for 10 sec. This was followed by 50 cycles of denaturation at 95 ˚C for 10 min and annealing at 61 ˚C for 45 sec. This was followed by a cycle of 95 ˚C for 1 min and 55 ˚C for 1 min.

Finally, the melt curve was produced through 80 cycles of 55 ˚C for 10 sec, increasing the temperature by 0.5 ˚C per cycle.

Each primer pair used was initially tested to ascertain what the optimum primer and cDNA concentrations would be. Each sample was run in triplicate and the average CT value calculated. This represents the number of PCR cycles when a product is first detected. Data was normalised to the expression of *Elongation Factor 1 Alpha* (*EF1α)*, a gene which has been used routinely as a housekeeping gene in both potato and rice (Jain *et al.*, 2006; Silver *et al.*, 2006). The primers used to obtain the expression profile of *EF1α* were EF1-RT FOR3 and EF1-RT REV3 (see Appendix 2 for sequences) at a concentration of 0.1 µM. Normalisation was achieved by dividing the expression of the gene of interest at a specific time-point by the expression of *EF1α* at that same time-point. Twenty-four hour averages of expression were calculated and standard errors included. Standard curves (using 10-fold serial dilutions) were plotted using cDNA synthesised from approximately 4 µg of RNA extracted from leaf material harvested at various time-points in a 24-hour period (as used for cDNA library construction, section 2.3.8.1).

2.3.4 Analysis of gene expression in SD and Intermediate day (ID) onion varieties grown under LD and SD photoperiods

Plants were grown from April to September 2007 at Wellesbourne (latitude 52º12'). Onion seeds from five different varieties were sown in modular trays (19th April 2007). The LD variety Ailsa Craig, the ID varieties Candy F_1 and Ramata di Milano and the SD varieties Agrifound Dark and Serrana were chosen. After 4 weeks, plants were potted up into 9 cm pots containing Levington M2 compost and left in natural conditions. Two plants were placed in each pot. One week later, plants were placed into either LD (16 hours) or SD (12 hours) conditions. Twelve hour SDs were used (as opposed to 8 hours used in the experiment described in section 2.3.3) as SD onion varieties require 12 hours of light to initiate bulbing. Plants were subjected to 8 hours of natural daylight plus a daylength extension of 8 hours (LD) or 4 hours (SD) using low-level incandescent light within a photoperiod chamber (Fig. 2.2). Plants were placed in specific locations using a Latin square design (Mead *et al.*, 1993). Leaf material was harvested over a 48-hr period 3-4

weeks later as described in section 2.3.3. The harvest times were ZT 0.5, 4, 7.5, 10, 13, 16 and 20.

Analysis of gene expression was carried out as described in section 2.3.3 except a MESA GREEN qPCR MasterMix for SYBR® green with fluorescein (Eurogentec, Cat. No. RT-SY2X-03+WOUFL) 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 from this experiment normalised to ß–tubulin. The primers used to obtain the expression profile for ß-tubulin were TUB FOR1 and TUB REV1 (see Appendix 2 for sequences) at a concentration of 0.4 µM and with an annealing temperature of 65 ˚C. All cDNA samples were diluted 1 in 3. Twenty-four hour averages of expression were calculated and standard errors included (Mead *et al.*, 1993).

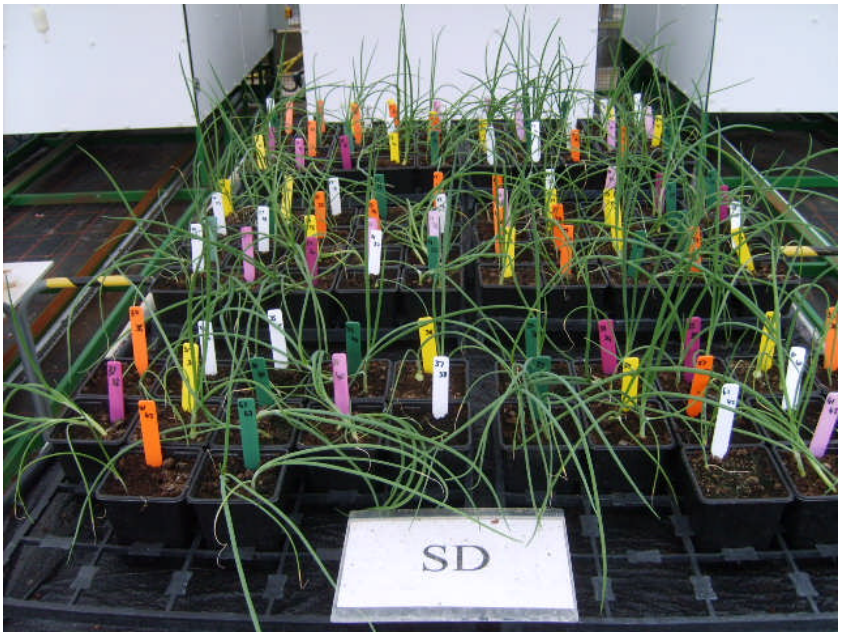


Fig. 2.2: Growth of different onion varieties to generate material for analysis. SD-grown plants are shown. A similar design was employed for plants grown in LDs. Different coloured sticks represent different varieties.

2.3.5 Study of gene expression in flowering onions

Leaf material was harvested from onion plants which had bolted at ZT5, 9 and 12.5, as described in section 2.3.3. Plants were at the unopened flower stage. RNA was extracted, DNase treated and first strand cDNA synthesised as described in

section 2.2.2. The cDNA was synthesised from 2 µg of total RNA. The expression of onion putative photoperiod response genes was then examined using RT-PCR with an annealing temperature of 61 °C and a cycle number of 30. The reactions were set up as described in section 2.2.1.

2.3.6 Functional analysis of onion putative photoperiod response genes using Arabidopsis transformations

Using Gateway® LR clonase® II (Invitrogen, Cat. No. 11791), *AcCOL* (*EST* BR20) and *AcFBox* (EST AV44) were cloned into a pB2GW7 vector to produce pBAcCOL and pBAcFBox vectors, following the manufacturer's guidelines. The pB2GW7 vector confers resistance to the herbicide Glufosinate-ammonium (via the *bar* gene) and contains a CaMV 35S promoter (Fig. 2.3). Following this, plasmid DNA was isolated and sequenced using gene-specific primers, following the protocols set out in section 2.2.5 and 2.2.8. Vectors pBAcCOL and pBAcFBox were then transformed into *Agrobacterium* c58pGV3101, a strain used routinely in *Arabidopsis* transformations.

The presence of the appropriate gene in the *Agrobacterium* was then confirmed using PCR with CO-RT and FKF1-RT primers (see Appendix 2 for sequences). Transformations were carried out using the Floral dip method (Clough and Bent, 1998). L*er* and *co-2* plants were transformed to express *AcCOL* and *fkf1-1* and Col-0 plants transformed to express *AcFBox*. Five plants were transformed for each ecotype. These were grown in LD conditions (16 hours of light, 22 °C) in growth chambers (Sanyo MLR Plant Growth Chamber) and allowed to flower and set seed. Seeds were collected and stored at 4 °C. T_1 generation plants were grown and sprayed with the herbicide Challenge® (Bayer CropScience, Cat. No. 05936136). This herbicide is also known as Basta. The active ingredient, Glufosinate-ammonium, was applied at a concentration of 12 mg l^{-1}. Plants were sprayed 3-4 times over a period of 10-14 days. Plants which survived were allowed to flower and T_2 generation seed collected. Genomic DNA was extracted from putative transgenics (using the protocol set out in section 2.2.4) and the presence of the transgene confirmed by PCR using gene-specific primers (described in sections 4.2.1.3 and 6.2.1.2). Flowering time was screened in the T_2 generation by recording

the number of days taken to produce a 1 cm bolt and the leaf number at this time in LD conditions. Either 14 or 15 independent lines were screened using 5/6 T_2 plants for each line. Averages were taken and one-way ANOVA's were carried out to assess the significance of any differences in flowering times using the software package Genstat (VSN International). The errors were calculated using the standard error of the difference (SED).

The presence of the transgene was tested using RT-PCR. A single leaf was harvested from a plant of 5 different transgenic lines and flash frozen in liquid nitrogen. The lines were chosen at random. RNA was then extracted, DNase treated and first strand cDNA synthesised as described in section 2.2.2 with a slight modification. Frozen leaf tissue was homogenised using a Dremel drill fitted with a specially adapted attachment designed to fit into a microfuge tube. PCR was then set up using gene-specific primers (described in sections 4.2.1.3 and 6.2.1.2).

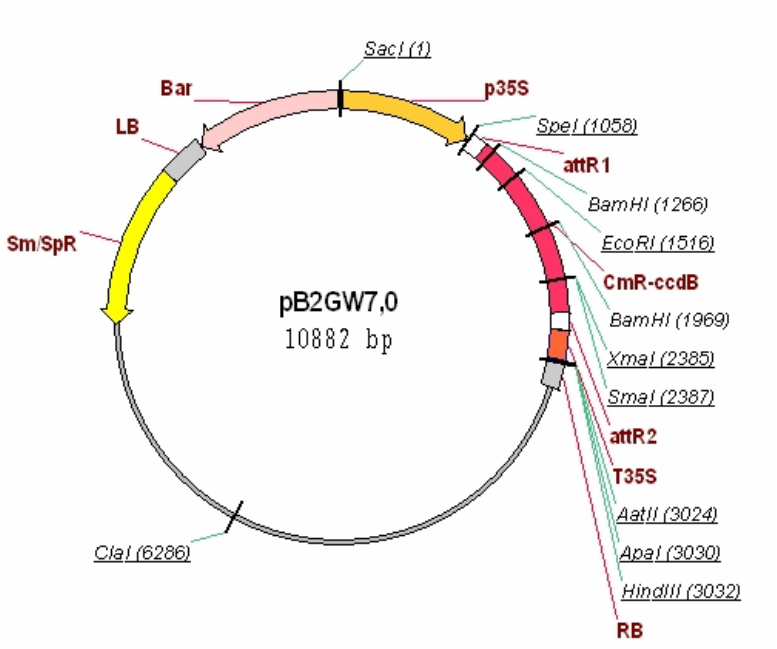


Fig. 2.3: Map of the pB2GW7 transformation vector

2.3.7 Construction of phylogenetic trees

The method described here was used to construct phylogenetic trees for *FKF1*, *GI*, *FT* and *PHYA* gene families. The analysis of *CO* and *CO*-like genes was more intensive and is described in section 4.2.2. Published sequences in FASTA

format were collated in a single document within Notepad. Alignments were carried out using Clustal X and edited using Jalview. Neighbour-joining trees (NJ-trees) were constructed and bootstrap values calculated using 1000 replicates using Clustal X. Phylogenetic trees were viewed and edited using NJPlot and TreeExplorer. A description of the software used can be found in Table 2.2.

Table 2.2: Software packages used for phylogenetic analyses

Package used	**Use**	**Reference**
ClustalX	Used to perform all alignments and construct NJ-trees	(Thompson *et al.*, 1997)
Jalview	Used to edit alignments	(Clamp *et al.*, 2004)
NJ Plot	Used to view trees	(Perrière and Gouy, 1996)
TreeExplorer	Used to view trees and edit taxa names	(Tamura, 2005)
Mesquite	All-purpose package, used specifically for ancestor reconstruction and collapsing branches on trees	(Maddison and Maddison, 2006)
PHYLIP	Constructing ML trees	(Felsenstein, 1989)

2.3.8 Normalised, full-length cDNA library

2.3.8.1 Library construction

A normalised full-length cDNA library was constructed from onion leaf and bulb tissue extracted at the time-points used for the gene expression experiment described in section 2.3.3. Material was harvested in foil packets and flash-frozen in liquid nitrogen before storage at -80 ºC. All plants were grown in LD conditions (16 hours of light). Material was pooled and total RNA extracted as described in section 2.2.2. Two separate extractions were carried out for leaf and bulb material. The protocol was scaled up to account for the large mass of material used. 35 ml of Trizol® was added to approximately 3 g of homogenised leaf tissue. 16 ml of Trizol® was added to approximately 1.5 g of bulb tissue. The rest of the protocol was scaled up accordingly. RNA was cleaned using RNeasy columns (QIAGEN RNeasy Mini Kit, Cat No. 74106). The integrity of the RNA was tested by running 1 µl on a bioanayser (carried out by Alison Jackson, Warwick HRI). Following this, leaf and bulb material was pooled to give approximately 150 µg of total RNA.

Twice as much leaf RNA was used. The RNA was posted as a dry pellet for library construction (Vertis Biotechnologie AG). Various quality controls were carried out on the library by the manufacturers, including PCR assessment of the relative abundance of GAPDH.

2.3.8.2 Library screening

The cDNA library was received as 8 aliquots of 540 µl. The titre was 750 CFU μl^{-1}. Freezer media (179.5 ml, see Appendix 1 for recipe) and 100 µg ml^{-1} ampicillin (Melford, Cat. No. A0104) were added to one aliquot of the cDNA library (12.5 % of the total library). This was then divided between the wells of six 384-well plates (75 µl per well, approximately 171 clones per well). If the number of genes in the onion genome is estimated to be 50,000, each gene should be present around 8 times in each library aliquot. The plates were placed at 37 ºC overnight without shaking. Positively charged nylon membrane (Roche, Cat. No. 1417240) was placed onto 24 x 24 cm plates containing LB (VWR International) and 100 µg ml^{-1} ampicillin (Melford). The wells were then spotted onto the membrane using a 384-well pin replicator (Nunc Inc.). The plates were placed at 37 ºC overnight without shaking.

Four plastic trays were placed on a bench and 3 mm Whatman® paper placed in each tray. The first 2 trays were soaked with denaturation solution (1.5 M NaCl, 0.5 M NaOH) and the second 2 with neutralisation solution (0.5 M Tris pH 7.4, 1.5 M NaCl). The membranes were placed on each tray for 2 min, air-dried for 30 min and baked at 120 ºC for 30 min. Membranes were processed by soaking in 5xSSC, 0.1% SDS, 1 mM EDTA for 2 hours at 42 ºC. The bacterial lysis material was removed by wiping a tissue over the surface. Membranes were washed in 5xSSC, 0.1% SDS 3 times, for 10 minutes each time, air-dried and stored at room temperature. All probing was carried out using digoxigenin (DIG) non-radioactive nucleic acid labelling (Roche, Cat No. 1636090), following the manufacturer's guidelines.

CHAPTER 3: THE PHOTOPERIODIC NATURE OF THE BULBING PROCESS

3.1 Introduction

The physiology of the onion plant is discussed in detail in section 1.4. Bulb initiation is a process which has been studied extensively and is photoperiodically driven in temperate onions (Mettananda and Fordham, 1997). These varieties require 16 or more hours of light to initiate bulbing (Brewster, 2008). This shows similarity to the photoperiodic control of flowering and hence it is hypothesised that the same network of genes controls both processes. Bulb initiation in LD onions is dependent on the length of day and shows an absolute requirement for far-red light (Lercari, 1982). The response of plants transferred from LD to SD conditions has been studied previously and indicates that the bulbing process is reversible (Sobeih and Wright, 1986).

This chapter described an experiment set up to measure the bulbing response of Renate F_1 onions grown in constant LD and SD conditions. In addition, plants were transferred between LD to SD conditions and vice-versa and the bulbing response monitored (measured by the bulbing ratio). The overall objective was to develop a system which could be used for future gene expression studies. As different varieties respond slightly differently to environmental cues, it is necessary to establish a system for the variety studied in this project.

3.2 Materials and Methods

Plants were grown from April to September 2007 at Wellesbourne (latitude 52º12'). Onion 'Renate' F_1 (Elsoms Seeds Ltd.) seeds were sown in modular trays, watered, and left in natural conditions within a glasshouse. Four weeks after sowing, 110 plants were potted-up into 9 cm pots containing Levington M2 compost. Two plants were placed in each pot. These plants were left in natural conditions for a further two weeks. Following this, half the plants (50) were placed in LDs (16 hr of light) and the other half in SDs (12 hr of light). Plants in both daylengths were subjected to 8 hours of natural light plus an extension using low level incandescent

light in photoperiod chambers (4 hr extension for SDs, 8 hr extension for LDs). Plants were then kept in the dark within the photoperiod chambers until the start of the next cycle. A small number of plants were also left in natural conditions within a glasshouse. Bulb and neck diameter measurements were taken weekly using callipers and 'Bulbing ratios' calculated by dividing the average bulb diameter by the neck diameter (Clark and Heath, 1962). Bulb diameters were measured twice (in two different planes) and an average taken. This is to allow for the fact that not all bulbs are uniformly round. Bulbing is considered to have been initiated when the bulbing ratio reaches a value greater than two. Means, standard deviations and standard errors were calculated using Microsoft Excel.

Two weeks after the plants had been placed in the photoperiod chambers, transfers were carried out. Each week, 8 plants were transferred from LDs to SDs and vice-versa over a 4 week period. Bulbing ratios were calculated as described above. Means, standard deviations and standard errors were also calculated. Natural logarithms were calculated for all data points and the significance of the differences between treatments assessed by using two-way ANOVA's. ANOVA's were carried out using Genstat.

3.3 Results and Discussion

3.3.1 Plants grown in constant LD and SD conditions

Plants grown in constant LD conditions showed an increase in bulbing ratio, past the threshold of 2 after around 70 days (Figs. 3.1-3.5). Plants grown in SD conditions showed no signs of bulb initiation. Plants grown in natural conditions produced a larger bulb than those grown in LDs (Fig. 3.1). This is probably due to higher light levels and longer days, conditions which have been shown to accelerate bulbing (Wright and Sobeih, 1986). ANOVA's (using log transformed data, Appendix 3, Tables A1 & A2) confirmed that the differences between LD and SD treatments were significant (Table 3.1). It was also confirmed that the number of days from sowing had a significant affect on bulbing ratio (Table 3.1). Finally, the interaction between days from sowing and daylength was also shown to be significant (Table 3.1), showing that the pattern of bulbing ratio over time is affected

by daylength. Mean square values demonstrated that it is daylength which has the most significant effect on bulbing ratio. Therefore, bulb initiation in 'Renate F_1' onions was shown to be controlled by daylength, with LDs stimulating bulbing. This is consistent with previous data published for other onion varieties (e.g. (Lancaster *et al.*, 1996) and shows distinct comparisons between bulb initiation and floral initiation. In addition, a system for studying the expression of putative photoperiod response genes in Renate F_1 onions was established.

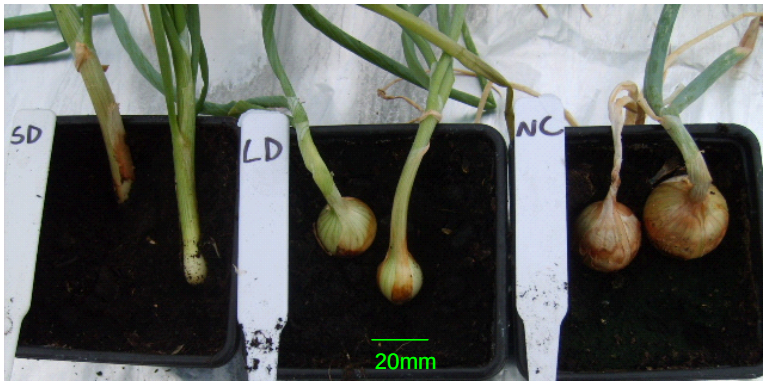


Fig.3.1: Comparison of Renate F_1 plants grown in SDs, LDs and NC (natural conditions) for 116 days.

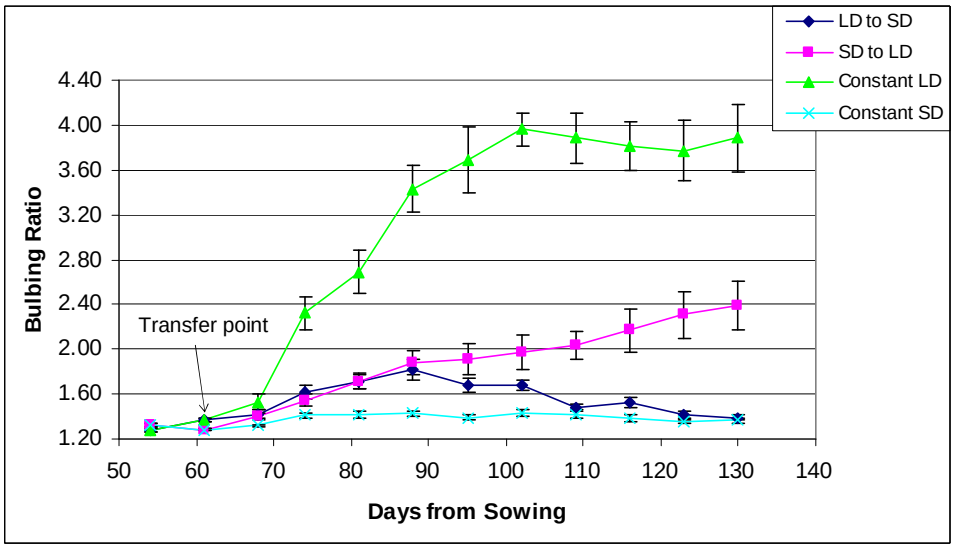


Fig. 3.2: The response of Renate F_1 onions to daylength, transfer 1. Error bars represent the SEM.

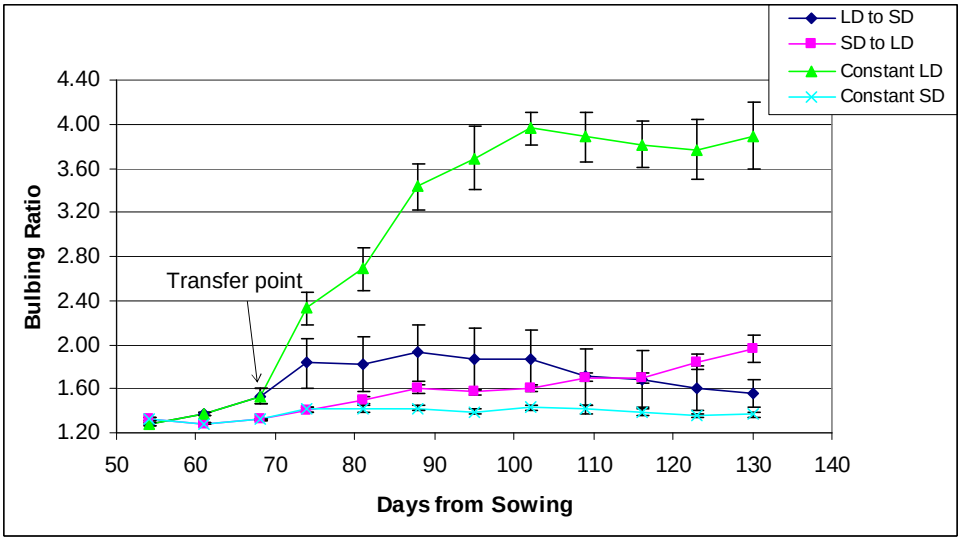


Fig. 3.3: The response of Renate F_1 onions to daylength, transfer 2. Error bars represent the SEM.

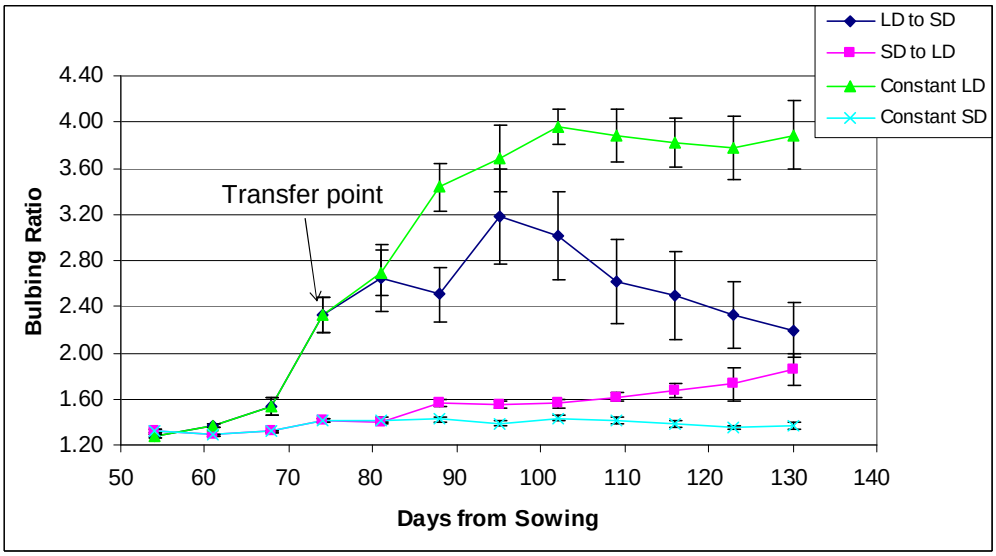


Fig. 3.4: The response of Renate F_1 onions to daylength, transfer 3. Error bars represent the SEM.

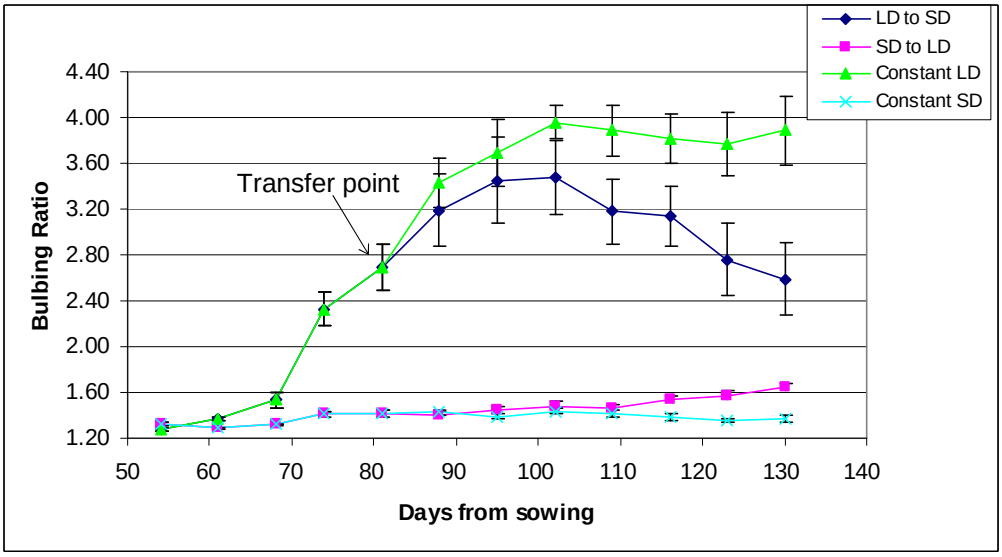


Fig. 3.5: The response of Renate F_1 onions to daylength, transfer 4. Error bars represent the SEM.

Table 3.1: Assessing the significance of the differences in bulbing ratio between treatments. The controls refer to differences between plants grown in constant LD and constant SD conditions. p=probability, M.S=mean square (green colour indicates stongest effect).

Transfer	Source of Variation	P	M.S	Degrees of Freedom
Control	Days from sowing	<0.001	0.81	12
Control	Daylength	<0.001	26.41	1
Control	Days from sowing x daylength	<0.001	0.65	12
1	Days from sowing	<0.001	36.83	11
1	Daylength	<0.001	566.83	3
1	Days from sowing x daylength	<0.001	12.93	33
2	Days from sowing	<0.001	12.02	10
2	Daylength	<0.001	383.83	3
2	Days from sowing x daylength	<0.001	5.70	30
3	Days from sowing	<0.001	3.22	9
3	Daylength	<0.001	296.77	3
3	Days from sowing x daylength	<0.001	2.21	27
4	Days from sowing	0.019	2.35	8
4	Daylength	<0.001	456.44	3
4	Days from sowing x daylength	<0.001	2.03	24

3.3.2 Plants transferred from SD to LD conditions

Plants transferred from SD to LD conditions showed no obvious increase in bulbing ratio for at least 2 weeks after transfer (Figs. 3.2-3.5). This was not expected as photoperiod has such a strong effect on bulbing. This delay may be due to the fact that bulb scales are formed quite quickly, but it takes longer to detect a swelling of the bulb. The development of leaves into bladeless bulb scales can be measured using the 'leaf ratio' (leaf blade length/sheath length) (Heath and Hollies, 1965). However, this requires destructive measurements to be taken and would have involved very large numbers of plants for an experiment such as this one. The bulbing ratio is routinely used as a non-destructive measurement of bulb initiation (Brewster, 2008). Following the slight delay, plants then began to show a very slow increase in bulbing ratio. It would appear that plants which have been grown in SDs for a long period of time are very slow to initiate bulbing (Figs. 3.4 & 3.5). This is the opposite of floral initiation, where plants which have been in SDs for a long period of time will flower rapidly upon transfer to LDs (Jack, 2004). The significance of the differences in bulbing ratios of transferred plants was tested using two-way ANOVA's carried out on log base 10 transformed data (Tables A1 and A2, Appendix 3). For all the transfers, it was shown that the observed differences were significant in terms of daylength and that the pattern over time was affected by treatment (Table 3.1). Mean square values showed that it is daylength which has the most significant effect on bulbing ratio.

Changes in bulb diameter were examined independently of bulbing ratio in plants from an early (transfer 1) and late (transfer 4) transfer. The swelling of the bulb was seen to be much slower in transferred plants than in those grown in constant LDs (Figs. 3.6 & 3.7). In fact, plants showed a similar increase in bulb diameter to those grown in constant SDs. The increase in bulbing ratios (especially evident in transfer 1, Fig. 3.2) must therefore have been due to a cessation of vegetative growth and hence the neck diameter remaining constant. Bulbing was initiated in plants from transfer 1 but not later transfers (Fig. 3.8). Bulb diameters for all transferred plants remained small throughout the experiment. The significance of the differences in bulb diameters was tested using two-way ANOVA's using log base 10 transformed data (Table A3, Appendix 3). For both transfers, daylength was shown to have a significant effect on bulb diameter (Table 3.2). Mean square values

showed that daylength has the most significant effect on bulb diameter. For transfer 1 the changes in bulb diameter observed over time were shown to be affected by daylength. However, for transfer 4, the pattern over time was shown to be independent of treatment. This suggests that after a certain point, plants which are transferred from SD to LD cannot initiate bulbing. The minor increases in bulbing ratio shown in plants from transfer 4 are due to a cessation of vegetative growth, leading to the neck diameter remaining fairly constant. This was further investigated by plotting the neck diameter data independently of bulbing ratio (Fig. 3.9). It is clear that the neck diameter stops increasing in transferred plants, even those transferred at a later date.

It is possible that the delay in bulb swelling is due to individual leaves becoming committed to a particular daylength response. However, this would be expected to lead to a continued increase in neck diameter. Another possible explanation is that irradiance levels and temperatures were very low in July 2007, possibly delaying the response. Both irradiance and temperature have been shown to affect bulbing (Steer, 1980; Wright and Sobeih, 1986). Therefore, it is possible that the bulbing process was initiated, but swelling of the bulb was delayed. However, this would also be expected to affect plants grown in constant LD conditions. The theory which is most consistent with the data is that plants which remain in SDs accumulate an inhibitor of bulb initiation. It would then take longer for plants to initiate bulbing as the inhibitor would have to be degraded.

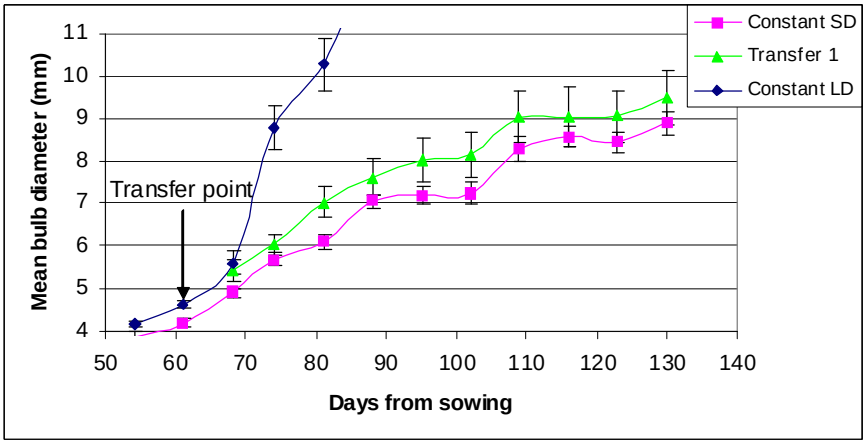


Fig. 3.6: Bulb diameters of Renate F_1 plants transferred from SD to LD conditions (transfer 1). Error bars represent the SEM. The mean bulb diameter of plants grown in LDs was 17.7 mm after 130 days.

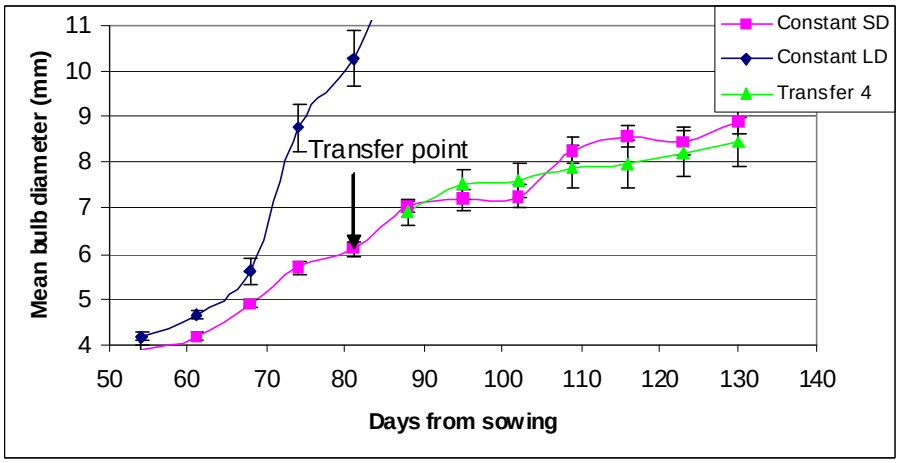


Fig. 3.7: Bulb diameters of Renate F_1 plants transferred from SD to LD conditions (transfer 4). Error bars represent the SEM. The mean bulb diameter of plants grown in LDs was 17.7 mm after 130 days.

Fig. 3.8: The response of Renate F_1 onions transferred from SD to LD conditions. Photograph shows plants from transfer 1, 77 days after transfer and plants from transfer 4, 56 days after transfer.

Table 3.2: Assessing the significance of the differences in bulb diameter between treatments. The controls refer to differences between plants grown in constant LD and constant SD conditions. p=probability, MS=mean square (green colour indicates strongest effect).

Transfer	Source of Variation	P	MS	Degrees of Freedom
Control	Days from sowing	<0.001	2.70	12
Control	Daylength	<0.001	14.86	1
Control	Days from sowing x daylength	<0.001	0.22	12
1	Days from sowing	<0.001	2.37	11
1	Daylength	<0.001	7.80	3
1	Days from sowing x daylength	<0.001	0.16	33
4	Days from sowing	<0.001	0.53	8
4	Daylength	<0.001	8.73	3
4	Days from sowing x daylength	0.745	0.02	24

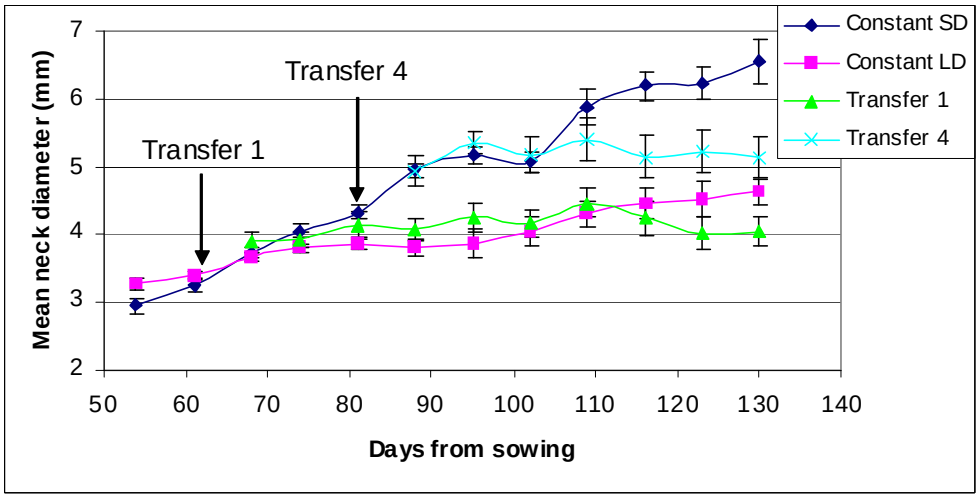


Fig. 3.9: Neck diameters of Renate F_1 plants transferred from SD to LD conditions. Error bars represent the SEM.

3.3.3 Plants transferred from LD to SD conditions

Plants transferred from LD to SD conditions respond quickly to the transfer, changes in bulbing ratio being visible as early as one week after transfer (Figs. 3.2-3.5). This suggests that individual leaves can resume vegetative growth very quickly

in non-inductive conditions and suggests that bulb scale production is inhibited rapidly in SD's. The effect of daylength transfer on bulbing ratio was shown to be significant for all transfers using two-way ANOVA's, as described in section 3.3.2 (Table 3.1).

When the neck diameter is considered independently of the bulbing ratio, it is clear that an increase in neck diameter is responsible for the decrease in bulbing ratio as illustrated by the data from transfer 2 (Fig. 3.10). A similar pattern was observed for all other transfers (Figs. A3-A5, Appendix 3). This suggests that Renate F_1 onions respond quickly to a transfer to non-inductive conditions. This is consistent with a theory that a repressor of bulb initiation accumulates in SDs, delaying the response after transfer from SD to inductive LD conditions. The return to vegetative growth following a transfer from LD to SD conditions is shown in Fig. 3.7. Two-way ANOVA's were carried out on log base 10 transformed data (Table A4, Appendix 3) to assess the significance of the changes in neck diameter between treatments. For all transfers, the treatment (daylength) was shown to have a significant effect on neck diameter (Table A6, Appendix 3). The pattern of change in neck diameter over time was also shown to be affected by daylength for all transfers. Mean square values showed that daylength has the most significant effect on neck diameter. ANOVA data for transfer 2 is displayed in Table 3.3.

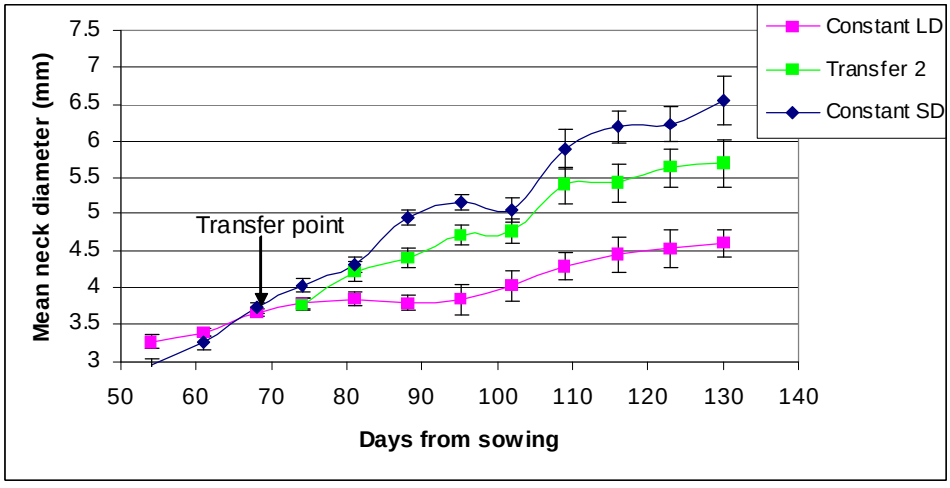


Fig. 3.10: Neck diameters of Renate F_1 plants transferred from LD to SD conditions (transfer 2). Error bars represent the SEM

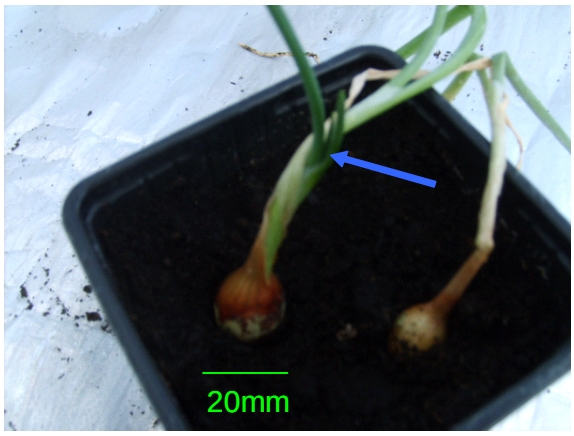


Fig 3.11: The resumption of vegetative growth following a transfer from LD to SD conditions (Transfer 2). The arrow indicates the production of new leaves which leads to an increase in neck diameter and therefore a decrease in the bulbing ratio.

Table 3.3: Assessing the significance of the differences in neck diameter between treatments. The controls refer to differences between plants grown in constant LD and constant SD conditions. p=probability, MS=mean square (green colour indicates strongest effect).

Transfer	Source of Variation	P	MS	Degrees of Freedom
Control	Days from sowing	<0.001	0.65	12
Control	Daylength	<0.001	1.65	1
Control	Days from sowing x daylength	<0.001	0.12	12
2	Days from sowing	<0.001	0.54	10
2	Daylength	<0.001	1.04	3
2	Days from sowing x daylength	<0.001	0.06	30

3.4 Conclusions

Bulb initiation in 'Renate F_1' onions is controlled by photoperiod. LDs of 16 or more hours of light will initiate bulbing. Bulbing is also a reversible process, drawing distinct comparisons with photoperiodic flowering (Summerfield *et al.*, 1991). However, there is a delay in response when plants are transferred from SD to LD conditions. A delay in response is not observed when plants are transferred from LD to SD conditions. This is consistent with the theory that an inhibitor of bulb

initiation accumulates when plants are in SD conditions, delaying initiation. The data presented in this chapter is consistent with data from field experiments and shows that LD onions require LDs throughout the period of bulbing to reach maturation (Kedar et al., 1975). However, it would be advantageous to undertake a larger scale experiment with transfer points throughout the development of the plants.

The experiment described here also allowed a system to be set up in order to study the expression of putative photoperiod response genes. The photoperiodic nature of bulb initiation leads to the hypothesis that the genes controlling photoperiodic flowering also control photoperiodic bulbing. The characterisation of several onion putative photoperiod response genes is discussed in chapters 4-6.

CHAPTER 4: THE SEARCH FOR AN ONION *CONSTANS* (*CO*) HOMOLOGUE

4.1: Introduction

This chapter discusses the search for and characterisation of onion *CO*/*CO*-like genes. Much emphasis was placed on the search for an onion *CO* orthologue as this gene is central to the photoperiodic flowering pathway in both *Arabidopsis* and rice (Jackson, 2008). In addition, it has been shown to have a role in a different response to photoperiod, namely tuberisation in potato (Martínez-García *et al.*, 2002). This led to the hypothesis that *CO* is involved in bulb initiation in onion.

4.1.1 The role of CO in the photoperiod control of flowering

CO is a circadian clock-regulated gene which controls flowering time in *Arabidopsis* (Suárez-López *et al.*, 2001). The role of *CO* in the photoperiodic flowering pathway is discussed in section 1.5.1, and is elaborated on in this section. The *Arabidopsis CO* gene controls flowering through induction of the floral integrator genes *FT, TSF and SOC1* (Samach *et al.*, 2000; Yamaguchi *et al.*, 2005; Yoo *et al.*, 2005). The expression of *CO* is controlled by several different genes. Recent work showed that FKF1 and GI form a protein complex which controls *CO* expression by degrading CYCLING DOF FACTOR 1 (CDF1), a transcriptional repressor of *CO* (Sawa *et al.*, 2007). In addition, other members of the *CDF* gene family have been shown to repress *CO* (Coupland, 2008). The GI/FKF1 protein complex has been shown to degrade two such proteins, CDF2 and CDF5. Other proteins which have been shown to repress CO include CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) and SUPPRESSOR OF PHYA-105 (SPA1), which have been shown to degrade the CO protein at night (Laubinger *et al.*, 2006; Jang *et al.*, 2008). In short, *CO* expression is at its highest towards the end of a long day (due to the degradation of CDF proteins in the afternoon). The CO protein is degraded at night. In SD grown plants, the expression of *CO* is seen to peak in the dark (Suárez-López *et al.*, 2001), when the protein would be degraded. This allows flowering to occur in the correct season in LD plants. The CO protein is expressed at

very low levels, making it the limiting factor in the photoperiodic control of flowering (Robson *et al.*, 2001).

An additional level of post-transcriptional control also allows for seasonal control of flowering. Photoreceptors have been shown to control the levels of CO protein, ensuring that levels only peak at the end of a LD (Valverde *et al.*, 2004). It is proposed that PHYB promotes the degradation of CO early in the day and the CRYPTOCHROMES (CRY1 and CRY2) and PHYA act antagonistically, stabilising the CO protein late in the day. The lower abundance of *CRY2* in SDs may also contribute to the inhibition of flowering under these conditions (El-Assal *et al.*, 2003). A summary of the precise role of *CO* in the photoperiod pathway is shown in Fig. 4.1. A recent study has also shown that the balance between *CO* and TEMPRANILLO (TEM1 and TEM2) genes controls the expression of *FT* (Castillejo and Pelaz, 2008). The *TEM* genes are repressors of *FT* and are downregulated during development. This downregulation leads to a higher ratio of *CO:TEM*, leading to *FT* transcription and hence floral induction. This indicates another level of control mediating seasonal control of flowering through CO.

4.1.2 The CO/CO-like gene family

CO belongs to a family of 17 genes in *Arabidopsis*, including 16 *CO*-like genes, all of which are putative transcription factors (Griffiths *et al.*, 2003). This family of genes is characterised by two conserved domains (Robson *et al.*, 2001). The first is a zinc-finger region near the amino terminus (B-box) and the second is a region near the carboxy terminus termed the CCT (*CO*, *CO*-like, *TOC1*) domain (Fig. 4.2). All *CO* and *CO*-like genes contain a CCT domain, but there is some variation in the B-box region (Table 4.1).

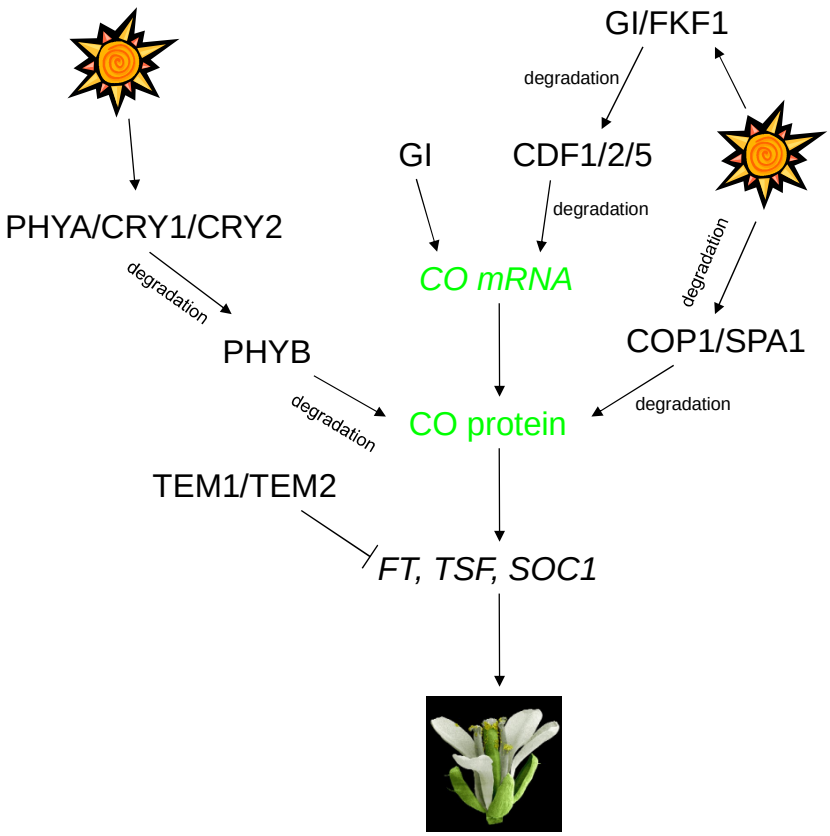


Fig. 4.1: The role of *CO* in the photoperiodic control of flowering. It is proposed that the balance between CO and TEM1/TEM2 controls *FT* expression. Arrows depict a promoting effect, bars depict a repression (adapted from Castillejo and Pelaz, 2008; Coupland, 2008 and Massiah, 2007). *Arabidopsis* flower picture copyright Juergen Berger, Max Planck Institute for Developmental Biology.

Fig. 4.2: Structure of the *CO* gene.

Table 4.1: The grouping of *CO* and *CO*-like genes in *Arabidopsis*.

Group	Members	B-box Structure
I	*CO, COL1-COL5*	Two B-boxes
II	*COL6-COL8, COL16*	One B-box
III	*COL9-COL15*	One B-box and one diverged zinc finger domain

B-boxes are also present in animal genes and act as protein-protein interaction domains (Borden, 1998). The CCT domain is specific to *CO*, *CO*-like and *TOC1* genes and functions in the nuclear localisation of the CO protein (Robson *et al.*, 2001). At present, the functions of only four *Arabidopsis CO*-like genes have been studied. *COL1* and *COL2* are circadian regulated but do not affect flowering time (Ledger *et al.*, 2001). Both genes show a peak in expression around dawn in LDs. This is in contrast to *CO* expression which is seen to peak around ZT 16 in LDs and ZT 20 in SDs (Suárez-López *et al.*, 2001). Overexpression of *COL1* affects circadian rhythms, so may have a role in a light input pathway. Expression of *COL9* is circadian regulated and its overexpression leads to delayed flowering (Cheng and Wang, 2005). Expression peaks of *COL9* are seen in the dark in LDs and at dusk in SDs. It has been suggested that *COL9* may regulate *CO* expression. Recently, *COL3* has been shown to be a positive regulator of red light signalling and root growth (Datta *et al.*, 2006). Unlike late flowering *co* mutants, *col3* mutants are early flowering.

CO and *CO*-like genes have also been identified in other species such as *Brassica napus* (Robert *et al*., 1998), *Pharbitis nil* (Liu *et al*., 2001), barley (Griffiths *et al.*, 2003), rice (Hayama *et al*., 2002; Hayama *et al*., 2003), *Lolium perenne* (Martin *et al*., 2004), wheat (Nemoto *et al*., 2003) pea (Hecht *et al*., 2005), maize (Miller *et al*., 2008) and the moss *Physcomitrella patens* (Zobell *et al.*, 2005). The presence of *CO*-like genes in short-day plants such as rice and *Pharbitis nil* suggests a conserved pathway that regulates flowering during an inductive daylength. It has also been shown that *Arabidopsis CO*, when overexpressed in potato, impairs tuberisation in short-day inductive conditions (Martínez-García *et al.*, 2002). This suggests a broader role for *CO* than simply controlling flowering in long-days and an involvement in bulb initiation can be hypothesised.

There are 16 *CO*-like genes in rice. *Heading date 1* (*Hd1*) is the rice homologue of *Arabidopsis CO* (Griffiths *et al*., 2003). However in rice, *Hd1* has the opposite function to *Arabidopsis CO*, suppressing the expression of *Heading date 3a* (*Hd3a*, the rice homolog of *FT*) and inhibiting flowering in LDs (Hayama *et al.*, 2003). This shows that *CO* is still an integral part of the photoperiod pathway even if the response to photoperiod is different. The presence of a *CO* homologue in rice is particularly interesting, as rice is more closely related to onion than *Arabidopsis* in terms of phylogenetic distances (Kuhl *et al*., 2004). A recent study described the

characterisation of one of the rice *CO*-like genes, *OsCO3* (Kim *et al*., 2008b). It was shown that this gene has a role in the photoperiod response in rice. It controls flowering by negatively regulating the expression of *FT*-like genes in SDs. It was also shown that *OsCO3* oscillates with a different phase to *Hd1*. This shows the involvement of *CO*-like genes in flowering. At present, the other members of the rice *CO*-like gene family have not been characterised.

The purpose of this study was to isolate and clone members of the *CO*/*CO*-like gene family in onion. The isolation and characterisation of an orthologue of *CO* was of particular interest as this gene is central to the photoperiod pathway. The starting point was the knowledge of the presence of at least one *CO*/*CO*-like gene (clone name BR20) in the *A.cepa* gene index (DFCI, 2008).

4.2 Materials and Methods

4.2.1 Characterisation of an onion CO-like gene

4.2.1.1 Sequencing and comparison with *CO* homologues

Sequence information for an onion *CO*-like gene (clone name BR20) was obtained as described in section 2.3.1. The gene was given the name *AcCOL* (for *Allium cepa CONSTANS*-like). A contig was constructed using the SeqMan package of DNAStar (DNAStar Inc.). Sequences of *CO* and *CO*-like genes from other plant species were downloaded from publicly available databases (NCBI, 2008). B-box and CCT domain regions were separated into different data files using the EditSeq package of DNAStar (DNAStar Inc.). B-box sequences were also stored in FASTA format in Notepad, alignments carried out and a NJ-tree constructed as described in section 2.3.7, using predicted amino acid sequences. Nucleotide and amino acid alignments were also carried out using the Megalign program of DNAStar, and percentage identities of *AcCOL* with *Arabidopsis CO*-like genes calculated.

4.2.1.2 Expression of *AcCOL*

The expression of *AcCOL* at different time-points and in different tissues was examined using RT-PCR as described in section 2.3.2. The primers used were CO-RT F1 & R1 (see Appendix 2 for sequences) with an annealing temperature of 55 °C and a cycle number of 35. PCR products were purified and sequenced using gene-specific primers. The relative expression of *AcCOL* in a LD onion variety (Renate F_1) over a 48-hour period was then examined using quantitative real-time PCR, as described in section 2.3.3. The expression was examined under LD (16 hours of light) and SD (8 hours of light) conditions. The primers used for quantitative RT-PCR were BR20-RT F2 & BR20-RT R2 at a concentration of 0.2 µM. All cDNA samples were diluted 1 in 5 and run in triplicate. Data was normalised to the expression of *EF1α* and standard errors calculated.

4.2.1.3 Over-expression of *AcCOL* in *Arabidopsis* plants

Transformations were carried out using the floral dip method described in section 2.3.6. *Arabidopsis co-2* mutant and L*er* wild-type plants were transformed to over-express *AcCOL*. Plants were allowed to flower and set seed under LD conditions. Flowering time in LDs was scored in the T_2 generation. The expression of the transgene was tested using RT-PCR as described in sections 2.2.1 and 2.2.2. TC2898 FOR and TC2898 REV primers (see Appendix 2 for sequences), with an annealing temperature of 55 ºC and a cycle number of 35, were used to test L*er* transgenic lines. BR20-RT F2 and BR20-RT R2 primers (see Appendix 2 for sequences), with an annealing temperature of 61 ºC and a cycle number of 30, were used to test *co-2* transgenic lines. PCR products were sequenced using gene-specific primers to confirm their identity.

4.2.2 Phylogenetic analysis of CO/CO-like genes

A detailed phylogenetic analysis of the *CO*/*CO*-like gene family was carried out. This analysis was based around the CCT domain and included other CCT domain containing genes (Table 4.2). Published sequences were collated in a single document within Notepad and converted to FASTA format (Accession numbers can

be found in Table A8, Appendix 4). Sequences were then aligned using the software package ClustalX (Thompson *et al.*, 1997). The alignment was edited using the Jalview software package to contain only the CCT domain (Clamp *et al.*, 2004). Sequences lacking a CCT domain were excluded at this stage. A preliminary NJ tree was then constructed using the software package Mesquite (Maddison and Maddison, 2006). Due to computational constraints, a maximum likelihood tree could not be constructed because of the large number of genes in this analysis. Therefore, an ancestor reconstruction was carried out on the outgroup produced in the NJ tree (using Mesquite) and this was transformed into one single representative sequence using a character collapser programme which employs a PAM scoring system (written by Robin Allaby, Warwick HRI). This single sequence was then used to form the outgroup of a maximum likelihood tree, which was constructed using the PROML package of PHYLIP (Felsenstein, 1989).

Table 4.2: Genes containing a CCT domain which were included in *CO/CO*-like phylogenetic analysis. *PRR=pseudo response regulators; ZIM=Zinc finger protein expressed in Inflorescence Meristem; ZML=ZIM-like; CIL/CIA2*=full names not specified; *ASML2= ACTIVATOR OF SPOMIN::LUC2; PPD-H1= Photoperiod-H1*.

Gene	Function	Reference
PRR gene family	Control of circadian clock	(Kato *et al*., 2007; Murakami *et al*., 2007)
TOC1 (important member of *PRR* gene family)	Control of circadian clock	(Strayer *et al*., 2000)
ZIM/ZML	GATA-type transcription factors	(Shikata *et al.*, 2004)
CIL/CIA2	Leaf-specific upregulation of chloroplast translocon genes	(Sun *et al*., 2001)
ASML2	Regulation of expression of sugar-inducible genes	(Masaki *et al*., 2005)
PPD-H1	Involved in photoperiod response in barley	(Turner *et al*., 2005)
ZCCT	Repression of flowering	(Yan *et al*., 2004)

4.2.3 Searching for onion CO/CO-like genes

Various strategies were employed in an attempt to isolate *CO*/*CO*-like genes from onion. This is summarised in Fig. 4.3.

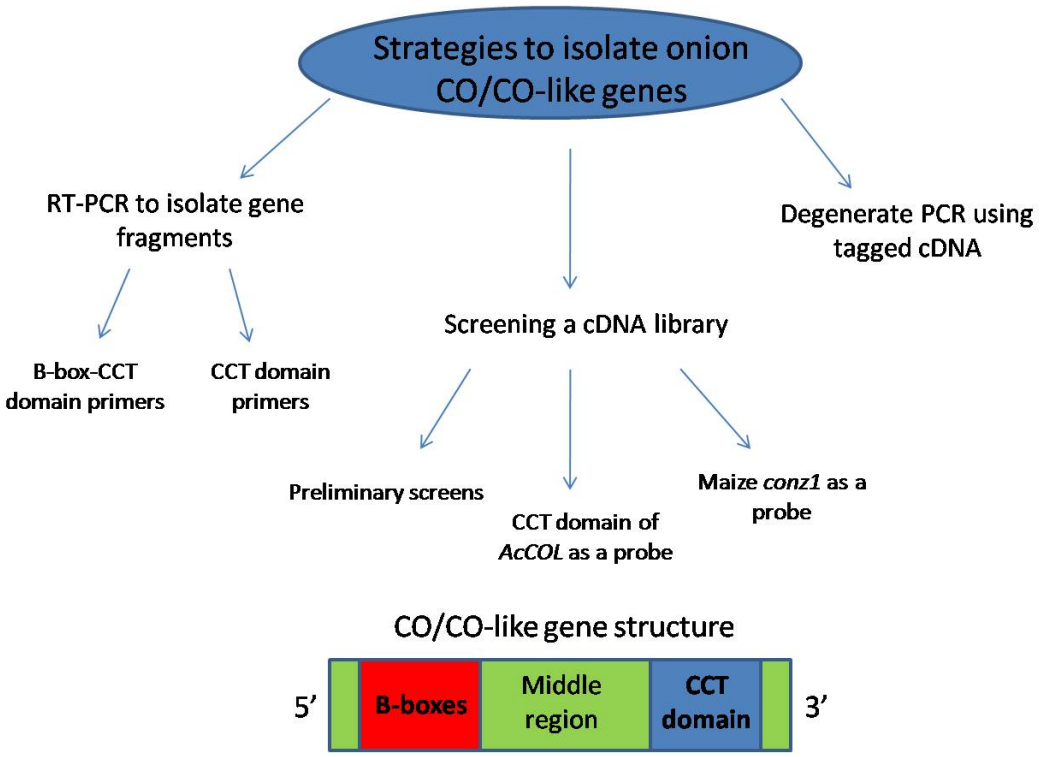


Fig. 4.3: The strategies employed to isolate onion *CO*/*CO*-like genes.

4.2.3.1 Amplification from B-box to CCT domain

Four degenerate primers were designed in an attempt to isolate onion *CO*. The two forward primers (B-box F1 and B-box R1) were designed in the B-box domain and the two reverse primers in the CCT domain (CCT F1 and CCT R1). Primer sequences can be found in Appendix 2. Primer design was carried out following the alignment of *CO* and *CO*-like genes from *Arabidopsis*, rice and barley with *AcCOL*. RNA was then extracted from leaf and bulb material (provided by Prof. Brian Thomas, Warwick HRI), DNase treated and first strand cDNA synthesised as described in section 2.2.2. PCR was then carried out (35 cycles) using the four combinations of primer pairs and the protocol in section 2.2.1. The annealing temperature for F1/R1 and F2/R1 was 55 °C. The annealing temperature

for F2/R1 and F2/F2 was 47 °C. *Arabidopsis* leaf cDNA and mRNA and plasmid DNA extracted from the *35S:CO* plasmid were received from Andrea Massiah/Karl Morris (Warwick HRI) and were used as additional PCR templates. PCR products were purified and cloned using a pMOS*Blue* blunt-ended cloning kit (Amersham Biosciences, Cat. No. RPN 5110), following the manufacturer's guidelines. The transformation method used was electroporation (section 2.2.9). The method of selection used was blue-white selection (Sambrook et al., 1989).

RNA was extracted from tissue harvested at ZT15, DNase treated and cDNA synthesised as described in section 2.2.2. Genomic DNA was also extracted, following the protocol described in section 2.2.3. RT-PCR was then carried out with the four primer pairs and cycling conditions mentioned earlier in this section. Products were purified and cloned into a pMOS*Blue* vector (Amersham Biosciences), following the manufacturer's guideline. The transformation method used was electroporation (section 2.2.9) and the selection method used was blue-white selection (Sambrook *et al.*, 1989). A second round of PCR was carried out using 1 µl of the previously purified products as a template. The same primers and cycling conditions were used. Products were purified and cloned using the method described above. Cultures were seeded, plasmid DNA isolated and sequencing carried out using T7 and U-19 vector primers (see Appendix 2 for sequences) as described in sections 2.2.5 & 2.2.8.

4.2.3.2 Amplification of the CCT domain

A forward primer was designed just outside the CCT domain (towards the 5' end) which contained 20 % degeneracy (preCCT, see Appendix 2 for sequence). A reverse primer was designed in the CCT domain and contained only 5 % degeneracy (CCTnew, see Appendix 2 for sequence). Primer design was based on previous sequence alignments of *CO* and *CO*-like genes (section 4.2.1.1). These primers were designed to amplify a 90 bp fragment. PCR was carried out with an annealing temperature of 54 °C and a cycle number of 35. A fragment was generated from cDNA and cloned using Gateway® BP Clonase® (Invitrogen Ltd., Cat. No. 11789), following the manufacturer's guidelines. PCR was set up to re-amplify this product, using 1 µl of the purified product as a template and the PCR cycling described. Both the original and re-amplified fragments were cloned into a pDONR222 vector

(Invitrogen Ltd., provided by Mahmut Tor, Warwick HRI), following the manufacturer's guidelines. Plasmid DNA was isolated and sequencing carried out using M13 forward and reverse primers (see Appendix 2 for sequences). A total of 32 clones were screened (16 from each fragment).

4.2.4 Normalised cDNA library

4.2.4.1 Preliminary screening of cDNA library

A normalised cDNA library was constructed from onion leaf and bulb tissue, as described in section 2.3.8.1. Plasmid DNA was extracted from a small fraction of the cDNA library, following the protocol set out in section 2.2.2. A 40 µl fraction of a single library aliquot was used to seed an overnight culture (approximately 1 % of the total library).

In order to work towards screening the library for onion *CO/CO*-like genes, primers were designed to amplify such genes. Two different types of probe were generated with a view to screening the library. Firstly, primers were designed specifically to amplify a region close to the CCT domain of monocot *CO* genes. One forward and two reverse primers were designed (MONCO F and MONCO R1/R2, see Appendix 2 for sequences). PCR was carried out using an annealing temperature of (40 cycles) using both primer pairs and maize genomic DNA as a positive control. The annealing temperatures used were 55 °C for MONCO F and R2 and 65 °C for MONCO F AND R1. A second set of primers were designed to specifically amplify the CCT domain of *AcCOL* (BR20 CCT F & BR20 CCT R, see Appendix 2 for sequences). PCR was carried out using 30 cycles and an annealing temperature of 61 °C with library plasmid DNA, genomic DNA and cDNA as templates. All products were purified (section 2.2.6) and cloned onto a pMOS*Blue* vector (Amersham Biosciences), following the manufacturer's guidelines. The transformation method used was electroporation (section 2.2.9) and the selection method used was blue-white selection (Sambrook *et al.*, 1989). Cultures were seeded, plasmid DNA isolated and sequencing carried out using T7 and U19 primers (sections 2.2.5 & 2.2.8, see Appendix 2 for primer sequences).

4.2.4.2 Screening a normalised cDNA library for onion *CO*/*CO*-like genes

4.2.4.2.1 *AcCOL* CCT domain as a probe

The protocol for screening the cDNA library is outlined in section 2.3.8.2. The probe was generated by PCR incorporation of DIG (Roche), following the manufacturer's guidelines. The enzyme used was KOD Hot Start DNA polymerase (Merck Bioscience). The primers used were BR20 CCT F & BR20 CCT R (see Appendix 2 for sequences), using the cycling conditions described in section 4.2.4.1. The template used was a previously purified product amplified from library plasmid DNA using the same primer pair.

A test hybridisation was carried out using 2 cm^2 discs of positively charged nylon membrane (Roche). Hybridisation was then carried out following the manufacturer's guidelines, with modifications (Roche, Cat No. 1636090). The pre-hybridisation step was carried out for 2 hr and the hybridisation carried out overnight. The hybridisation temperature used was 42 ºC with a probe concentration of 3 µl per ml of hybridisation solution. The high stringency washes were carried out using 0.5x SSC, 0.1 % SDS at 60 ºC. Following the addition of CSPD, the membranes were exposed to X-ray film (Kodak Biomax MR film, Sigma-Aldrich, Cat No. 891 2560) for 2 hours and overnight at room temperature. The film was then passed through a hyperprocessor (Amersham Life Science). Membranes were then stripped and stored in 2xSSC, following the protocol provided with the kit.

Positively hybridising clones were tracked back to the original 384-well plate and PCR's carried out using BR20-CCT F & R primers (see Appendix 2 for sequences) or a combination of one BR20 CCT primer or one vector primer (M13 forward or reverse, see Appendix 2 for sequences) to confirm the presence of a positive clone. PCR's were carried out as described in section 2.2.1 using an appropriate annealing temperature for the primer pair and 25-35 PCR cycles. Products were purified and sequenced using both forward and reverse primers (sections 2.2.6 & 2.2.8).

4.2.4.2.2 Maize *conz1* as a probe

The maize *conz1* gene (provided by Dr. Theresa Miller, Marquette University, Milwaukee) was cut out of its vector (pGEM-T, Invitrogen Ltd.) using the restriction enzyme EcoR1 (Invitrogen Ltd.). The product was run on a 1 % agarose (w/v) gel and the insert (1279 bp) purified. Random primed labelling (Dig-High Prime DNA Labelling and Detection Starter Kit II, Roche, Cat. No. 1585614) was carried out using 300 ng of the purified product and following the manufacturer's guidelines. Hybridisation and detection were carried out following the manufacturer's guidelines, using approximately 25 ng of probe per ml of hybridisation solution. A hybridisation temperature of 35 ºC was used and the high stringency wash carried out using 0.5x SSC, 0.1 % (w/v) SDS at 58 ºC. Membranes were exposed to X-ray film for 5 min, 25 min and 2 hr.

Following the library screen, positively hybridising clones were tracked back to the original 384-well plate. One microlitre of wells K13 (plate 2) and K9 (plate 4) were mixed with 1.5 ml of LB media (VWR International). Fifty microlitres was then spread on LB plates containing 100 µg ml^{-1} ampicillin (Melford). The plates were left at 37 ºC overnight. Single colonies (168 for each of the 2 wells) were then used to seed cultures on a 384-well plate containing 75 µl of freezer media (see Appendix 1 for recipe) per well & 100 µg ml^{-1} ampicillin. Further cultures on the plate were seeded using 1 µl of all of the wells which produced positive hits on the initial screen. A colony containing a clone of the maize *conz1* gene (in the pGEM-T vector) was used to seed the positive control wells. Negative control wells were grown from wells which produced no spot with the previous screen. The plate was placed at 37 ºC overnight without shaking. A 384-well pin replicator (Nunc Inc.) was used to spot the wells onto positively charged nylon membrane (Roche) as described in section 2.3.8.2. The membranes were processed and probed as described in the same section. Membranes were exposed to X-ray film (Sigma-Aldrich) for 12, 26 and 60 min.

4.2.5 Degenerate PCR using tagged cDNA

Degenerate PCR was carried out using a protocol adapted from the 3' RACE PCR method (Borson *et al.*, 1992), communicated by Dr. Ken Manning, Warwick HRI. A poly T primer with a specific tag sequence was designed (named PolyT Adapter, see Appendix 2 for sequence) and cDNA synthesised using this primer in place of Oligo DT (following the protocol set out in section 2.2.2). Degenerate primers were then designed to amplify from the B-box (B-box 1 DEG/B-box 2 DEG, see Appendix 2 for sequences) or CCT (CCT DEG 1/CCT DEG 2, see Appendix 2 for sequences) domains. Primers were designed with a specific TAG sequence attached to allow for PCR re-amplification of weakly amplified products. Primer design was based on the large scale alignment of *CO* and *CO*-like genes described in section 4.2.2. PCR was set up using a forward degenerate primer in conjunction with the reverse TAG primer. All primers were tested with an annealing temperature of 60 ºC and a cycle number of 35. PCR products which matched the estimated product size (CCT primers only) were cloned into a pMOS*Blue* vector (Amersham Biosciences). The method of transformation was electroporation as described in section 2.2.9. Positive clones were selected using blue-white selection (Sambrook *et al.*, 1989). Plasmid DNA was isolated from 47 individual clones and sequencing carried out using T7 and U-19 vector primers. Sequences were then BLASTED against publicly available databases (NCBI, 2008).

4.3 Results and Discussion

4.3.1 Characterisation of AcCOL

4.3.1.1 Sequencing analysis

Sequence analysis revealed that the clone containing *AcCOL* was full-length and comprised a coding region of 954 bp (318 amino acids, Fig. 4.4). This is slightly different from *Arabidopsis CO* which has a coding region spanning 1122 bp (374 amino acids). Further analysis of the sequence of *AcCOL* revealed that the gene contains B-Box and a CCT domain regions, the conserved domains which are present

in all *CO* and *CO*-like genes (Robson *et al*., 2001). Amino acid alignments show that this gene shares the highest percentage identity with *Arabidopsis COL4*, a hitherto uncharacterised *CO*-like gene (Table 4.3). A closer examination of the B-Box structure reveals that this gene is a group I *CO*-like gene as it contains 2 B-Boxes (Griffiths *et al*., 2003). This group contains the genes which are most closely related to *CO* itself.

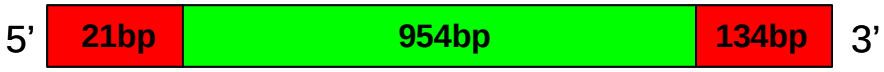


Fig. 4.4: Structure of *AcCOL* mRNA. The green box represents the coding region; red boxes represent the untranslated regions.

Table 4.3: Amino acid identity of *AcCOL* with *Arabidopsis CO* and *COL1-5*.

***Arabidopsis* Gene**	**Percentage Amino Acid Identity**
CO	34.9
COL1	39.3
COL2	37.7
COL3	40.0
COL4	48.1
COL5	43.1

A method of distinguishing *CO* from *CO*-like genes by amino acid sequence has been previously described (Griffiths *et al*., 2003). This involves the presence or absence of certain amino acids within the B-box. Using this method, group I genes can be further subdivided by the presence or absence of certain amino acids (illustrated in Fig. 4.5). Analysis of *AcCOL* would suggest it is a *CO*-like gene as it contains amino acids that distinguish *COL3-COL5* from *CO*, *COL1* and *COL2* (Fig. 4.5). A further issue involves the *co-6* mutant. This mutant flowers 21 days later than the wild-type under LDs but is not late flowering under SDs and is not affected by vernalisation (TAIR, 2009). The *co-6* mutant has an amino acid substitution, where a valine replaces an alanine. *AcCOL* has a threonine in this position which may be significant.

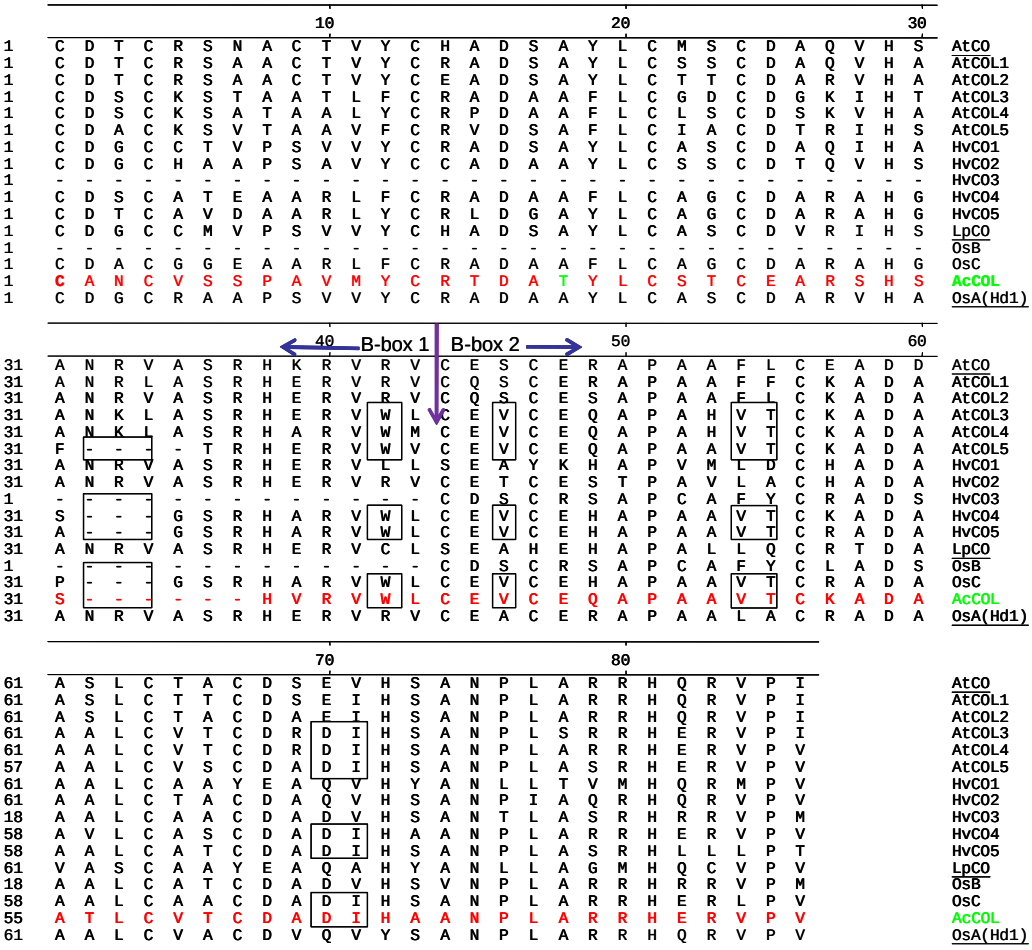


Fig. 4.5: Amino acid alignment of the B-Boxes of *CO* and *CO*-like genes showing the residues used to distinguish *CO*-like genes from *CO*. Boxed residues distinguish *COL3* to *COL5* from the most *CO*-like group (*CO*, *COL1* and *COL2* in *Arabidopsis*). The boxed region showing a gap of 3 amino acids is only found in *CO*-like genes and may disrupt B-box function The green coloured amino acid shows a substitution in *AcCOL* at the same site that a substitution is seen in the *co-6* mutant. Genes which are underlined are *CO* orthologues. *HvCO1* and *HvCO2* are the barley genes which are the closest relatives of *CO*. At=*Arabidopsis thaliana*, Hv=*Hordeum vulgare*, Os=*Oryza sativa*, Lp=*Lolium perenne*. Accession numbers can be found in Table A8, Appendix 4.

A phylogenetic tree was constructed using the B-box sequences of several Group I *CO*/*CO*-like genes (section 4.2.1.1, Fig. 4.6). This tree shows a clear separation of what can be referred to as Group Ia and group Ib genes. Two separate clades are formed with strong support from high bootstrap values. *AcCOL* sits in the

clade containing Group Ib genes. This supports the observations from Fig. 4.5 and suggests that *AcCOL* is a *CO*-like gene and not a *CO* orthologue. A more detailed phylogenetic analysis (based around the CCT domain) was carried out and is discussed in section 4.3.2.

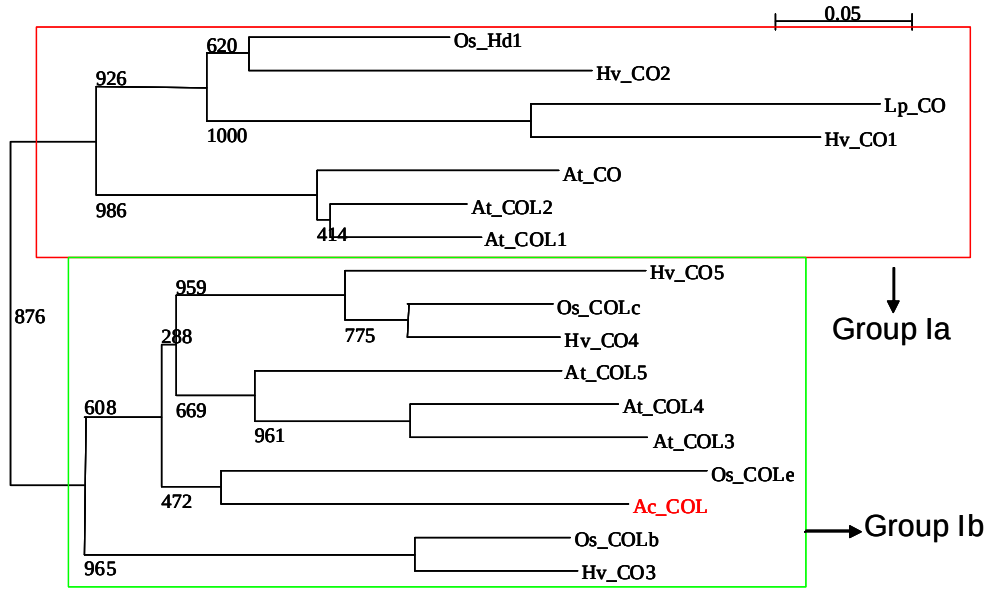


Fig. 4.6: NJ tree showing the relationships between Group I *CO*/*CO*-like genes. Numbers represent the bootstrap values calculated using 1000 bootstrap replicates. Os=*Oryza sativa*, Hv=*Hordeum vulgare*, At=*Arabidopsis thaliana*, Lp=*Lolium perenne*, Ac=*Allium cepa*. Accession numbers can be found in Table A8, Appendix 4.

4.3.1.2 Expression analysis

The results of the preliminary gene expression experiment (sections 2.3.2 & 4.2.1.2) showed that *AcCOL* is expressed at all time-points in the leaf material (Fig. 4.7). Expression would appear to be higher at ZT11.5 and 15 than at ZT8. Expression of *AcCOL* was also seen in the material described as the bulb. The plants used for harvests were not yet bulbing, so bulb material was hard to distinguish from leaf material and some expression would be expected. No expression was seen in the roots (data not shown). Initial results suggest that *AcCOL* is differentially expressed over time. *Arabidopsis CO* is expressed highest at ZT16 (Suárez-López *et al*., 2001). *Arabidopsis COL1* and *COL2* are expressed highest around dawn and *COL9* around

dusk (Ledger *et al.*, 2001; Cheng and Wang, 2005). The expression of other members of the *CO*-like gene family has yet to be investigated.

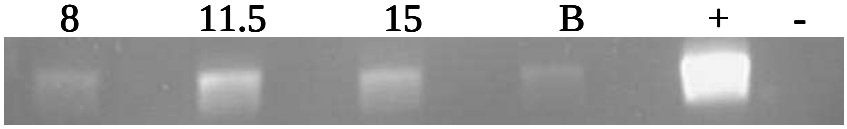


Fig. 4.7: Expression of *AcCOL* in Renate F_1 onions at different time-points and in different tissues. Numbers represent zeitgeber time in leaf tissue, B=bulb, positive control is plasmid DNA isolated from a pCMV.SPORT.*AcCOL* vector, negative control is water.

The expression of *AcCOL* was further investigated using quantitative real-time PCR (sections 4.2.1.2 & 2.3.3). The expression profile for this gene shows no obvious trend; various peaks are seen in LD and SD (Fig. 4.8). The expression pattern in the first day is also different from that of the second day, suggesting no circadian or diurnal regulation of the expression of this gene. This is the case for both LD and SD grown plants. *Arabidopsis CO* shows clear expression peaks around ZT16 in LDs and ZT20 in SDs (Suárez-López *et al*., 2001). The timing of the peaks are closely related to the function of this gene within the photoperiod pathway. *CO* belongs to a gene family consisting of 17 genes in *Arabidopsis* and rice (Griffiths *et al.*, 2003). The data presented here suggests that *AcCOL* is a CO-like gene and not the *CO* homologue. This gene may still be of interest as *CO*-like genes have been shown to affect flowering time (Cheng and Wang, 2005). In addition to this, different members of this gene family may be involved in bulbing and/or flowering in onion. Phylogenetic analyses suggest that *AcCOL* is more closely related to *Arabidopsis COL4* (section 4.3.2). This gene has not been characterised, but data from microarrays show that its expression increases prior to bolting and decreases upon floral initiation (Hruz *et al*., 2008). This suggests a role for *COL4* in the control of flowering in *Arabidopsis*.

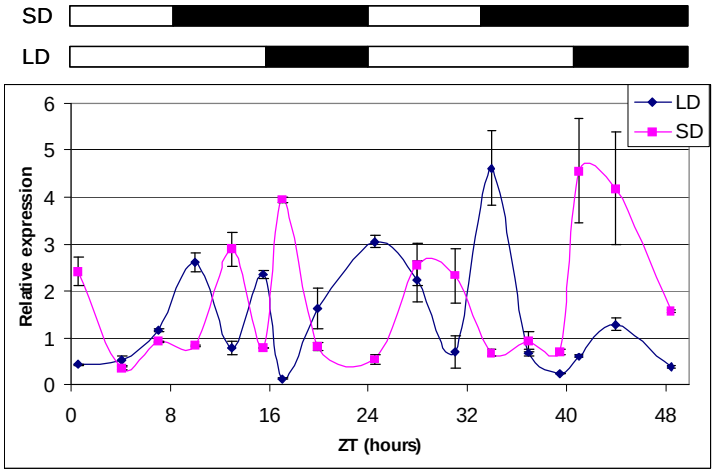
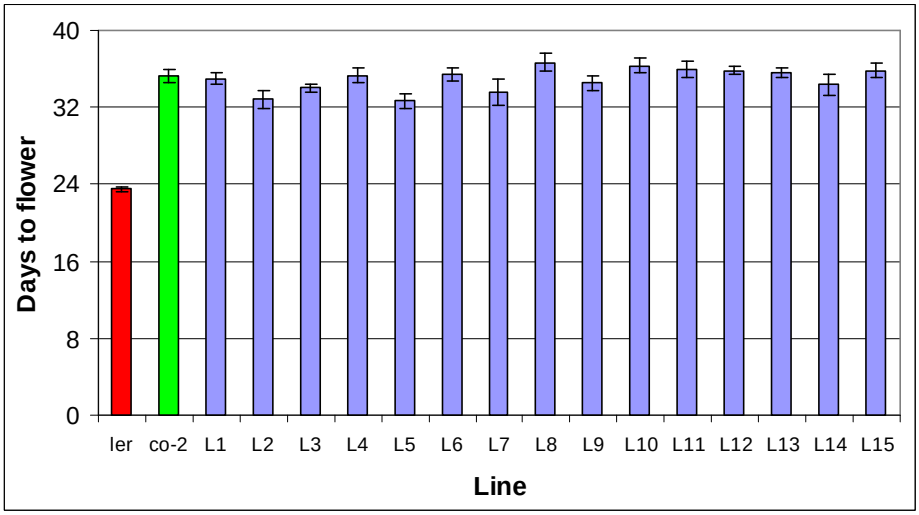


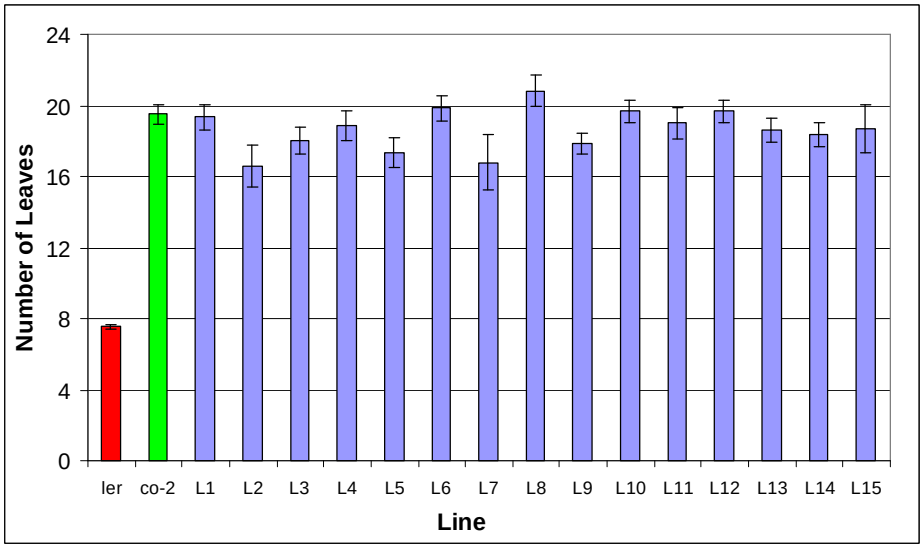
Fig. 4.8: The expression of *AcCOL* in Renate F_1 leaf tissue over a 48-hour period, relative to *EF1α*. White and black bars denote light/dark cycles. Error bars represent the SEM.

4.3.1.3 Expression of *AcCOL* in *Arabidopsis* plants

Arabidopsis co-2 mutant plants were transformed to over-express *AcCOL* (section 4.2.1.3). This mutant produces flowers 16 days later than the wild-type (L*er*) in LDs and at the same time as the wild-type in SDs (TAIR, 2009). It also has an increased number of rosette leaves. If *AcCOL* is an orthologue of *Arabidopsis CO*, then a restoration of wild-type flowering time would be expected. This has been successfully carried out using a monocot *CO* homologue, suggesting that a copy of a *CO* orthologue from a distantly related species can complement a mutant phenotype (Martin *et al*., 2004). Minor differences were observed between the flowering time of *co-2* mutant plants and transgenic lines (Fig. 4.9). ANOVA's were carried out to assess the significance of these differences. The differences in flowering time were shown to be significant for number of days to flower (p=0.013, SED=1.13 d.f.=79) but not for leaf number (p=0.052, SED=1.20, d.f.=79). This suggests that any differences observed in the transgenic lines only have a minor effect on flowering time. It is clear that wild-type flowering time was not restored as L*er* plants flowered with a mean of only 7.5 leaves (23.5 days). The earliest mean flowering time of any transgenic line was 16.6 leaves (32.7 days). In addition, transgenic plants had the co-2 phenotype of an increased number of rosette leaves (Fig 4.9c). This suggests that *AcCOL* is not the onion *CO* orthologue. However, there is still a question of whether an onion *CO* gene would be functional in *Arabidopsis.*



(a)

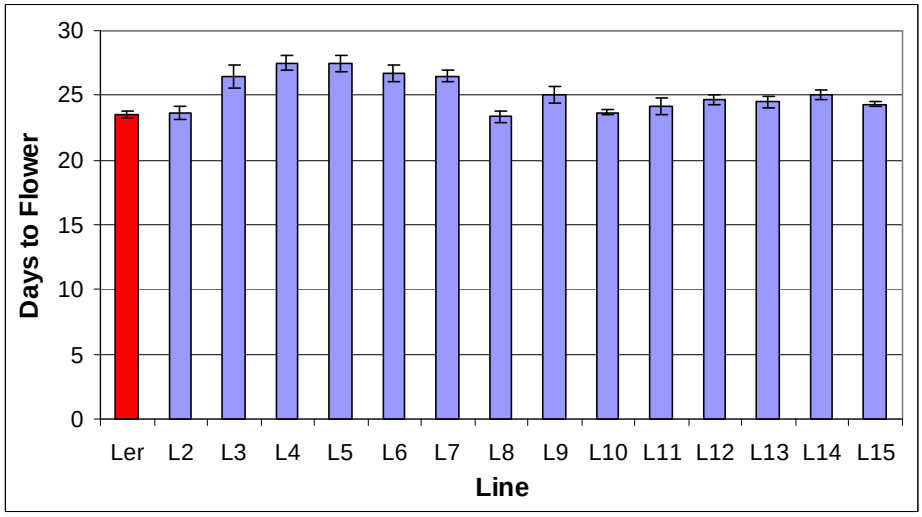


(b)

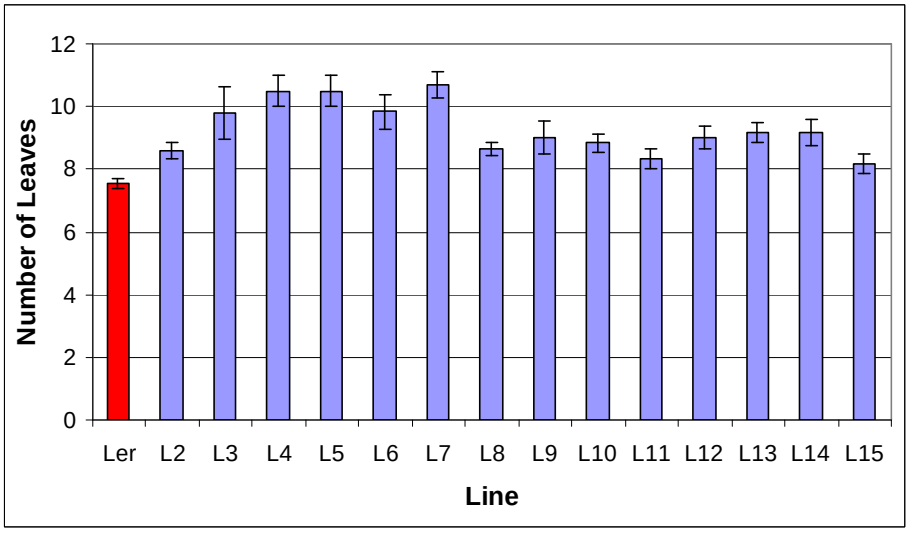
(c)

Fig. 4.9: Mean flowering time (in LDs) of 15 independent *Arabidopsis co-2* transgenic lines (T_2 generation), transformed to express onion *AcCOL*. (a) number of days taken to produce bolt; (b) number of leaves upon initiation; (c) comparing a transgenic and non-transgenic plant at the bolting stage. Error bars represent the SEM. L*er*= Landsberg *erecta.*

Arabidopsis L*er* wild-type plants were also transformed to over-express *AcCOL*. If *AcCOL* has a role in the photoperiod response, an early flowering phenotype (in LDs) would be expected since over-expression of *Arabidopsis CO* in wild-type plants creates an early flowering phenotype (Putterill *et al.*, 1995). However, many of the lines tested showed a slightly later flowering phenotype than wild-type plants (Fig. 4.10). ANOVA's showed that this difference was significant for both days to flower ($p=<0.001$, SED=0.70, d.f.=80) and number of leaves ($p=<0.001$, SED=0.57, d.f.=80). This suggests a potential inhibitory effect of *AcCOL* on flowering. This is in contrary to the data from *co-2* transgenic lines which showed a slightly earlier flowering phenotype (in terms of number of days taken to flower). This makes assigning a function to this gene difficult, but it does appear that *AcCOL* is not a direct *CO* orthologue. Gene knock-out studies in onion would be the true test of this theory (Eady *et al.*, 2008), but this is a lengthy process which is not feasible for a PhD project.



(a)



(b)

(c)

Fig. 4.10: Mean flowering time (in LDs) of 14 independent *Arabidopsis* L*er* transgenic lines (T_2 generation), transformed to express onion *AcCOL*. (a) number of days taken to produce bolt; (b) number of leaves upon initiation; (c) comparing a transgenic and non-transgenic plant at the bolting stage. Error bars represent the SED. L*er*= Landsberg *erecta.*

The expression of *AcCOL* in transgenic lines was tested using RT-PCR. Expression of the transgene was detected in all of the *co-2* transgenic lines tested except L5 of the *co-2* transgenics (Fig. 4.11). It is possible that the transgene was lost in the T_2 generation as the expression of this gene was apparent in the T_1 generation (data not shown). This transgenic line showed a very similar flowering time to the non-transformed *co-2* mutant plant (Fig. 4.9). Expression of the transgene was detected in all the L*er* transgenic lines tested (Fig. 4.12). The presence of transcripts from the transgene shows that transformations were successful and supports the hypothesis that *AcCOL* is not an orthologue of *Arabidopsis CO.*

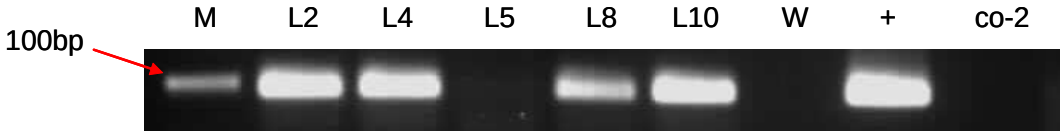


Fig. 4.11: Expression of *AcCOL* in *Arabidopsis co-2* mutant plants. M=marker, L=line number, W=water control, co-2=*co-2* cDNA, positive control is *AcCOL* plasmid DNA.

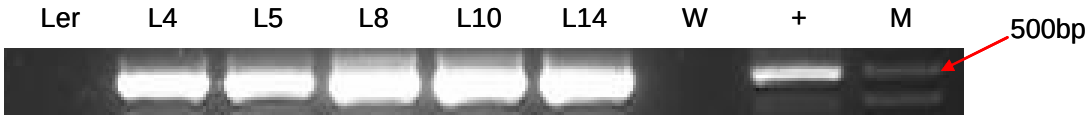


Fig. 4.12: Expression of *AcCOL* in *Arabidopsis* L*er* wild type plants. M=marker, L=line number, W=water control, co-2=*co-2* cDNA, positive control is *AcCOL* plasmid DNA.

4.3.2 Phylogenetic analysis of CO/CO-like genes

A large-scale phylogenetic analysis of *CO* and *CO*-like genes from many species was carried out (section 4.2.2). The purpose of this was not only to investigate the evolutionary relationships in this gene family, but also to set up a system for rapid characterisation of any *CO*-like genes isolated from onion. The initial part of this analysis led to the production of a NJ-tree showing the relationship between *CO* and *CO*-like genes (Fig. 4.13). This is a simple, distance-based approach to phylogenetic analysis. However, it is useful for making certain

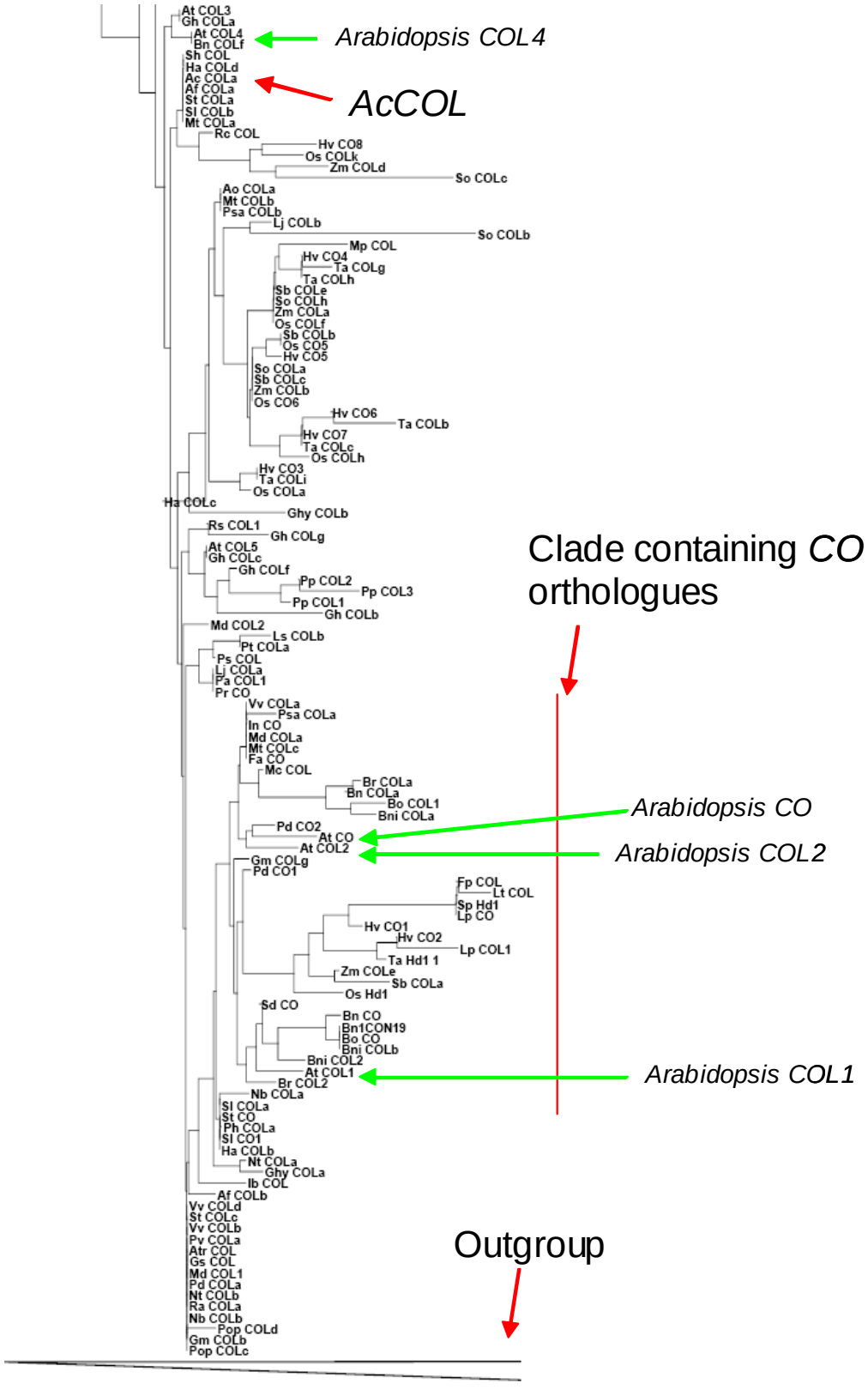


Fig. 4.13: Section of a NJ tree showing the evolutionary relationships between *CO* and *CO*-like genes. The outgroup has been collapsed and contains other CCT domains described in Table 4.3. Accession numbers can be found in Table A8, Appendix 4.

conclusions. Firstly, the *CO* orthologues and *CO*-like 1 and 2 cluster to a specific clade within the tree. This shows a distinct separation within the group I *CO/CO*-like genes, as has been reported previously (Griffiths *et al*., 2003). Secondly, *AcCOL* sits in a clade which includes *Arabidopsis COL4*, suggesting that this gene is a *CO*-like gene not *CO*. This confirms previous observations discussed in section 4.3.1. A more detailed analysis was carried out, resulting in the construction of a maximum likelihood (ML) tree.

A ML analysis selects the tree that is most likely to have generated the sequences which are observed (Thornton and DeSalle, 2000). ML trees are important for deep phylogenies and allow for evolution rate variation between amino acid sites to be taken into account. ML algorithms are much more computationally demanding, the limiting factor in the use of ML trees. Therefore, an ancestor reconstruction was carried out in order to convert the outgroup into a single representative sequence. Following the ancestor reconstruction, a maximum likelihood tree was constructed (Fig. 4.14).

Interesting conclusions can be drawn from this tree. Firstly, many groups are poorly resolved. Therefore, it is dangerous to assume that they form specific clades as the NJ tree may suggest. There seems to be around five clades which are well supported. Clade 2 contains the *Brassica CO* homologues and may form a well resolved clade as four different Brassica species are included in this analysis. The *CO*/*CO*-like genes in these species are almost identical. Clade 5 contains *Arabidopsis COL9-15*, suggesting these genes are closely related. Clade 3 contains *Arabidopsis COL7, COL8 and COL16* suggesting a close relationship among these genes. This clade also contains the *CIL* genes which are involved in chloroplast protein import (Sun *et al.*, 2001). These genes are placed in a strange location in the tree as it would be expected that they would form part of the outgroup. This may be due to the fact that this analysis is based around the CCT domain only. This domain is proposed to function in the nuclear localisation of the protein (Robson *et al*., 2001) so a similar structure may reflect a similar function of the domain in a completely different gene. The other *Arabidopsis CO*-like genes are scattered throughout the tree. The *CO* orthologues are also found throughout the backbone of the tree. This is in contrary to previous studies, which show clear groupings of genes, especially the *CO* orthologues (Griffiths *et al*., 2003). However, this study includes a much larger number of genes so new observations may well be expected. The monocot *CO*

orthologues form a fairly well resolved clade within the tree (clade 4). It would be expected that an onion *CO* orthologue would fall in this clade of the tree. This supports the information obtained from the NJ tree.

An interesting clade which is well resolved within this tree contains the *ZCCT* genes (clade 1). These genes contain a CCT domain but do not function like *CO*. The *ZCCT* genes act to repress flowering in wheat (Yan *et al*., 2004). This is a process which is down-regulated by vernalisation. The *ZCCT* genes in barley have been mapped to the *VRN2* locus and hence are often referred to as *VRN2*. The *VRN2* gene is also thought to have a similar function in barley (Trevaskis *et al*., 2006). *VRN2* has a function which is analogous to *Arabidopsis FLC*, repressing flowering until vernalisation occurs. However, the *VRN2* genes referred to here are different genes to the *Arabidopsis VERNALISATION 2* (*VRN2*) gene. *Arabidopsis VRN2* is a Polycomb group transcription factor which functions in the repression of *FLC* following vernalisation, allowing flowering to occur (Wood *et al*., 2006). The fact that the *ZCCT* genes form a clade in the main tree and not the outgroup suggests that the CCT domain is very similar to *CO* and *CO*-like CCT domains. The CCT domain of the *ZCCT* genes is predicted to have a similar function to the *CO*/*CO*-like CCT domain, nuclear localisation of the protein (Yan *et al*., 2004). There is no *Arabidopsis* gene (or any other dicot gene) in this clade, suggesting that the ancestor of *ZCCT* originated in the monocots.

The results presented here suggest that the CCT domain is very well conserved both within and across species. Grouping *CO*-like genes by B-Box structure may be more advantageous as there is a lot more diversity in structure. The presence or absence of particular amino acids could prove a more useful tool for annotating *CO*-like genes (Robson *et al*., 2001). A parallel analysis using B-Box sequences of all the *CO*-like genes studied here would provide a useful comparison. However, this was slightly beyond the aims of this PhD project.

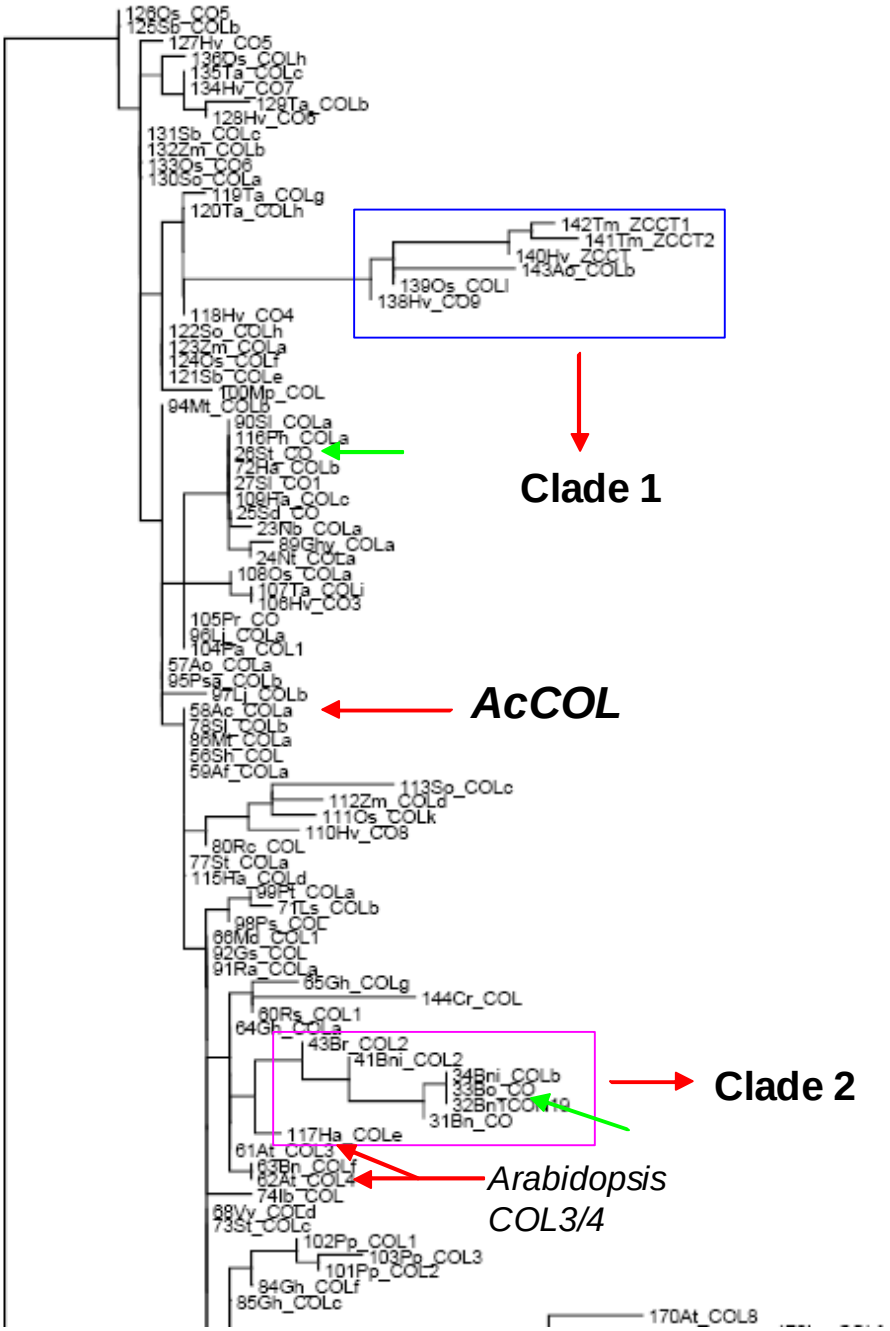
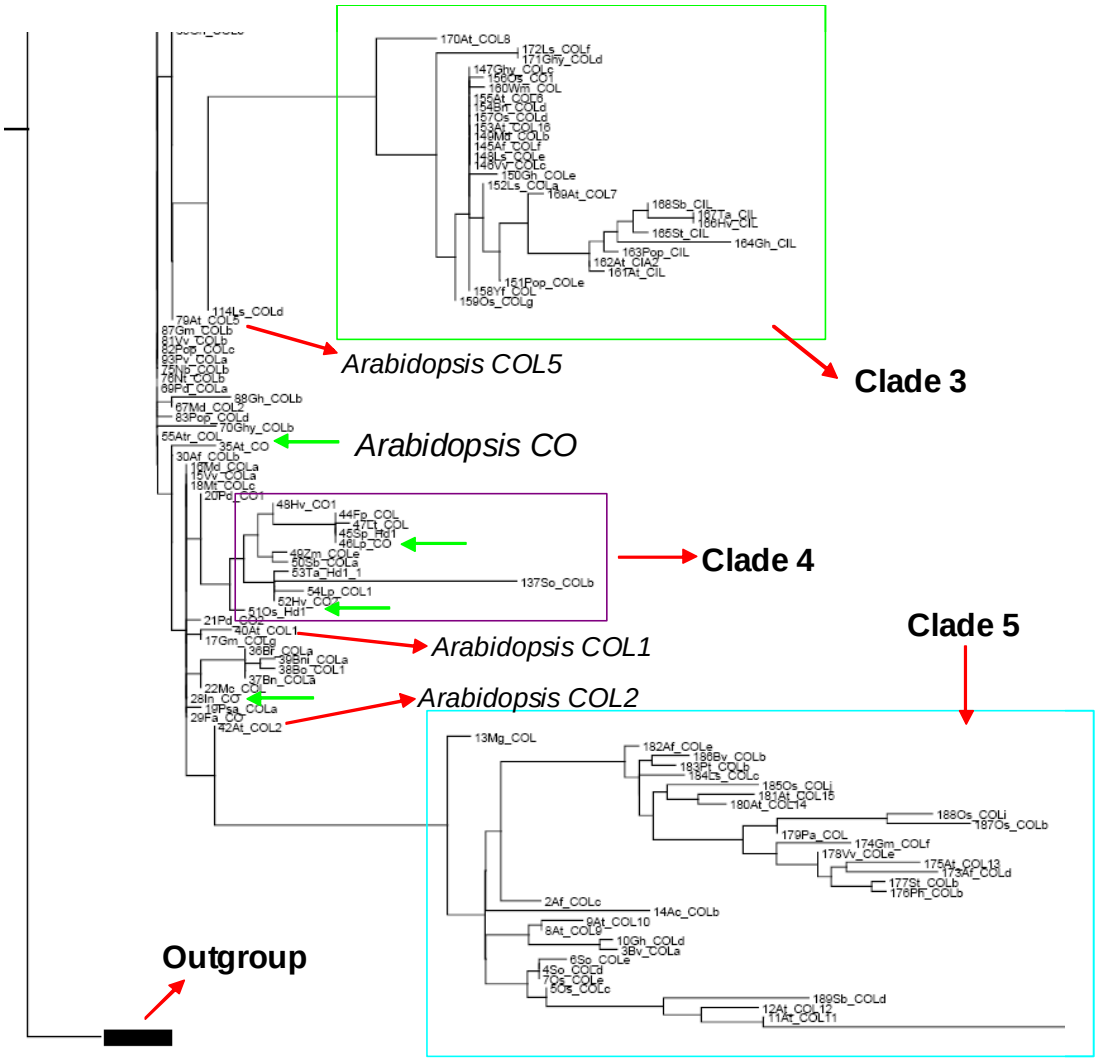

126Os_CO5
125Sb_COLb
127Hv_CO5
136Os_COLh
135Ta_COLc
134Hv_CO7
129Ta_COLb
128Hv_CO6
131Sb_COLc
132Zm_COLb
133Os_CO6
130So_COLa
119Ta_COLg
120Ta_COLh
142Tm_ZCCT1
141Tm_ZCCT2
140Hv_ZCCT
143Ac_COLb
139Os_COLl
138Hv_CO9
118Hv_CO4
122So_COLh
123Zm_COLa
124Os_COLf
121Sb_COLe
100Mp_COL
94Mt_COLb
90Sl_COLa
116Ph_COLa
26St_CO
72Ha_COLb
27Sl_CO1
109Ta_COLc
25Sd_CO
23Nb_COLa
89Ghy_COLa
24Nt_COLa
108Os_COLa
107Ta_COLi
106Hv_CO3
105Pr_CO
96Li_COLa
104Pa_COL1
57Ao_COLa
95Psa_COLb
97Li_COLb
58Ac_COLa
78Sl_COLb
86Mt_COLa
56Sh_COL
59Af_COLa
113So_COLc
112Zm_COLd
111Os_COLk
110Hv_CO8
80Rc_COL
77St_COLa
115Ha_COLd
99Pt_COLa
71Ls_COLb
98Ps_COL
66Md_COL1
92Gs_COL
91Ra_COLa
65Gh_COLg
144Cr_COL
60Rs_COL1
64Gh_COLa
43Br_COL2
41Bni_COL2
34Bni_COLb
33Bo_CO
32BnTCONT9
31Bn_CO
117Ha_COLe
61At_COL3
63Bn_COL4
62At_COL4
74Ib_COL
69Vv_COLd
73St_COLc
102Pp_COL1
103Pp_COL3
101Pp_COL2
84Gh_COLf
85Gh_COLc
170At_COL8
Clade 1
AcCOL
Clade 2
Arabidopsis
COL3/4

Fig. 4.14 (a)



(b)

Fig. 4.14: ML analysis of the *CO*/*CO*-like gene family. (a) Top half (b) bottom half. Green arrows represent the location of *CO* orthologues.

4.3.3 Searching for onion CO/CO-like genes using degenerate PCR

Following the conclusion that *AcCOL* is not the onion *CO* orthologue, a PCR-based method was undertaken in search of the *CO* orthologue and other members of the gene family (section 4.2.3). Initially, alignments were carried out using *Arabidopsis*, rice and barley *CO*/*CO*-like gene sequences. All the primers which were designed were biased towards rice, as this is closer to onion than *Arabidopsis* in terms of phylogenetic distances (Kuhl *et al*., 2004). RT-PCR using degenerate primers (described in section 4.2.3.1) resulted in the amplification of many different

non-specific products (Fig. 4.15). Several of these products were cloned, but no *CO*/*CO*-like genes were isolated. Further attempts to amplify *CO*/*CO*-like genes resulted in products which could be routinely re-amplified (Fig. 4.16). On this occasion, the templates used were cDNA from RNA harvested at ZT15 to coincide with the peak in expression of *Arabidopsis CO* and genomic DNA to alleviate any problems of low expression levels of *CO* (Suárez-López *et al*., 2001). However, following cloning, the inserts were shown to be chloroplast DNA/ribosomal RNA sequences. Two possible causes of the problems encountered were postulated. Firstly, the primers may have been too degenerate, allowing for the amplification of hundreds of different genes. Secondly, the B-Box is a region which is not as well conserved as the CCT domain (Griffiths *et al.*, 2003). It is possible that the B-Box primers were not able to amplify any onion CO homologues. The next approach was to design primers around the CCT domain.

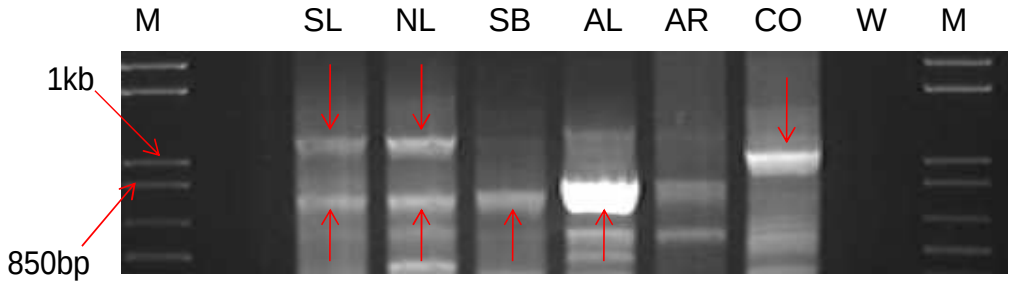


Fig. 4.15: Amplification of products using B-box F2 and CCT R1 primers. M=marker, SL=SD grown onion leaf, NL= onion leaf grown in natural conditions SB=SD grown onion bulb, AL=*Arabidopsis* leaf cDNA, AR=*Arabidopsis* mRNA, CO=35S:CO plasmid, W=water. Arrows indicate products which were excised and purified.

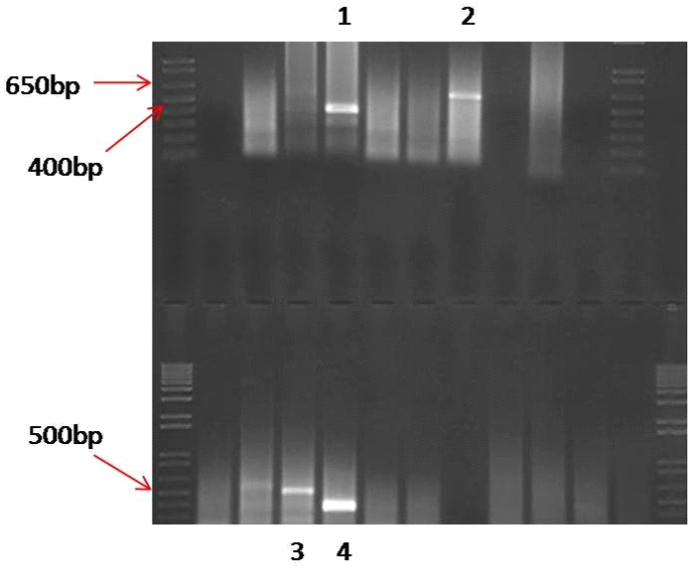


Fig. 4.16: Re-amplification of four previously purified products. 1, 3 and 4 originated from cDNA and 2 from genomic DNA. 1 and 2 were generated with B-box F1 and CCT R1 primers, 3 and 4 with B-box R2 and CCT R1.

RT-PCR using new degenerate primers (section 4.2.3.2) resulted in the amplification of a 90 bp product (Fig. 4.17). This product was expected to span a conserved region which starts just outside the CCT domain (towards the 5' end) and has been described in other monocots (Martin *et al*., 2004). Analysis of clones revealed different CCT domain sequences (Fig. 4.18). Only minor differences were observed but the CCT domain is very well conserved across *CO* and *CO*-like genes so minor differences may be significant. These results give support to the hypothesis that a *CO*/*CO*-like gene family exists in onion (as is observed in *Arabidopsis*). However, it is more likely that the minor sequence differences observed simply reflect the fact that the onion variety used is an F_1 hybrid. The differences could be due to a slightly different copy of the *AcCOL* gene being present in each of the parents. In addition to this, parent plants of F_1 hybrids are not completely inbred in onion so this variety may contain up to four copies of *AcCOL*. In addition, some of the differences were observed in positions where the amino acid is conserved in all CCT domains previously studied (Griffiths *et al*., 2003). This suggests that these differences may be due to sequencing errors. In the case of clone P8, amino acid translation in 2 different reading frames is displayed. The fact that different parts of

the sequence align to *AcCOL* in different reading frames suggests that this may reflect a mutation which causes a frame-shift.

Fig. 4.17: Amplification of 90bp fragment from cDNA using new degenerate primers. M=marker, C1r/C2r=cDNA fragment, re-amplified, C1/C2=cDNA, E1/E2=*AcCOL* plasmid DNA, W=water. C2 and C2r were cloned.

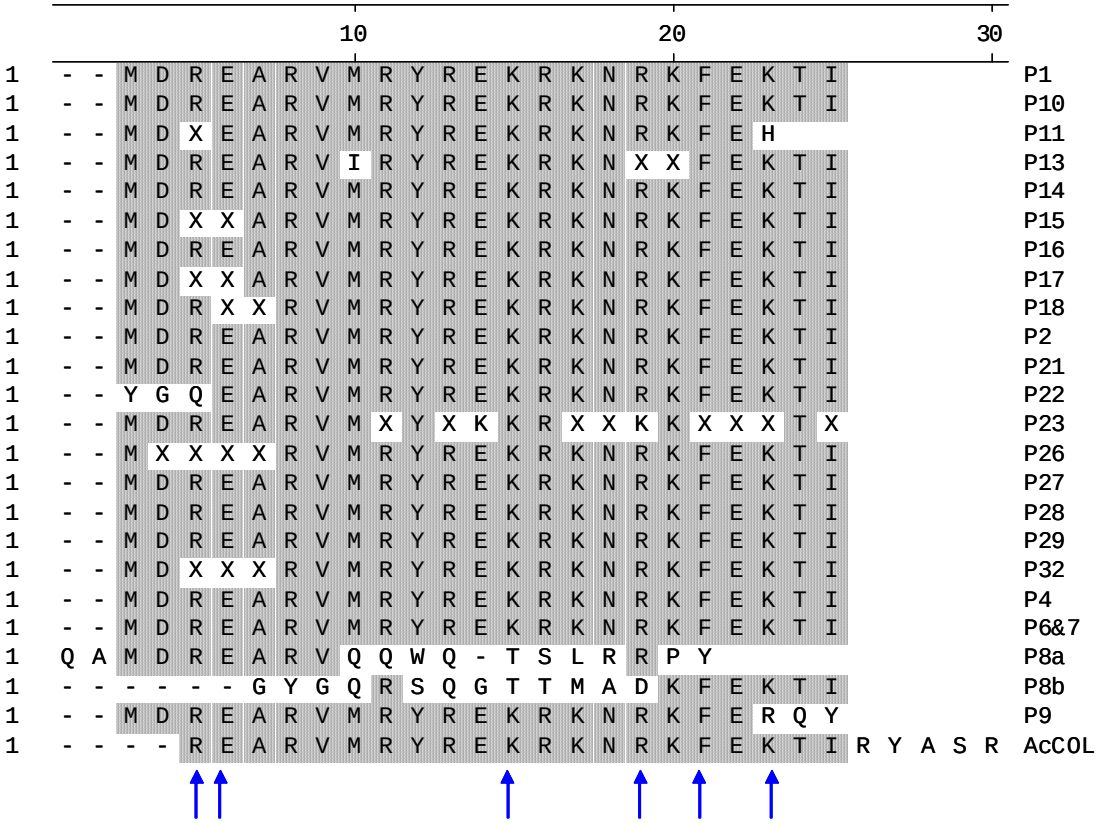


Fig. 4.18: Sequence alignment of the predicted amino acid sequences of CCT domain genes, isolated from Renate F_1 cDNA and cloned using the Gateway BP Reaction. Shaded residues are those which match *AcCOL*. Arrows indicate amino acids which are conserved in all CCT domains previously examined (Griffiths *et al.*, 2003).

4.3.4 Screening a normalised cDNA library for onion CO/CO-like genes

Because of the lack of success with a PCR-based approach to isolate onion *CO*, a normalised cDNA library was constructed (section 2.3.8.1). This approach was taken as *Arabidopsis CO* is expressed at very low levels so the normalisation in the library construction should help with this problem (Suárez-López *et al.*, 2001). It was decided that the CCT domain sequence which was previously obtained (described in section 4.3.3) would not be used as a library probe as it would appear that this fragment only contains *AcCOL* CCT domain fragments.

4.3.4.1 Preliminary library screens

In order to work towards screening the library for onion *CO/CO*-like genes, primers were designed to amplify such genes (section 4.2.4.1). It was noted that the monocot *CO* genes formed a specific clade within the NJ tree produced during phylogenetic analysis of *CO* and *CO*-like genes (also includes some other group I *CO*-like genes). Therefore, MONCO primers were designed specifically to these monocot genes. Included in this clade is a maize gene which is referred to in the tree as ZmCOL. A recent paper has shown that this gene is a *CO* orthologue and it has been named maize *conz1* (Miller *et al*., 2008). RT-PCR using MONCO primers led to the amplification of products of the expected size which were cloned (Fig. 4.19). Sequencing analysis showed that the product cloned from genomic DNA was from the PGT2 gene, a glycoprotein which is abundant in potato tubers (Köster-Töpfer *et al.*, 1989) and no CCT domain sequence was obtained. The product cloned from library plasmid DNA contained either a POLYA binding gene or glycosyl hydrolase. The products obtained using maize genomic DNA were sequenced and shown to contain the *conz1* gene. This means that these primers can routinely amplify a *CO*-like gene from maize but do not amplify *CO*/*CO*-like genes from onion.

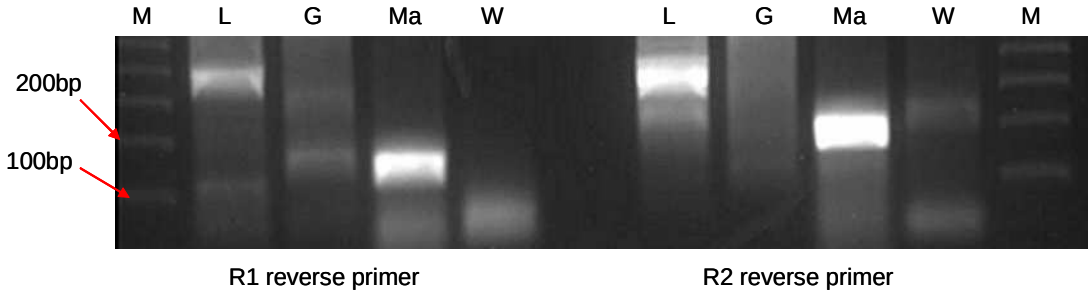


Fig. 4.19: Amplification of products using MONCO primers. L=onion cDNA library plasmid DNA, G=onion genomic DNA, Ma=maize genomic DNA, W=water, M=marker.

Due to the limited success in amplifying different *CO*/*CO*-like genes from onion, it was decided to probe the library at low stringency with a product from the CCT domain of *AcCOL*. PCR using BR20-CCT primers led to the amplification of a 100bp product (Fig. 4.20a). The cDNA and genomic DNA products contained only the *AcCOL* CCT domain but the library plasmid DNA product was mixed. Therefore, this product was cloned and sequencing analysis showed that two different genes had been amplified (Fig. 4.20b). The second gene amplified did not translate well, translation being interrupted by stop codons. It is possible that this is a pseudogene, a copy of a gene which does not produce a functional, full-length protein (Vanin, 1985). It has been reported that some pseudogenes are expressed and one particular gene has been shown to be involved in the regulation of mRNA stability in mice (Hirotsune *et al*., 2003). It would be expected that this gene would be isolated in a library screen and analysis of a possible full-length sequence will help elucidate its function.

(a)

(b)

Fig. 4.20: Towards a library screen for onion *CO*. (a) amplification of a 100bp CCT domain product, (b) amino acid alignment showing the products amplified from library plasmid DNA. L=onion cDNA library plasmid DNA, C=onion cDNA, G=onion genomic DNA, Ma=maize genomic DNA, A=*Arabidopsis* genomic DNA, W=water, M=marker.

4.3.4.2 Screening a cDNA library with a CCT domain probe

Initially, the cDNA library was screened using a section of the CCT domain from *AcCOL* (section 4.2.4.2.1). This method was employed as the CCT domain is very well conserved across the *CO*/*CO*-like gene family (Griffiths *et al.*, 2003). A total of 21 positive hits was obtained, 15 of which were present on both the replica filters (Fig. 4.21). Fourteen of the hits proved to be *AcCOL* and 1 hit was shown to be slightly different. However, the sequence obtained from the CCT domain of this gene was identical and only two amino acids were different in the region between the CCT domain and the 3' end of the gene (Fig. 4.22). It was concluded that this gene was a different copy of *AcCOL*, present because the onion variety used for this analysis was an F_1 hybrid (as described in section 4.3.3). PCR products could not be obtained for the remaining 7 hits (using various primer pair combinations), suggesting that these were false spots or non-specific genes. Of these 7, only 2 were present on both replicates. The fact that at least 14 copies of *AcCOL* were detected in this screen shows that this gene is abundant in the onion genome and may be duplicated several times. It was expected that other CCT domain containing genes would have been detected using this method. This was not the case, suggesting that there may be fewer CCT-domain genes in onion than in *Arabidopsis*, rice and barley.

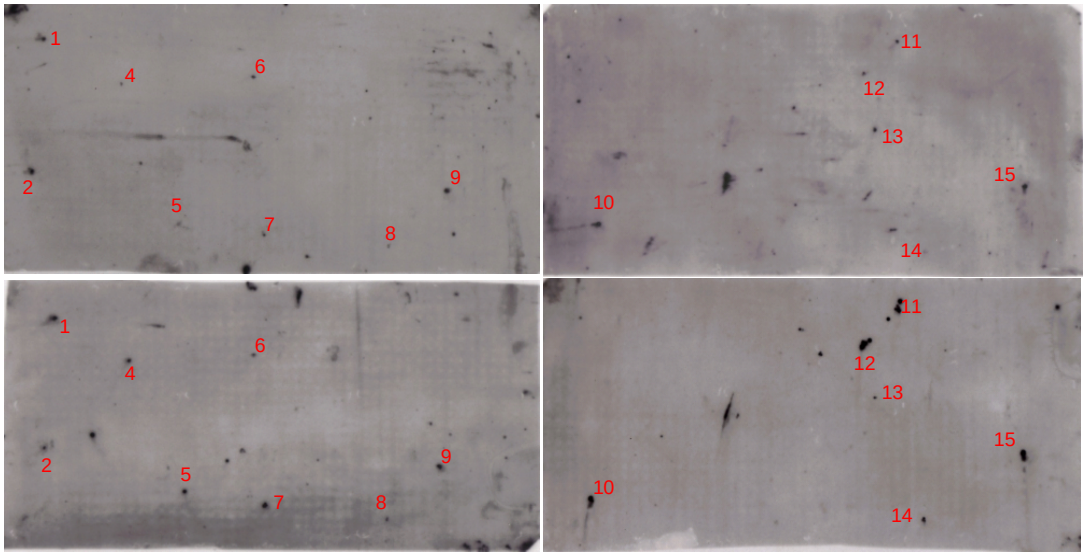


Fig. 4.21: Screening an onion normalised cDNA library using a CCT domain probe. Numbers indicate spots appearing on both of the replica filters.

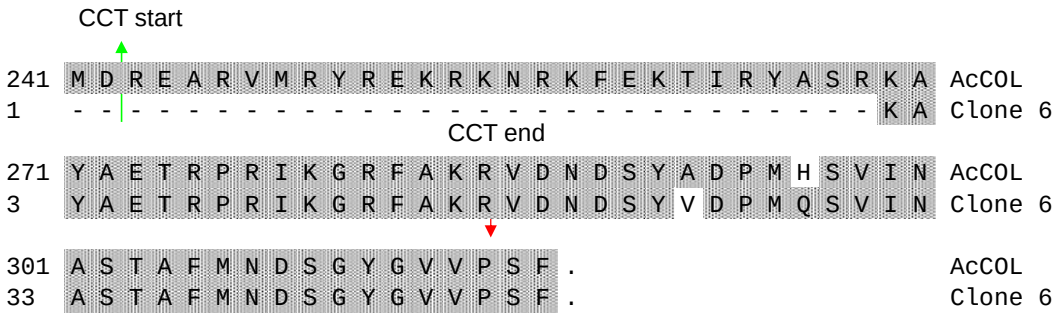


Fig. 4.22: Amino acid alignment of a gene isolated (clone 6) by screening an onion normalised cDNA library with *AcCOL*. Shading represents amino acids which are identical to *AcCOL*. The alignment was carried out using the MegAlign package of DNAStar (DNAStar Inc.).

4.3.4.3 Screening a cDNA library with the maize *conz1* gene as a probe

Following the lack of success with the previous screen, a second library screen was set up using the maize *conz1* gene as a probe (section 4.2.4.2.2). This was chosen as maize is a monocot species and is therefore closer to onion than *Arabidopsis* in evolutionary terms (Stevens, 2001 onwards). Probing with this gene resulted in nine positive hits, indicated in Fig. 4.23. Five of these hits were from

adjacent wells and could be due to only one true positive hit. Closer analysis revealed that one of the five spots in a row (K13, plate 2) was also observed as a positive hit on the CCT domain screen. This was one of the hits for which a PCR product could not be obtained so was of particular interest. Therefore, single clones were obtained from the original well and used to seed 168 wells of a new plate. Well K9 (plate 4) also produced a strong hit so single colonies were obtained from this well and also used to seed 168 wells of the new plate. Only one spot appeared on both replica filters. This was from a colony originating from well K13 (plate 2). Spots were not evident in the wells which were replicated from those which produced the positive hits shown in Fig. 4.24. This suggests that the hits may have been false. The single clone producing the single positive spot was sequenced and analysis revealed that it did not contain a *CO*-like gene. This gene would appear to be a calcium binding protein (NCBI, 2008). Isolating *CO*/*CO*-like genes from onion proved just as difficult with a library screening method as it was with a PCR-based method.

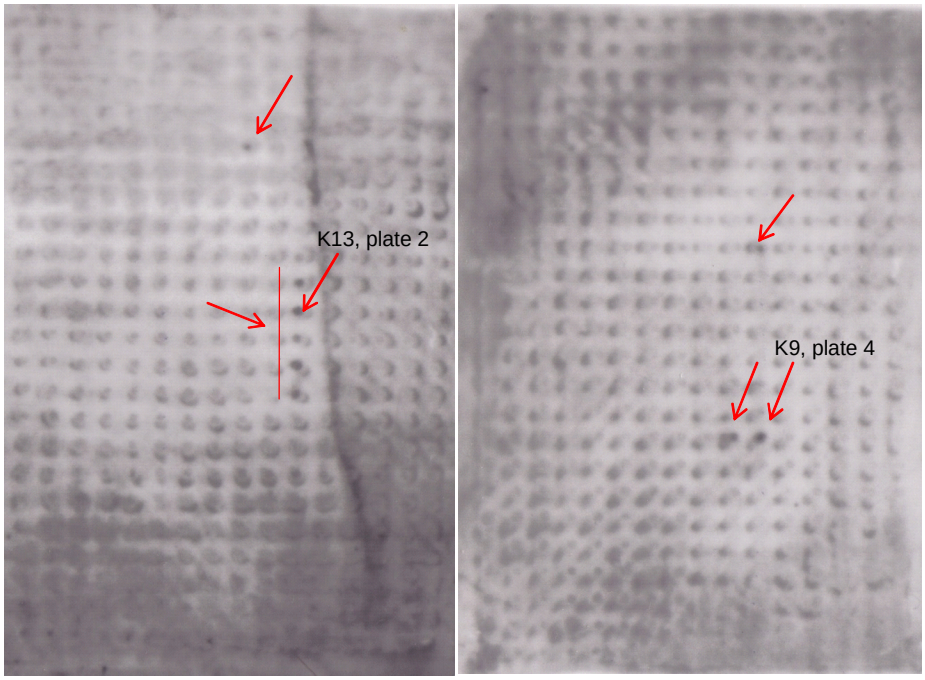


Fig. 4.23: Screening an onion normalised cDNA library with the maize *conz1* gene as a probe. Arrows indicate positive hits.

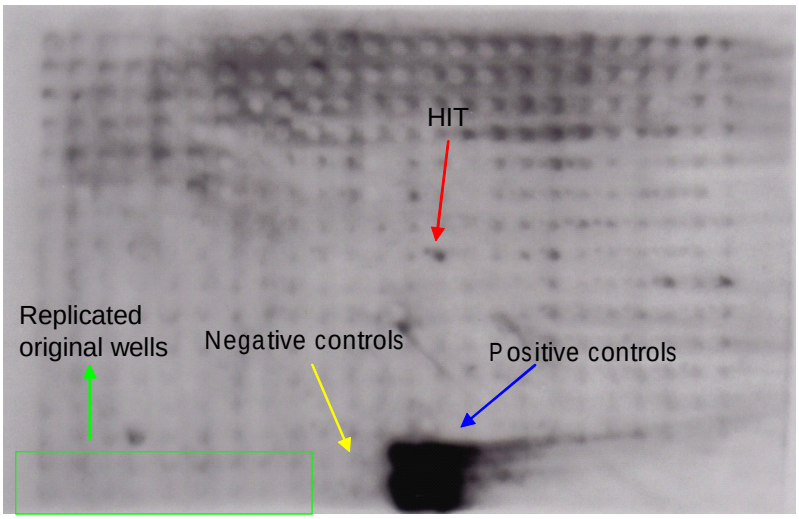


Fig. 4.24: Secondary screen of an onion normalised cDNA library using the maize *conz1* gene as a probe.

4.3.5 Degenerate PCR using tagged cDNA

One further attempt was made to isolate *CO*/*CO*-like genes from onion. The method of using only one degenerate primer with a known TAG primer was attempted as more specificity can be obtained if only one primer is degenerate. This should reduce amplification of non-specific products. PCR using a B-box degenerate primer did not produce any products of the expected size (data not shown). This is probably due to the fact that the B-box is quite variable among species so degenerate primer design is challenging (Robson *et al.*, 2001; Griffiths *et al.*, 2003). PCR using 2 different CCT domain degenerate primers resulted in the amplification of products around the expected size (Fig. 4.25). Data from *Arabidopsis CO* and rice *Hd1* suggest a PCR product of around 300-400 bp in size would be expected. Products were cloned and sequencing revealed an array of different genes had been amplified. Unfortunately, in all the clones sequenced, the only *CO*-like gene which was found was *AcCOL*.

Fig. 4.25: Amplification of products from onion cDNA using a forward degenerate primer designed to amplify from the CCT domain and a reverse TAG primer. M=marker, c=tagged cDNA, W=water, negative control= untagged cDNA. Green arrows indicate products which were purified and cloned.

4.4 Conclusions

CO is a gene which controls flowering time in many plant species and is central to the photoperiodic flowering pathway (Jackson, 2008). This gene has been shown to control the photoperiod response in many species and is very well conserved across the plant kingdom (Griffiths *et al.*, 2003). A *CO*-like gene, which cannot functionally complement *Arabidopsis CO*, was characterised in onion (named *AcCOL*). The function of this gene is hitherto unknown as it shares the highest sequence identity with *Arabidopsis COL4*, an uncharacterised gene. In addition, *AcCOL* does not show a diurnal expression pattern, a pattern which is characteristic of photoperiod response genes (Imaizumi and Kay, 2006). Additional members of the *CO*-like gene family in onion were not isolated. It is possible that many of these genes were non-functional and were lost as the plant evolved. It is highly likely that a *CO* orthologue exists in onion as much of the gene network controlling photoperiod response seems to be conserved. A large-scale phylogenetic analysis of *CO* and *CO*-like genes from many plant species was carried out. This provided some insights into the evolutionary relationships between these genes and set up a system for characterising any onion *CO*-like genes which may be isolated in the future. The lack of success in isolating onion *CO* from cDNA sources could well be due to a very low expression level, as seen in *Arabidopsis CO* (Putterill *et al.*, 1995). Problems with isolating this gene from genomic DNA may well be related to the huge genome size in onion (McCallum *et al.*, 2001; Kuhl *et al.*, 2004). However, the possibility of an altered photoperiod pathway in onion cannot be ruled out.

CHAPTER 5: CHARACTERISATION OF AN ONION *GIGANTEA* (*GI*) HOMOLOGUE

5.1 Introduction

GIGANTEA (*GI*) was first identified as a late flowering mutation in *Arabidopsis* (Redei, 1962). It is a circadian clock-controlled gene which regulates photoperiodic flowering in *Arabidopsis* by positively regulating the expression of *CO* (Fowler *et al*., 1999; Mizoguchi *et al*., 2005). Overexpression of *GI* leads to an increase in *CO* expression and precocious flowering in both LDs and SDs whilst *gi* mutant plants are severely late flowering (Suárez-López *et al.*, 2001; Mizoguchi *et al.*, 2005). *GI* mRNA shows a peak in expression around 8-10 hours after dawn (Fowler *et al.*, 1999). The GI protein has been shown be tightly controlled by light/dark cycles, peak levels occurring at a similar time to *GI* mRNA (David *et al.*, 2006). One mechanism regulating GI protein levels in the dark involves proteolysis by the 26S proteosome (David *et al.*, 2006). The timing of GI expression is critical for the seasonal control of flowering. Protein accumulation and degradation is tightly regulated by the circadian clock, ensuring the GI protein is available in the late afternoon under inductive, LD conditions.

GI regulates flowering by controlling *CO* expression through at least two different mechanisms (Coupland, 2008, Fig. 5.1). Recent studies have shown that the GI protein forms a complex with FKF1 under blue light (Sawa *et al.*, 2007). This complex binds to CDF1 (a repressor of *CO*) and forms on the *CO* promoter, regulating *CO* expression. This occurs in the late afternoon in LDs, leading to CO protein expression and eventually flowering (through regulation of *FT* expression, as described in section 1.5.1). It has also been shown that the GI/FKF1 complex also inhibits other repressors of *CO* from the same gene family (Coupland, 2008). The complex has been shown to degrade *CYCLING DOF FACTOR 2* and *5* (*CDF2*/*CDF5*). *GI* can also activate *CO* directly, without forming a complex with FKF1 (Coupland, 2008). The exact mechanism of this activation is currently unknown. The GI protein has been shown to be nuclear localised (Mizoguchi *et al.*, 2005). Hence, it is proposed that the site at which *GI* controls flowering is the nucleus. This is supported by the fact that the CO protein is also nuclear localised (Robson *et al.*, 2001).

GI also has an additional role, related to the control of flowering time. It is involved in the maintenance of circadian rhythms, a function which is distinct from its control of *CO* expression (Mizoguchi *et al*., 2005). This is achieved through an interaction with ZEITLUPE (ZTL) under blue light conditions (Kim *et al*., 2007). GI is required to establish and maintain the oscillation of the ZTL protein. This is achieved through protein-protein interactions which are enhanced by blue light. This blue light reaction occurs through the LOV domain of ZTL and allows ZTL to maintain a normal circadian period. This is achieved by controlling the proteosome-dependent degradation of TIMING OF CAB EXPRESSION 1 (TOC1). *GI* has been shown to have several other distinct roles aside from its roles in photoperiodic flowering and controlling circadian rhythm. Such roles include regulating starch accumulation and phytochrome A-mediated photomorphogenesis (Eimert *et al.*, 1995; Mizoguchi *et al.*, 2005; Martin-Tryon *et al.*, 2007; Oliverio *et al.*, 2007). In addition, work on the model species *Medicago truncatula* has shown that *GI* has a role in de-etiolation in blue light and early flowering in SDs (Paltiel *et al*., 2006). The implications are that *GI* is a multi-functional gene, a role in photoperiodic flowering being one of many distinct functions.

The majority of the work carried out on *GI* has centred on *Arabidopsis*. However, *GI* homologues have also been isolated and characterised in monocots such as rice, wheat and barley *(*Hayama *et al., 2003;* Dunford *et al., 2005;* Xiang *et al*., 2005) as well as dicots such as radish and *Medicago truncatula* (Curtis *et al*., 2002; Paltiel *et al*., 2006). In addition to this, *GI* has also been shown to be present in the gymnosperm loblolly pine (Mizoguchi *et al*., 2005). A recent study reports the isolation of a *GI* homologue from pea (Hecht *et al.*, 2007). This gene is referred to as *PEA LATE BLOOMER1* (*LATE1*). *LATE1* mutants are late flowering in inductive LDs and have been shown to prevent the induction of a pea *FT* homologue. This gene was also shown to have a role in the control of circadian rhythms, showing that *LATE1* has similar roles to *Arabidopsis GI*.

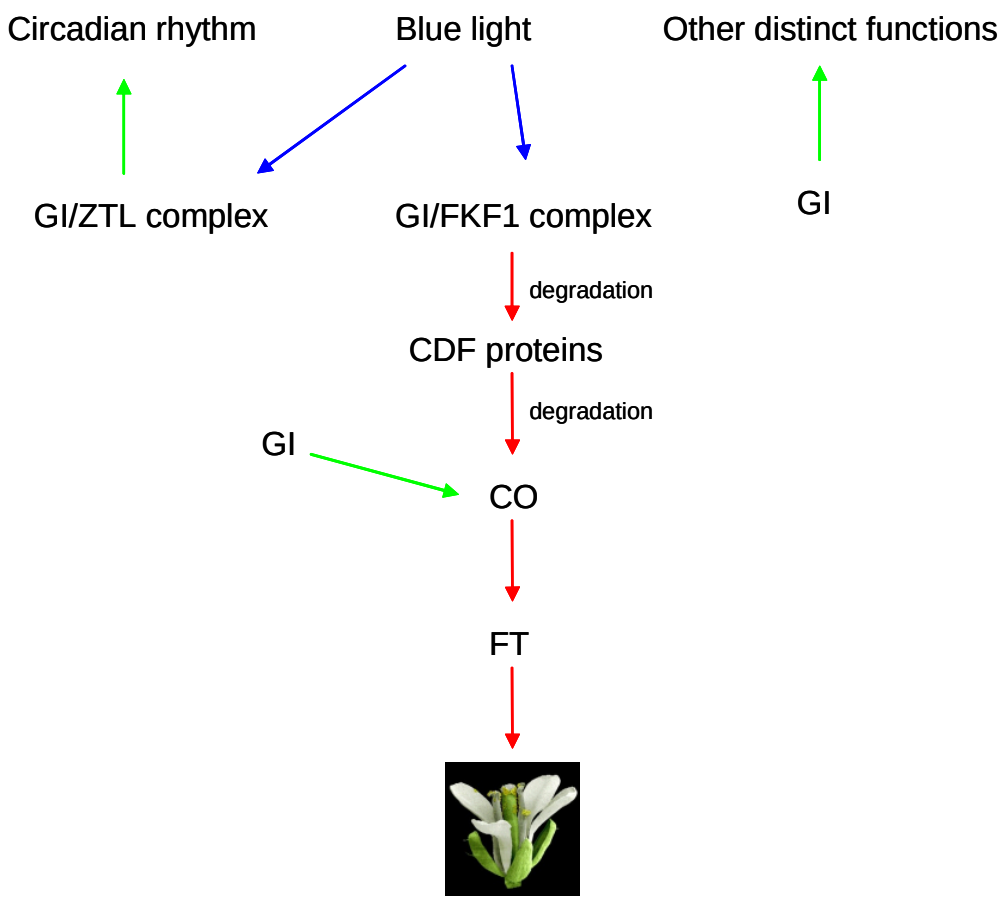


Fig. 5.1: A summary of the functions of *GI*, with emphasis on the photoperiodic control of flowering. The GI/FKF1 complex represses the CDF proteins, thus allowing *CO* transcription to occur (adapted from Coupland, 2008 and Kim *et al.*, 2007). *Arabidopsis* flower picture copyright Juergen Berger, Max Planck Institute for Developmental Biology.

The *GI* gene encodes a 1174 amino acid protein containing six putative transmembrane domains, strongly suggesting that GI is a membrane protein (Park *et al.*, 1999). The gene itself appears to be expressed in every organ of the plant and throughout the entire developmental cycle (Fowler *et al.*, 1999). The GI protein is encoded by a single gene and is not a member of a gene family. It does not show homology with any gene of known function (Fowler *et al.*, 1999). This chapter covers the characterisation of an onion *GI* homologue. The starting point was an EST, found in the *A.cepa* gene index (DFCI, 2008), which shows sequence similarity with *Arabidopsis GI*. This gene was further characterised through expression and phylogenetic studies and a full-length copy sought.

5.2 Materials and Methods

5.2.1 AcGI

Screening the *A.cepa* gene index resulted in the discovery of an EST showing sequence similarity with *Arabidopsis GI*. This gene was given the name *AcGI* (for *Allium cepa GIGANTEA*). The clone was obtained (Mike Havey, USDA, University of Wisconsin), plasmid DNA isolated and sequencing carried out using M13 forward and reverse primers as described in sections 2.2.5 and 2.2.8. Several attempts were made to obtain the correct clone as there seemed to be a mis-labelling issue in the EST collection. On each occasion, clones were sequenced using M13 forward and reverse primers (see Appendix 2 for sequences).

5.2.2 Expression of AcGI

5.2.2.1 RT-PCR

The expression of *AcGI* was examined using RT-PCR. The primers used were GI-RT FOR and GI-RT REV (see Appendix 2 for sequences) with an annealing temperature of 61 ºC and a cycle number of 30. Both cDNA and genomic DNA templates originated from the Renate F_1 variety. The same primer pair was used to assess the expression of *AcGI* in the leaves of onions which had been induced to bolt (unknown variety, see section 2.3.5). Plants were at the unopened flower stage. The annealing temperature was 61 ºC and the cycle number 35. Products were purified and sequenced (using both forward and reverse primers) to confirm their identity, as described in section 2.2.8.

5.2.2.2 Relative expression in a LD variety

The relative expression of *AcGI* in a LD onion variety (Renate F_1) was examined using quantitative real-time PCR, as described in section 2.3.3. Plants were grown in LD (16 hrs of light) and SD (8 hrs of light) conditions and leaf material harvested over a 48-hour period. The primers used for quantitative RT-PCR were GI-RT FOR and GI-RT REV (see Appendix 2 for sequences) at a concentration

of 0.4 µM. All cDNA samples were diluted 1 in 3 and run in triplicate. A selection of the PCR products were purified and sequenced to confirm their identity (using GI-RT FOR and GI-RT REV primers). The expression was normalised to the expression of *EF1α* as described in section 2.3.3. Twenty-four hour averages of expression were calculated and standard errors included.

5.2.2.3 Relative expression in SD/ID varieties

Quantitative real-time PCR was carried out to assess *AcGI* expression in SD and ID onion varieties, as described in section 2.3.4. Plants were grown in LD (16 hrs of light) and SD (12 hrs of light) conditions and leaf samples taken over a 48-hour period. The primers used for quantitative RT-PCR were GI-RT FOR and GI-RT REV at a concentration of 0.4 µM with an annealing temperature of 61 ºC. All cDNA samples were used undiluted and run in triplicate. Expression was normalised using the housekeeping gene ß-tubulin as described in section 2.3.4. Twenty-four hour averages of expression were calculated and standard errors included. A selection of the PCR products were purified and sequenced to confirm their identity (using GI-RT FOR and GI-RT REV primers).

5.2.2.4 Statistical analyses

Statistical analyses were carried out to assess the significance of the differences in *AcGI* expression between plants grown in LDs and SDs and between different varieties. All analyses were carried out using the 24-hour average data. Gaussian curves were fitted to the data, an analysis which is based on the normal distribution (Mead *et al.*, 1993). Non-linear regression analyses were then used to assess the significance of the differences between data sets. The significance relates to differences in two parameters, the spread of the curve (S) and the mean (M). The M value relates to the timing of the peak in gene expression.

5.2.3 Screening a cDNA library for a full-length clone of AcGI

PCR products were labelled to be used as probes for a cDNA library screen, following the protocol outlined in section 2.3.8.2. The primer pair used was GI

FOR2 & GI REV (see Appendix 2 for sequences). The template used for the labelling reaction was 1 µl of a previously purified PCR product using the same primer pair. The labelling reaction was set up following the manufacturer's guidelines, with the exception that the annealing temperature was 61 ºC. The labelled product was run on a 2 % (w/v) agarose gel to check the labelling efficiency. Hybridisation and detection were carried out as described in section 2.3.8.2. A hybridisation temperature of 46 ºC was used with a probe concentration of approximately 2.5 µl per ml of hybridisation solution.

Following hybridisation and detection, filters were exposed to X-ray film (Sigma-Aldrich) for 3.5 hr and overnight. Positively hybridising clones were traced by culturing single colonies from the original well of interest (from the 384-well plates grown up from a single library aliquot). Colony PCR was carried out (as described in section 2.2.1) on 46 colonies using GI FOR2 and GI REV primers (see Appendix 2 for sequences) with an annealing temperature of 61 ºC and a cycle number of 30. Colonies which produced a positive PCR product were cultured and plasmid DNA isolated (as described in section 2.2.5). Sequencing was carried out using M13 forward and reverse primers and a T7 promoter primer. Contigs were constructed using the Seqman package of DNAStar (DNAStar Inc.).

5.2.4 Sequencing the 5' end of AcGI by RACE PCR

In order to obtain information on the full-length gene sequence of *AcGI*, 5' RACE PCR was carried out (Invitrogen Ltd., Gene Racer kit, Cat No. L1502-02) following the manufacturer's guidelines, with slight modifications. The dephosphorylation reaction was set up using 3 µg of total RNA extracted from leaf material harvested throughout a 24-hour period (from library construction, section 2.3.8.1). The cDNA synthesis step was set up using the oligo dT primer. The following PCR cycling conditions were used: 1 cycle of 94 °C for 2 min; 5 cycles of 94 °C for 15 sec, 72 °C for 4 min; 5 cycles of 94 °C for 15 sec, 70 °C for 4 min; 20 cycles of 94 °C for 15 sec, 68 °C for 30 sec, 72 °C for 4 min; 1 cycle of 72 °C for 10 min. The gene specific primer used was named GI GSP (see Appendix 2 for sequence). All PCR reagents were used at the concentrations described in the protocol and the enzyme used was KOD Hot Start DNA Polymerase (Merck Bioscience). PCR products were purified as described in section 2.2.6.

Nested PCR was carried out following the protocol provided with the kit and using the PCR cycling described in section 2.2.1. The nested primer used was named GI GSP NEST (see Appendix 2 for sequence). An annealing temperature of 65 °C was used and the cycle number was 25. Nested PCR products were purified and cloned into pCR®4Blunt-TOPO® (Invitrogen Ltd.), the vector provided with the Gene Racer kit, following the protocol provided. The chemical transformation method was followed and selection carried out on LB plates containing 100 μg ml^{-1} ampicillin (Melford). Colonies were cultured, plasmid DNA isolated and sequenced using T7 and T3 primers as described in sections 2.2.5 and 2.2.8. Contigs were constructed using the Seqman package of DNAStar (DNAStar Inc.).

5.*2.5 Amplifying and sequencing the full-length AcGI gene*

Primers were designed to amplify from the 5' and 3' untranslated regions of *AcGI* (GI 5'UTR 3 AND GI 3'UTR 1/2, see Appendix 2 for sequences). RT-PCR was carried out using 30 cycles and an annealing temperature of 59 °C. PCR templates were onion cDNA and genomic DNA, originating from the Renate F_1 variety. Products were purified, sequenced and cloned into a pMOS*Blue* vector (Amersham Biosciences), following the manufacturer's guidelines. The transformation method used was electroporation (described in section 2.2.9). Plasmid DNA was isolated and sequencing carried out using gene-specific primers and T7/U-19 vector primers. Contigs were constructed using the Seqman package of DNAStar (DNAStar Inc.). One contig was constructed from sequences at the 3' end of the gene and a second contig constructed from sequences at the 5'end of the gene. Primers were designed in order to sequence out from 5' and 3' contigs. Further primers were designed until one single overlapping sequence was obtained (see Appendix 2 for all primer sequences). The sequence was confirmed by obtaining double-stranded sequence for the entire gene.

Alignments were constructed using publicly available sequences of *GI* homologues. Both nucleotide and amino acid alignments were constructed, percentage identities calculated and a NJ tree constructed as described in section 2.3.7. The tree was based on predicted amino acid sequences.

5.2.6 The genomic structure of AcGI

PCR was carried out using various primer pair combinations in order to assess the genomic structure of *AcGI*. The DNA used for PCR reactions was onion DH genomic DNA (unknown variety). The primer pair which revealed some information on the genomic structure of *AcGI* was GI 3'UTR1 and GI FOR 2 (see Appendix 2 for sequences). This pair was used with an annealing temperature of 60 ºC and a cycle number of 30. PCR products were purified and sequenced using the same primers, as described in sections 2.2.6 and 2.2.8.

5.2.7 Expressing AcGI in Arabidopsis plants

RT-PCR was used to generate a full-length *AcGI* PCR product containing att sites in order to allow Gateway® cloning. The primers used were GI 5'UTR 4 att and GI 3'UTR 1 att (see Appendix 2 for sequences). PCR cycling was as described in section 2.2.1 with an annealing temperature of 59 ºC and a cycle number of 30. PCR products were purified and cloned into a Gateway® pDONR™207 vector (Invitrogen Ltd., provided by Nicki Adams, Warwick HRI), using the Gateway BP reaction (Invitrogen Ltd., Cat. No. 11789), following the manufacturer's guidelines. Electrocompetant EC100 cells (Cambio Ltd) were transformed using electroporation, as described in section 2.2.9. Plasmid DNA was isolated from positive clones, and their identity confirmed by PCR using GI 5'UTR 4 and GI 3'UTR 1 primers. The vector containing *AcGI* was then cloned into a pB2GW7 vector using Gateway® LR clonase® II (Invitrogen, Cat. No. 11791) to create a pB*AcGI* vector, as described in section 2.3.6. Plasmid DNA was isolated and clone identity confirmed by PCR using GI 5'UTR 4 and GI 3'UTR 1 primers (see Appendix 2 for sequences). *Agrobacterium* transformations and floral dips were carried out as described in section 2.3.6. A slightly modified protocol was followed for floral dips, following the release of an updated protocol (Zhang *et al.*, 2006). *gi-3* mutant and L*er* wild-type plants were transformed to over-express *AcGI*. Following the application of Basta (Bayer CropScience), the flowering time of transgenic plants was assessed in the T_1 generation, as described in section 2.3.6.

The expression of the transgene was tested in five *gi-3* transformed lines using RT-PCR (section 2.2.1 and 2.2.2). The primers used were GI-RT FOR and

REV with an annealing temperature of 61 ºC and a cycle number of 30. Products were purified and sequenced using both forward and reverse primers, as described in sections 2.2.6 and 2.2.8.

5.3 Results and Discussion

5.3.1 Expression of AcGI

5.3.1.1 RT-PCR

Expression of *AcGI* was detected in a cDNA sample (Fig. 5.2). The expression levels appear to be high in this particular sample (originating from RNA extracted from pooled leaf material harvested at multiple time-points). The expression in cDNA shows that *AcGI* is functional and is not a pseudogene. Sequencing confirmed the PCR product was amplified from *AcGI*. A PCR product could not be obtained from genomic DNA using this primer pair. This may be due to the huge genome size in onion (Kuhl *et al*., 2004). It is also possible that the primer pair used amplifies a region containing an intron, leading to a different product size. If an intron is present part way through where the primer anneals then no PCR product would be obtained. The fact that *AcGI* is expressed allowed for quantitative RT-PCR to be undertaken.

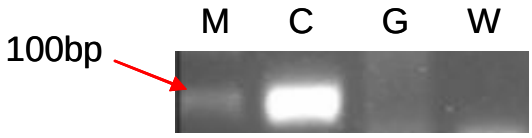


Fig. 5.2: Expression of *AcGI* in the onion Renate F_1 variety. M=marker, C=cDNA, G=genomic DNA, W=water control.

5.3.1.2 Relative expression in a LD variety

A detailed expression profile for *AcGI* was obtained using quantitative RT-PCR (described in section 5.2.2.2). The expression profile discussed in this section was obtained using Renate F_1 leaf material (a LD onion variety). The expression of

AcGI in this variety is seen to peak around ZT10 in LDs compared with ZT7 in SDs (Figs. 5.3 and 5.4). This expression pattern is clearer to see in the average 24-hour trace (Fig. 5.4) than the 48-hour trace (Fig. 5.3). It is very similar to the expression of *Arabidopsis GI*, which peaks at ZT10 in LDs and ZT8 in SDs (Fowler *et al.*, 1999). It shows that *AcGI* has a diurnal expression pattern, characteristic of genes involved in the photoperiod response (Jackson, 2008). It also suggests that this gene is circadian regulated although experiments involving growing plants in constant light conditions would be required to confirm this. The difference in timing of peaks in LD and SD grown plants gives further evidence that this gene is involved in the photoperiod response in onion. In *Arabidopsis*, the timing of expression of *GI* is an essential component of the photoperiodic control of flowering. Recent data indicates that the complex which forms between GI and FKF1 directly regulates CDF1 stability in the afternoon under LD conditions, resulting in its breakdown (Sawa *et al.*, 2007). This leads to an increased expression of *CO* and induction of flowering when daylength increases. Therefore, the peaks of expression observed in *AcGI* could facilitate the initiation of bulbing in LD conditions, via a similar mechanism.

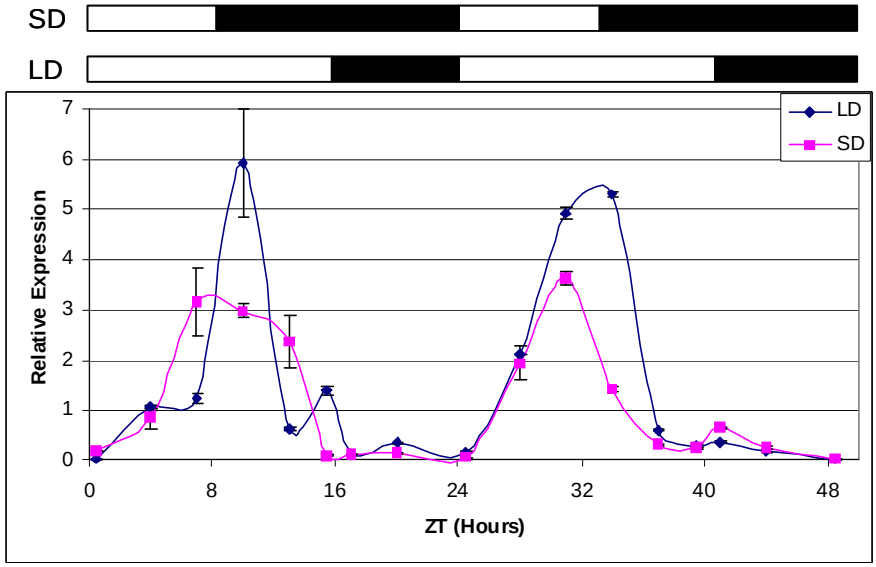


Fig. 5.3: Expression of *AcGI* in a LD onion variety (Renate F_1) over a 48-hour period, relative to *EF1α*. White and black bars represent light/dark cycles. Error bars represent the SEM.

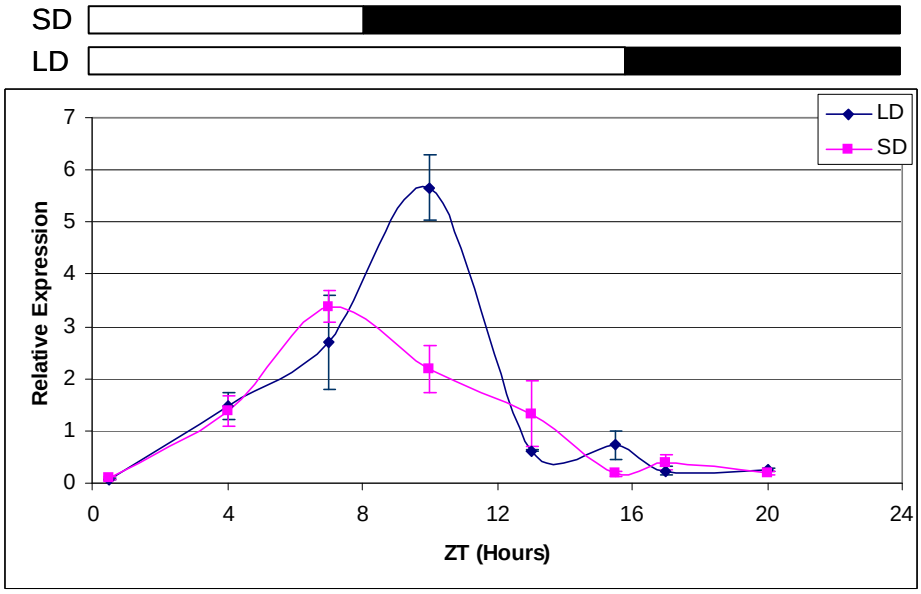


Fig. 5.4: Average expression of *AcGI* over 24 hours in a LD onion variety (Renate F_1), relative to *EF1α*. Error bars represent SEM. White and black bars represent light/dark cycles.

5.3.1.3 Expression in flowering onions

It was also shown that *AcGI* is expressed in flowering onions (Fig. 5.5). This was expected as *Arabidopsis GI* is expressed throughout development (Fowler *et al*., 1999). Once again, this highlights the similarities between *AcGI* and *Arabidopsis GI*. It is difficult to see any expression pattern with only 3 time-points of harvest and quantitative real-time PCR would provide a more detailed analysis of the expression of *AcGI* in onions which have bolted. However, this would require a large number of flowering onion plants, an experiment which would take a large amount of time and was deemed beyond the aims of this project.

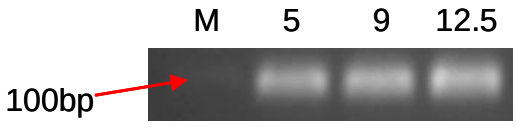


Fig. 5.5: Expression of *AcGI* in the leaves of onions which have bolted (unknown variety). M=marker, numbers represent zeitgeber time.

5.3.1.4 Expression in SD and ID onion varieties

Although temperate onions respond to LDs, many other onion varieties exist around the world that respond to shorter daylengths (Brewster, 2008). Onions are broadly classified as LD, SD or ID varieties. The expression of *AcGI* was examined in a SD and an ID onion variety (section 5.2.2.3). Both varieties were grown in both LD and SD conditions. Under LD conditions (Figs. 5.6/5.7), the varieties tested showed a very similar expression pattern with a peak around ZT10. Under SD conditions (Figs. 5.8/5.9), the varieties tested also showed a very similar expression pattern, with a peak around ZT7-8. The expression patterns for the SD and ID varieties matched the pattern seen in Renate F_1, a LD variety (Figs. 5.3 and 5.4, section 5.3.1.2). This suggests that if a difference in daylength response is associated with a change in the photoperiod pathway, this change occurs downstream of *GI*. It also suggests that the circadian rhythm component of the photoperiod pathway is active in LD, ID and SD onion varieties.

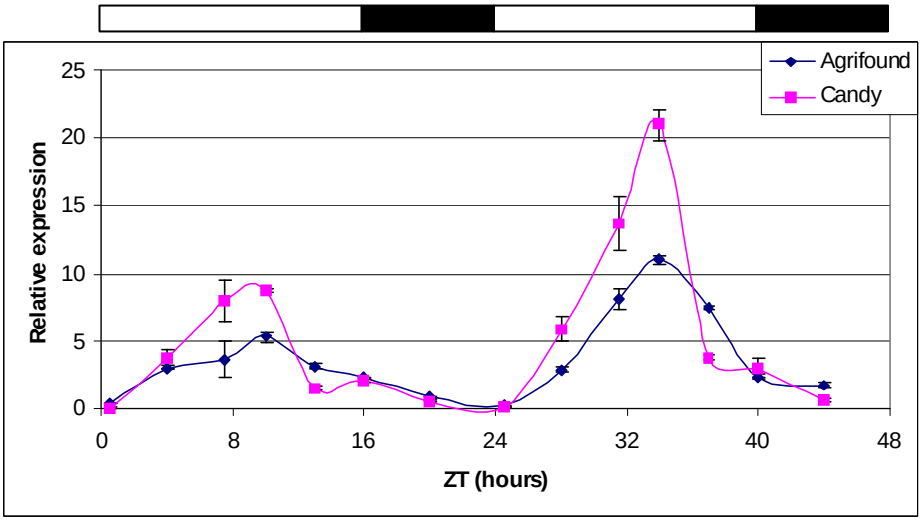


Fig. 5.6: LD expression of *AcGI* over a 48-hour period in the leaves of a SD (Agrifound) and ID variety (Candy), relative to ß-*tubulin*. White and black bars represent light/dark cycles.

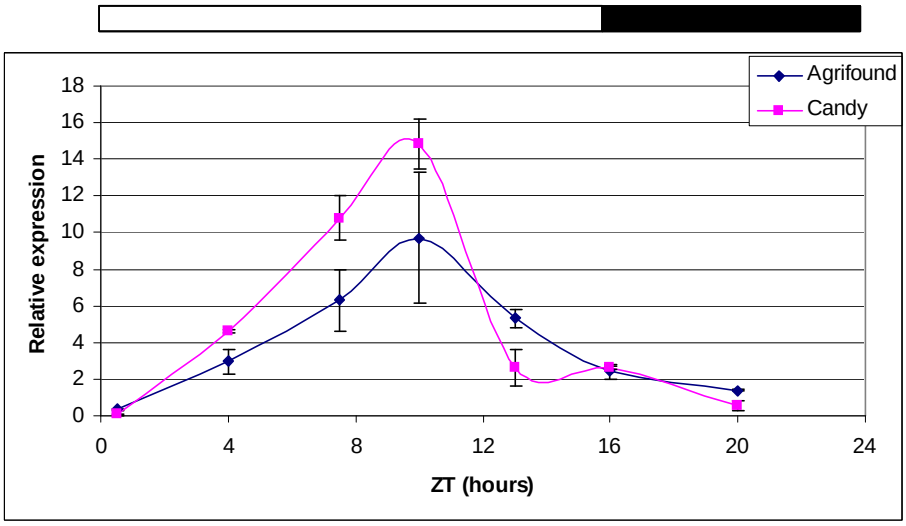


Fig. 5.7: Average LD expression of *AcGI* over a 48-hour period in the leaves of a SD (Agrifound) and ID variety (Candy), relative to ß-*tubulin*. White and black bars represent light/dark cycles.

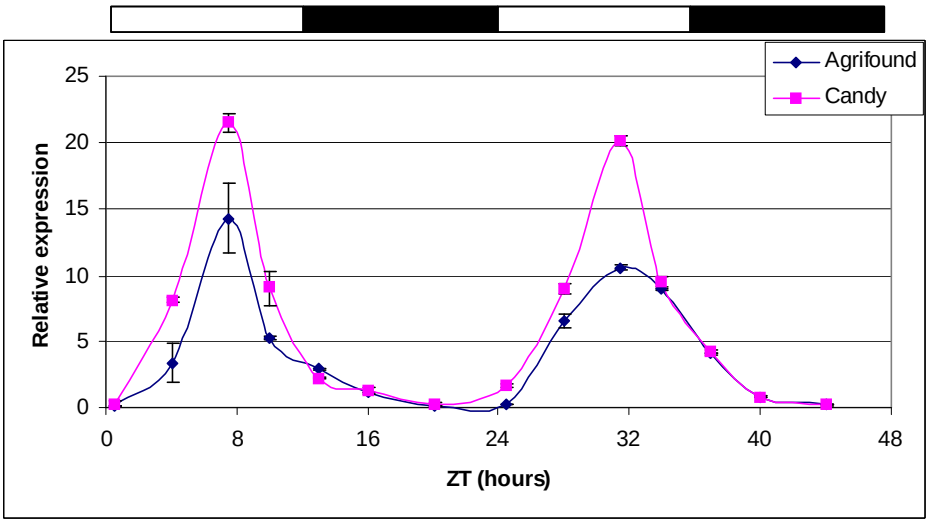


Fig. 5.8: SD expression of *AcGI* over a 48-hour period in the leaves of a SD (Agrifound) and ID variety (Candy), relative to ß-*tubulin*. White and black bars represent light/dark cycles.

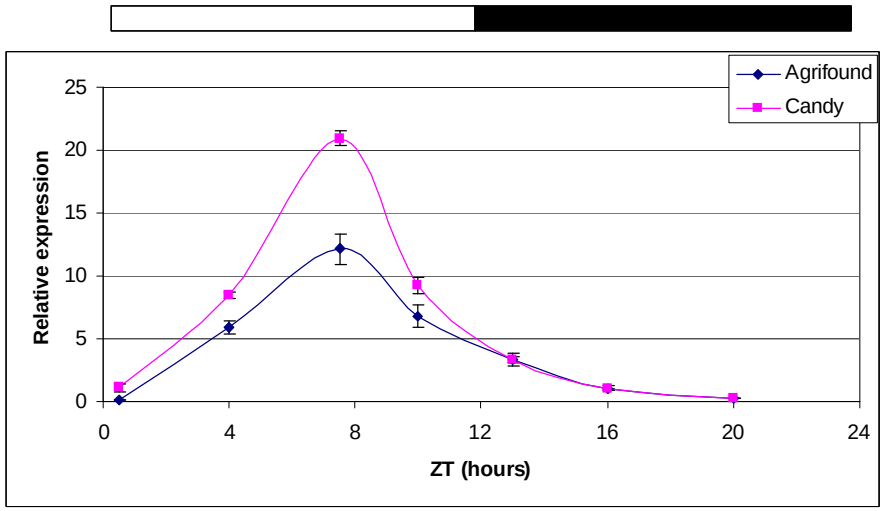


Fig. 5.9: Average SD expression of *AcGI* over a 48-hour period in the leaves of a SD (Agrifound) and ID variety (Candy), relative to ß-*tubulin.* White and black bars represent light/dark cycles.

5.3.1.5 Statistical analyses

The significance of the differences between the expression of *AcGI* in LD and SD conditions as well as between varieties were assessed by fitting a Gaussian curve to the data and carrying out non-linear regression analyses (section 5.2.2.4). It was found that the difference in expression between LD and SD grown plants was significant for all varieties tested (Table 5.1). This supports the hypothesis that a difference in the timing of *AcGI* expression controls bulb initiation in a similar way that *Arabidopsis GI* controls floral initiation. It was also shown that there was no significant difference between varieties in terms of either LD or SD expression. This supports the assertion that if the difference in daylength response between varieties is associated with a change in the photoperiod pathway, this change occurs downstream of *GI.*

Table 5.1: Significance of the differences between *AcGI* expression in SD and LD grown plants and between different onion varieties.

	LD mean	**SD mean**	**P**
Agrifound Dark	9.74	7.55	0.018
Candy F_1	8.90	7.01	0.008
Renate F_1	9.04	7.86	0.025
All varieties LD			0.60
All varieties SD			0.34

5.3.2 Searching for a full-length copy of AcGI

5.3.2.1 Screening a cDNA library

A normalised cDNA library was screened in an attempt to isolate a full-length copy of *AcGI*. PCR using primers specific to *AcGI* (section 5.2.3) resulted in amplification of a single product from cDNA (Fig. 5.10). Sequencing confirmed that this product contained a fragment of *AcGI*. No amplification was seen in library plasmid DNA or genomic DNA (data not shown). This may be due to the fact that the library plasmid DNA only represents a very small proportion of the library and GI is a single-copy gene (Fowler *et al*., 1999). Problems were often encountered when attempting to amplify from genomic DNA, probably due to the very large genome size of onion. Sequencing confirmed the identity of the cDNA product; therefore this was used as a template for the labelling PCR in order to obtain maximum specificity of template.

Fig. 5.10: Amplification of a PCR product from *AcGI* in order to screen an onion normalised cDNA library. C=cDNA, W=water, M=marker.

The cDNA library was screened using the *AcGI* gene-specific probe (section 5.2.3). This resulted in just 1 positive hit, which appeared on only one of the replicate filters (Fig. 5.11). The single clone was obtained from the original well and sequencing confirmed that this clone did indeed contain the *AcGI* gene. Unfortunately, the clone was not full-length. However, it did contain around 1 kb of the *AcGI* gene, including information on the 3' untranslated region. The *Arabidopsis GI* gene is around 4 kb in size (Fowler *et al.*, 1999), and full-length sequence information for *AcGI* could potentially be obtained by 5'RACE PCR.

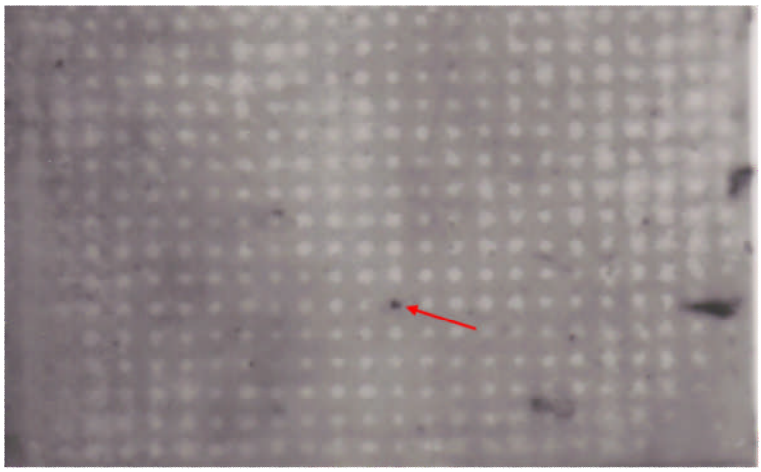


Fig. 5.11: Screening an onion normalised cDNA library for *AcGI*. The arrow indicates the single positive hit which was obtained.

5.3.2.2 RACE PCR and sequencing the full-length AcGI gene

In order to obtain full-length gene sequence information for *AcGI*, 5'RACE PCR was employed (section 5.2.4). Following nested PCR, a product of an approximate size of 3 kb was obtained (Fig. 5.12). This was cloned and sequencing confirmed that this product contained the *AcGI* gene. Now that sequence information was available for both 5' and 3' UTR's, primers were designed which amplified a product containing the full coding region of the *AcGI* gene, and some of both UTR's (Fig. 5.13). This was cloned, and sequencing with various different primers revealed the sequence of the full-length *AcGI* gene. Upon analysing the sequencing data, it was found that (at least) 2 slightly different copies of the *AcGI* gene exist in the Renate F_1 variety (Fig. 5.14). The two genes were designated

AcGIa and *AcGIb* and differed by 14 nucleotides, resulting in a single amino acid change from a glutamine in *AcGIa* to an arginine in *AcGIb*. Both genes have a coding region of 3330 bp (1110 amino acids, Fig. 5.15). This is very similar to the *Arabidopsis GI* gene, which has a coding region of 3522 bp (1174 amino acids). The presence of two slightly different alleles of *AcGI* was thought to reflect the fact that the onion variety used is a hybrid, the 2 alleles representing each of the parent lines used to create this hybrid. It is predicted that both copies are functional as they are so closely matched.

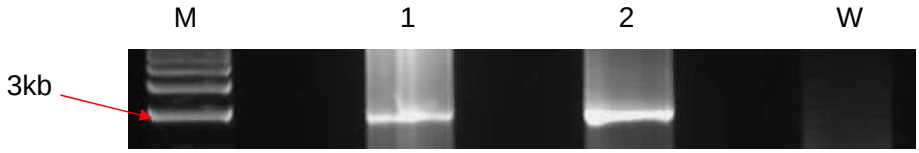


Fig. 5.12: Amplification of the 5' end of *AcGI* by nested RACE PCR. M=marker, 1=1µl of original PCR product as a template for nested PCR, 2=2µl of original PCR product as a template, W=water control.

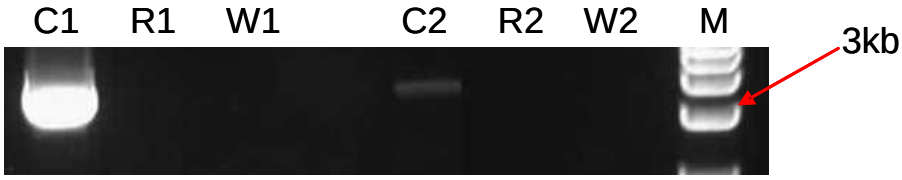


Fig. 5.13: Amplification of the full-length *AcG*I gene from onion cDNA with 2 different primer pairs. C=cDNA, R=RACE cDNA, W=water control. Numbers represent primer pair used (1=GI 5'UTR 3 & GI 3'UTR 1, 2=GI 5'UTR 3 & GI 3' UTR 2)

```
1    M S V V C E K W I D G L Q Y S S L L W P P P Q D E H Q R Q A  AcGIa
1    M S V V C E K W I D G L R Y S S L L W P P P Q D E H Q R Q A  AcGIb

31   Q I L A Y V E Y F G Q F T S E Q F P E D V A Q L I Q N H Y P  AcGIa
31   Q I L A Y V E Y F G Q F T S E Q F P E D V A Q L I Q N H Y P  AcGIb

61   S K E Q R L L D E V L A T F V L H H P E H G H A I V H P I L  AcGIa
61   S K E Q R L L D E V L A T F V L H H P E H G H A I V H P I L  AcGIb

91   S C I I D G T L V Y D K H D P P F S S F I S L F N Q N S E K  AcGIa
91   S C I I D G T L V Y D K H D P P F S S F I S L F N Q N S E K  AcGIb

121  E Y S E Q W A L A C G E I L R V L T H Y N R P I F K A E H Q  AcGIa
121  E Y S E Q W A L A C G E I L R V L T H Y N R P I F K A E H Q  AcGIb

151  N K I E R L S S C D Q A T T S D P K E E K V H H S S M P E N  AcGIa
151  N K I E R L S S C D Q A T T S D P K E E K V H H S S M P E N  AcGIb

181  D R K P V R A L S P W I A D I L I T S P L G I R S D Y F R W  AcGIa
181  D R K P V R A L S P W I A D I L I T S P L G I R S D Y F R W  AcGIb

211  C G G V M G K Y A A G G E L K P P I T S S S R G S G K H P Q  AcGIa
211  C G G V M G K Y A A G G E L K P P I T S S S R G S G K H P Q  AcGIb

241  L M Q S T P R W A V A N G A G V I L S V C D E E V A R Y E T  AcGIa
241  L M Q S T P R W A V A N G A G V I L S V C D E E V A R Y E T  AcGIb
```

Fig. 5.14: Alignment report showing the single amino acid difference between AcGIa and AcGIb. Shading represents amino acids which match AcGIa. The alignment was carried out using the MegAlign package of DNAStar (DNAStar Inc.)

Fig. 5.15: The structure of *AcGI* mRNA. The green box represents the coding region and the red boxes represent the untranslated regions.

Percentage identities with other *GI* homologues were calculated following nucleotide and amino acid alignments (Table 5.2). The percentage identities relate to *AcGIa*, but the values displayed are almost identical to those observed for *AcGIb*. These values show that *AcGI* is highly homologous to the other *GI* homologues, especially the monocot *GI* homologues. Amino acid identities of up to 76 % are observed, strongly indicating that this gene is the onion *GI* homologue. This very high level of conservation also suggests a conservation of function, a concept which

was investigated by expressing *AcGI* in *Arabidopsis gi* mutant and wild-type plants (section 5.3.5).

Table 5.2: Comparing *AcGI* with some other *GI* homologues in terms of percentage identity.

Plant Species	Accession Number	Percentage Identity (nucleotide)	Percentage Identity (amino acid)
Arabidopsis	NM_102124	70.2	69.0
Barley	AY740523	73.2	76.0
Lolium	DQ534010	72.2	74.6
Rice	NM_001048755	73.8	75.8
Wheat (GI1)	AF543844	73.2	75.5
Maize (GI1A)	BK006299	73.5	75.9
Maize (GI1B)	BK006298	73.1	75.5

5.3.3 Phylogenetic analysis of GI homologues

A phylogenetic tree was constructed to confirm the identity of *AcGI* (Fig. 5.16, described in section 5.2.5). It is clear from the tree that *AcGI* is closely related to other *GI* homologues. It clusters very close to the other monocot *GI* homologues, with the support of high bootstrap values. It is difficult to draw definitive conclusions from this analysis as *GI* is not part of any gene family and does not show homology with any gene of known function (Fowler *et al.*, 1999). This means the tree is without an outgroup, making the analysis less robust. However, it is clear that *AcGI* is a close relative of other *GI* homologues, and as *GI* does not share homology with any other known gene, *AcGI* can be classed as the onion *GI* homologue. It has been shown that the pea and radish *GI* homologues displayed in this tree have similar functions to *Arabidopsis GI* (Curtis *et al.*, 2002; Hecht *et al.*, 2007). It remains to be seen whether or not function is conserved across all *GI* homologues.

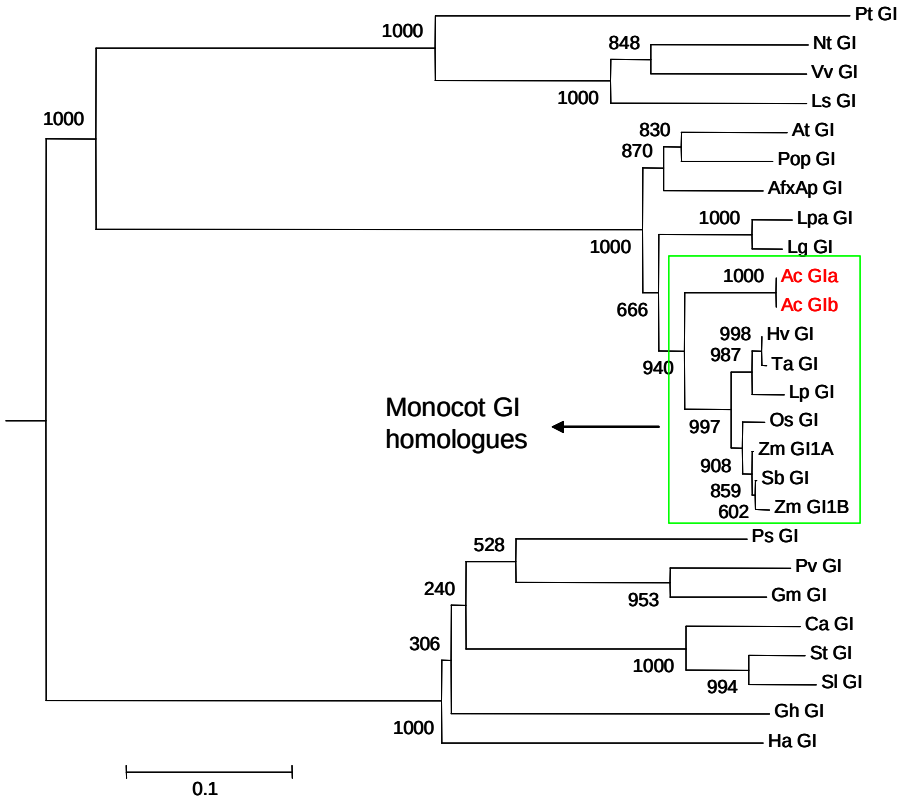


Fig 5.16: NJ-tree showing the relationship between *GI* genes from different plant species. Tree was constructed based on amino acid sequences and bootstrap values calculated using 1000 bootstrap replicates. Pt=*Pinus taeda*, Nt=*Nicotiana tabacum*, Vv=*Vitis vinifera*, Ls=*Lactuca sativa*, At=*Arabidopsis thaliana*, Pop=*Populus*, AfxAp= *A.formosa_x_A.pubescens*, Lpa= *Lemna paucicostata*, Lg= *Lemna gibba*, Ac=*Allium cepa*, Hv= *Hordeum vulgare*, Ta= *Triticum aestivum*, Lp= *Lolium perenne*, Os= *Oryza sativa* , Zm=*Zea mays*, Sb= *Sorghum bicolour*, Ps= *Pisum sativum* , Pv=*Phaseolus vulgaris*, Gm=*Glycine max*, Ca= *Capsicum annuum*, *St= Solanum tuberosum*, *Sl= Solanum lycopersicum*, Gh= *Gossypium hirsutum*, Ha= *Helianthus annuus* (see Table A9, Appendix 4 for accession numbers).

5.3.4 The genomic structure of AcGI

Partial information was obtained on the genomic structure of *AcGI*. One primer pair (GI 3'UTR1 & GI FOR2) resulted in the amplification of a PCR product of around 3.5 kb in size from DH genomic DNA (compared to 800 bp from cDNA, Fig. 5.17). This product was sequenced and showed that *AcGI* contains a very large intron (close to 3 kb in size) near to the 3' end of the gene. This shows that the gene is a minimum of 6.5 kb long and probably considerably longer. The *Arabidopsis GI*

gene contains 13 introns and is 6149 bp in size. Preliminary findings suggest that *AcGI* is significantly larger.

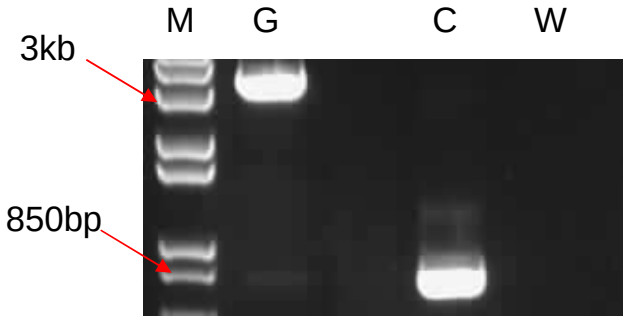


Fig. 5.17: Analysis of the 3' end of *AcGI*. M=marker, G=genomic DNA (double haploid), c=cDNA (Renate F_1), W=water control.

5.3.5 Expressing AcGI in Arabidopsis plants

Transformations were carried out in order to assess the effect of *AcGI* in *Arabidopsis gi-3* mutant and L*er* wild-type plants (section 5.2.7). The *gi-3* mutant phenotype is late flowering in LD's and normal flowering in SDs (TAIR, 2009). Mutant plants also show an increased number of rosette leaves. Due to time constraints, flowering time could only be recorded in transgenic *gi-3* T_1 plants. Analysis of T_2 generation plants is currently in progress. It would appear that the flowering time of these plants was very similar to the non-transgenic control (Fig. 5.18). However, flowering time in this generation may be affected by application of Basta and the initial sowing density of the plants. The true test of phenotype would be in the T_2 generation. The expression of *AcGI* in the transgenic lines was tested. Expression was detected in five out of the six lines tested, although the expression was very weak in two of the lines. Further work is required to complete this experiment and assess the phenotype of L*er* plants transformed to over-express *AcGI*.

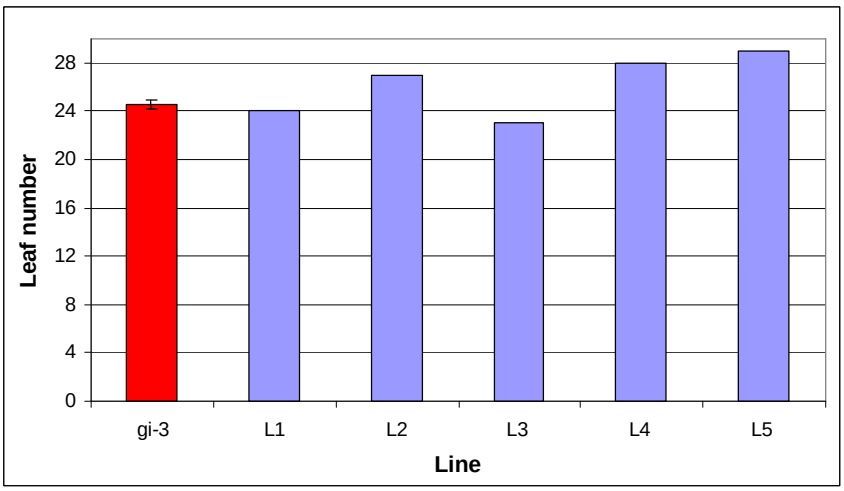


Fig. 5.18: LD flowering time (in terms of leaf number) of five *gi-3* T_1 transgenic lines, transformed to over-express *AcGI*.

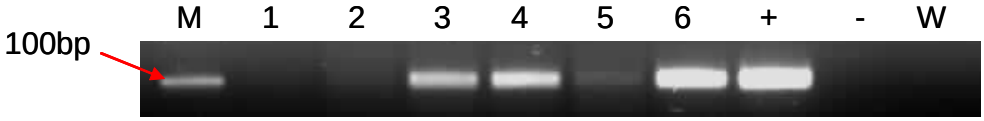


Fig. 5.19: Expression of *AcGI* in transgenic *Arabidopsis gi-3* plants. Numbers 1-5 refer to the lines in Fig. 5.18. Line 6 was yet to flower. The positive control was onion cDNA and the negative control was *Arabidopsis gi-3* cDNA. M=marker, W=water.

5.4 Conclusions

The data presented in this chapter details the characterisation of *AcGI*, a homologue of the *Arabidopsis* flowering time gene *GI*. A full-length copy of this gene was obtained through a combination of methods. Data from expression and phylogenetic studies support the concept that *AcGI* is homologous to *Arabidopsis GI* and suggest a conservation of function. The precise timing of *GI* expression in *Arabidopsis* controls seasonal flowering (Sawa *et al.*, 2007). Therefore, the similar pattern observed for *AcGI* supports the hypothesis that the genes controlling daylength response have a similar role in onion bulb initiation. The expression pattern of *AcGI* was found to be very similar in onion varieties which respond to different daylengths. This suggests that if these varieties have a modified

photoperiod pathway, the modifications occur downstream of *GI*. It remains to be seen whether *AcGI* interacts with the same genes as *Arabidopsis GI*. Homologues of *ZTL* and *FKF1* have been isolated (Chapter 6) and a similar role for *AcGI* is predicted.

CHAPTER 6: SEARCHING FOR ONION *FKF1* AND *ZTL* HOMOLOGUES

6.1 Introduction

6.1.1 *FLAVIN-BINDING, KELCH REPEAT, F-BOX* (*FKF1*)

FKF1 is a circadian-regulated gene, which controls flowering time by regulating *CO* expression (Imaizumi *et al*., 2003). FKF1 protein has been proposed to function as a photoreceptor for the light controlled expression of *CO (Nelson et al*., 2000). The FKF1 protein has three characteristic domains: the light, oxygen and voltage (LOV)-sensing domain, the F-box and the Kelch repeat (Nakasako *et al*., 2005). The F-box is located in the central region of the protein and is believed to function in targeting proteins for degradation through ubiquitination (Patton *et al*., 1998; Mizoguchi and Coupland, 2000). The Kelch repeat may act as the protein-protein interaction domain, which recruits proteins for degradation. Perhaps the most interesting feature of this protein is the LOV domain. This domain is homologous to the LOV domains of phototrophins, which work as blue light receptors in plants (Briggs and Christie, 2002). The LOV domains are a subfamily of the PER-ARNT-SIM (PAS) superfamily (Zikihara *et al*., 2006). PAS domains are widely distributed in both eukaryotes and prokaryotes and function in the sensing of light, oxygen and some other stimuli (Taylor and Zhulin, 1999). It has now been shown that the *FKF1* LOV domain is a flavoprotein which binds to a flavin mononucleotide chromophore, suggesting that this domain does have light-sensing properties (Imaizumi *et al*., 2003).

It has been shown that FKF1 protein mediates the cyclic degradation of CDF1, a repressor of *CO* (Imaizumi *et al*., 2005). Mutant studies indicated that the CDF1 protein is more stable in plants lacking a functional *FKF1* gene. Recent advances have shown that FKF1 protein forms a complex with GI, specifically under blue light conditions (Sawa *et al*., 2007). This interaction is induced by blue light absorption within the LOV domain and confirms previous proposals that *FKF1* is a blue light photoreceptor, acting in the photoperiod pathway (Imaizumi *et al.*, 2003). It has also been shown that the FKF1/GI complex regulates the expression of *CO* during the day (Sawa *et al.*, 2007). This regulation is achieved through degradation

of CDF1. The degradation of CDF1 at least partly accounts for the regulation of *CO* by the FKF1/GI complex. It is thought that *FKF1* controls *CO* through at least one more unknown mechanism (Coupland, 2008). A summary of the function of *FKF1* within the photoperiodic flowering pathway is given in Fig 6.1.

At present, *FKF1* orthologues have not been characterised from any other species. However, various databases contain EST's which show a high level of homology with *Arabidopsis FKF1*. This includes putative orthologues in rice and wheat (NCBI, 2008).

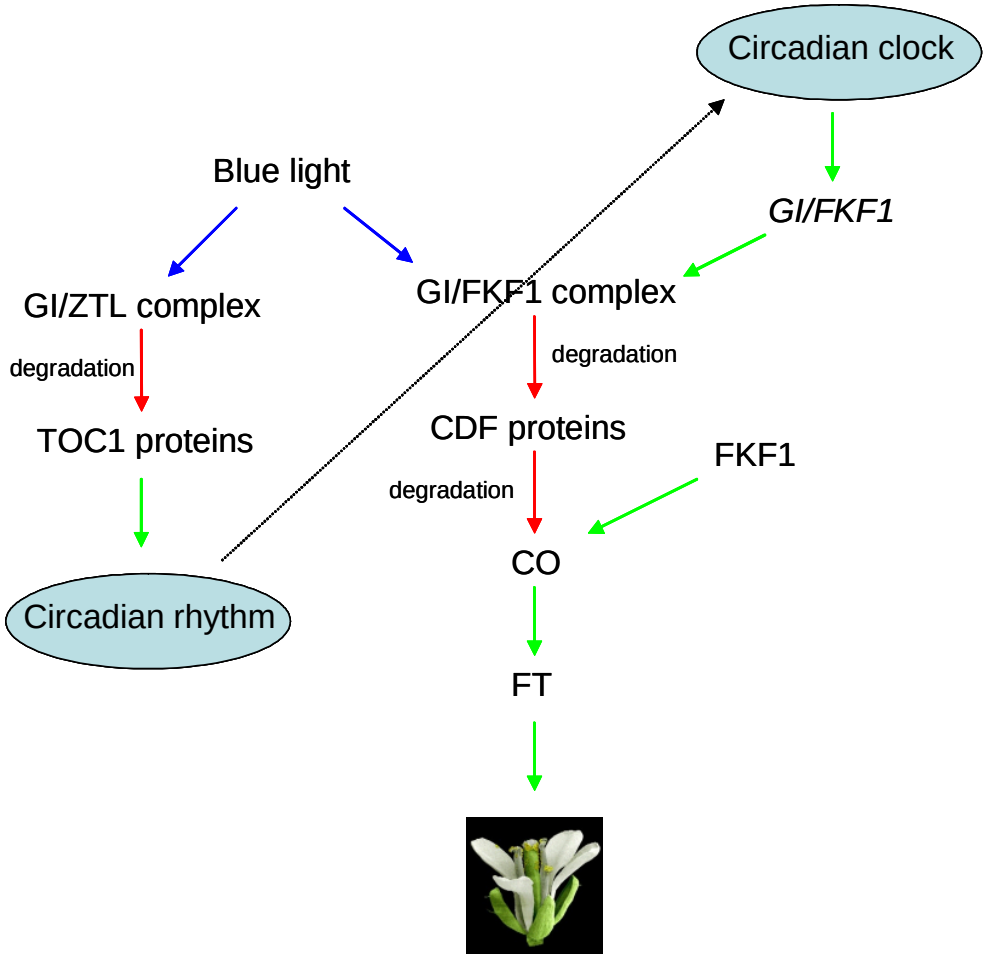


Fig. 6.1: A summary of the functions of *FKF1* and *ZTL* in the photoperiodic control of flowering time in *Arabidopsis*. Red arrows indicate a degradation, green arrows indicate a promotion. Adapted from Coupland, 2008 and Kim *et al*., 2007. *Arabidopsis* flower picture copyright Juergen Berger, Max Planck Institute for Developmental Biology.

6.1.2 *ZEITLUPE* (*ZTL*)

ZTL was first identified as a circadian clock associated protein. In *Arabidopsis*, It was found that mutations at the *ZTL* locus caused an alteration in various clock-controlled processes, including flowering in LDs (Somers *et al*., 2000). The ZTL protein has a structure which is similar to FKF1. It consists of a LOV domain, an F-box and six kelch repeats. There is a strong interaction between ZTL and TOC1, as indicated by yeast two-hybrid studies (Mas *et al*., 2003). Further analysis reveals that ZTL is required for the targeted degradation of TOC1. The degradation of TOC1 is not evident in *ztl* mutant plants, leading to constitutive expression of *TOC1*. This leads to a disrupted circadian rhythm as *TOC1* is part of a feedback loop within the circadian clock (Strayer *et al.*, 2000). The circadian clock controls flowering time in *Arabidopsis* through the regulation of the expression of photoperiod response genes such as *CO, GI* and *FKF1* (described in section 1.5.2).

Recent work has revealed more detail on the exact function of *ZTL*. Firstly, it was shown that whilst *ZTL* mRNA is constitutively expressed, the protein levels oscillate with up to a four-fold change (Kim *et al*., 2007). The peak in protein abundance is seen around ZT10-13. This suggests a posttranscriptional level of control. Further analysis has revealed that GI is required to establish and maintain the ZTL protein oscillations. This is achieved through protein-protein interactions which are enhanced by blue light. This blue light reaction occurs through the LOV domain of ZTL. Current research shows ZTL to be a blue light photoreceptor which controls circadian rhythm through TOC1 and is itself postranscriptionally regulated by GI. A summary of the function of *ZTL* within the photoperiodic flowering pathway is given in Fig 6.1.

ZTL has also been shown to have another distinct function. It has been shown to have a physical interaction with both *CRY1* and *PHYB* (Jarillo *et al*., 2001). The interaction with *PHYB* is thought to facilitate developmental responses to red light which have been shown to be controlled by *ZTL* (Kevei *et al*., 2006). Such responses include cotyledon function and stem elongation, processes which are not controlled by the circadian clock. This indicates that *ZTL* has a broader role than simply as a blue light photoreceptor controlling circadian rhythm. To date, *ZTL* orthologues have not been characterised in other plant species. However, databases of publicly

available sequences contain several *ZTL* homologues which are predicted to function in a similar way to *Arabidopsis ZTL*.

This chapter describes the work undertaken in search of onion genes homologous to *FKF1* and *ZTL*. The starting point was an EST which showed sequence similarity with *Arabidopsis FKF1*. The available sequence for this gene indicated that it contained an F-Box, a domain which is present in both FKF1 and ZTL proteins (Nelson *et al*., 2000; Somers *et al*., 2000). This EST was characterised before further work was carried out in search of other members of this gene family.

6.2 Materials and Methods

6.2.1 Characterisation of an onion FKF1-like gene

6.2.1.1 Sequencing and expression analyses

The clone containing an onion *FKF1*-like gene (clone name AV44) was obtained (Prof. Mike Havey, USDA, University of Wisconsin). This gene was assigned the name *AcFBox* (for *Allium cepa* F-Box protein). *AcFBox* was sequenced using M13 forward and reverse primers (see Appendix 2 for sequences) as described in section 2.2.8. Gene-specific primers were used to confirm the double-stranded sequence (named FKF1-FKF4, see Appendix 2 for sequences).

The expression of *AcFBox* was examined in cDNA samples originating from material harvested at 3 different time-points using RT-PCR (described in section 2.2.1). The primers used were FKF-RT F1 & R1 (see Appendix 2 for sequences) with an annealing temperature of 55 °C and a cycle number of 35. PCR products were purified and sequenced using both forward and reverse primers.

The relative expression of *AcFBox* in a LD onion variety (Renate F_1) was examined using quantitative real-time PCR as described in section 2.3.3. The expression was examined in plants grown in LD (16 hours of light) and SD (8 hours of light) conditions. The primers used were AV44 RT2 FOR and AV44 RT2 REV (see Appendix 2 for sequences) at a concentration of 0.4 µM. The cDNA used was undiluted and all samples run in triplicate. Data was normalised using the housekeeping gene *EF1α*, as described in section 2.2.3. Errors were calculated in

terms of the standard error of the mean (SEM). A selection of PCR products were purified and sequenced using both forward and reverse primers.

6.2.1.2 Complementation analysis

Arabidopsis transformations were carried out using the floral dip method described in section 2.3.6. *Arabidopsis fkf1-1* mutant (late flowering) and Col-0 wild-type plants were transformed to over-express *AcFBox*. Plants were allowed to set seed and T_1 generation plants sprayed with Basta (Bayer CropScience). T_2 seeds were collected and flowering time scored in the T_2 generation (described in section 2.3.6). All plants were grown in LD conditions. Averages were taken and the SEM calculated (Mead *et al.*, 1993). One-way ANOVA's were carried out to assess the significance of any differences in flowering times (using Genstat). The expression of the transgene was tested using RT-PCR, as described in section 2.2.1. AV44-RT2 FOR and AV44-RT2 REV primers (see Appendix 2 for sequences), with an annealing temperature of 55 ºC and a cycle number of 35, were used for both the *fkf1-1* and Col-0 transgenic lines. PCR products were sequenced to confirm their identity (using forward and reverse primers, as described in section 2.2.8).

Sequences of *Arabidopsis FKF1* and related genes from other plant species were collated from publicly available sequences (NCBI, 2008). Alignments were carried out and a phylogenetic tree constructed based on predicted amino acid sequences as described in section 2.3.7. This analysis was expanded to include *ZTL* homologues and is described in section 6.2.2.3.

6.2.2 Searching for other FKF1-like genes

6.2.2.1 Degenerate PCR

In an attempt to isolate any other *FKF1*-like genes, a forward degenerate primer (FKF DEG 1, see Appendix 2 for sequence) was designed using previous alignments of publically available sequences (section 6.2.1.2). RT-PCR was carried out using Tagged cDNA and a TAG-specific primer (TAG primer) along with FKF DEG 1, following the protocol described in section 4.2.5, with an annealing temperature of 60 ºC and a cycle number of 35. Products were purified (section

2.2.6) and cloned into a pCR®4Blunt-TOPO® vector (Invitrogen Ltd.) following the manufactures guidelines and using electroporation as the transformation method (section 2.2.9). Positive clones were selected on plates containing LB & 100 µg ml^{-1} ampicillin (Melford). Plasmid DNA was isolated and sequenced using M13 forward and reverse primers (sections 2.2.5 & 2.2.8). Sequences were BLASTED against publicly available sequences (NCBI, 2008), alignments carried out and a phylogenetic tree constructed as described in section 2.3.7.

6.2.2.2 Obtaining full-length sequence information for putative *FKF1* homologues

5' RACE PCR was carried in order to obtain full-length gene sequence information on the two genes obtained by the method described in section 6.2.2.1. The two genes were assigned the names *AcFKF1* (for *Allium cepa FLAVIN-BINDING, KELCH REPEAT, F-BOX*) and *AcZTL* (for *Allium cepa ZEITLUPE*). Primers were designed (FKF GSP, FKF GSP NEST, ZTL GSP, ZTL GSP NEST, see Appendix 2 for sequences) and the protocol in section 5.2.4 followed with the exception of the extension cycles which were carried out for 2 min.

Following nested PCR, products were purified and cloned into a pCR®4Blunt-TOPO® vector (Invitrogen Ltd.), following the manufacturer's guidelines. Transformations were carried out using electroporation (as described in section 2.2.9) and positive clones selected on plates containing LB & 100 µg ml^{-1} ampicillin (Melford). Plasmid DNA was isolated and sequencing carried out using M13 forward and reverse primers, as described in sections 2.2.5 and 2.2.8.

The sequence information obtained by degenerate PCR and RACE PCR was used to construct contigs using the SeqMan package of DNAStar (DNAStar Inc.). Alignments were carried out using previously obtained sequence information for *FKF1* and related genes (section 6.2.1.2), and a phylogenetic tree constructed using amino acid sequences as described in section 2.3.7.

6.2.3 Expression analysis of AcFKF1 and AcZTL

The expression of *AcFKF1* and *AcZTL* in Renate F_1 leaf material was investigated using RT-PCR. The primers used were pFKF-RT FOR3 and pFKF-RT REV3 for *AcFKF1* and ZTL-RT FOR1/ZTL-RT REV for *AcZTL* (see Appendix 2

for all primer sequences). Both primer pairs were used with an annealing temperature of 65 ºC and a cycle number of 30. Products were purified and sequenced using forward and reverse primers (section 2.2.8). The expression of these 2 genes was further investigated using quantitative real-time PCR as described in section 2.3.4. The primers used for *AcFKF1* were pFKF-RT FOR3 and pFKF-RT REV3 at a concentration of 0.4 µM. The primers used for *AcZTL* were ZTL RT FOR1/ZTL RT REV at a concentration of 0.2 µM. All cDNA samples were used undiluted and run in triplicate. The expression profile of *AcFKF1* was examined in Renate F_1, Agrifound Dark and Candy F_1 varieties. Renate F1 plants were grown in LD (16 hours of light) and SD (8 hours of light) conditions (described in section 2.3.3). Agrifound Dark and Candy F1 plants were grown in 16 hour LDs and 12 hour SDs (described in section 2.3.4). The expression of *AcZTL* was examined in Agrifound Dark only. All data were normalised to the level of expression of ß-tubulin. Twenty-four hour averages of expression were calculated and standard errors included (Mead *et al.*, 1993). A selection of PCR products were purified and sequenced to confirm their identity.

Statistical analyses were carried out to assess the significance of the differences in *AcFKF1* expression between plants grown in LDs and short days and between different varieties. All analyses were carried out using the 24-hour average data. Gaussian curves were fitted to the data, an analysis which is based on the normal distribution (Mead *et al.*, 1993). Non-linear regression analyses were then used to assess the significance of the differences between data sets, as described in section 5.3.1.5.

6.3 Results and Discussion

6.3.1 Characterisation of AcFBox

6.3.1.1 Gene Structure

Sequencing analysis revealed that the *AcFBox* clone obtained covered the entire coding region of this gene and the 3'UTR sequence. Sequence information for the 5'UTR was absent. This gene has a coding region which spans 1314 bp (438

amino acids, Fig. 6.2). This is significantly smaller than *Arabidopsis FKF1* which contains a coding region spanning 1860 bp (620 amino acids), providing evidence that *AcFBox* may not be the onion *FKF1* homologue. In addition, close analysis of the predicted protein sequence shows that *AcFBox* lacks a LOV domain, a characteristic domain present in *FKF1* genes (Nakasako *et al*., 2005).

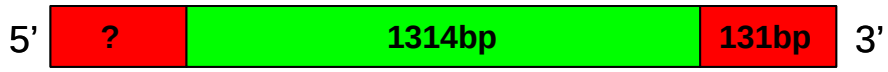


Fig. 6.2: Structure of *AcFBox* mRNA. The green box represents the coding region; red boxes represent the untranslated regions.

6.3.1.2 Expression analysis

The results of the initial gene expression experiment (using RT-PCR, section 6.2.1.1) showed that *AcFBox* is expressed at all time-points in the leaf material (Fig. 6.3). Amplification was very weak, but initial results suggest that the highest expression levels are at ZT11.5. Expression of *AcFBox* was also seen in the material described as the bulb. The plants used for harvests were not yet bulbing, so bulb material was hard to distinguish from leaf material and some expression would be expected. No expression was seen in the roots. Initial results suggested that *AcFBox* is differentially expressed over time. *Arabidopsis FKF1* is expressed highest at ZT10, suggesting that this EST could be the onion homologue of *FKF1* (Imaizumi *et al*., 2005). A larger scale time-course experiment was set up to further investigate these results.

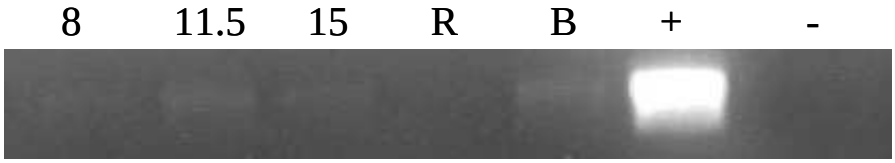


Fig. 6.3: Expression of *AcFBox* in Renate F_1 onions. Numbers represent time of harvest in zeitgeber time. B=bulb, R=root, positive control is *AcFBox* plasmid DNA and negative control is water.

Quantitative real-time PCR (section 6.2.1.1) provided more detailed information on the expression of *AcFBox* in light/dark cycles. The expression profile for this gene shows various peaks in LDs without an obvious pattern (Fig. 6.4). Peaks and troughs of expression are evident, but the timing of these is not consistent in the two separate days. This suggests that this gene does not show a diurnal expression pattern, a pattern which is evident in many photoperiod pathway genes (Jackson, 2008). The expression levels are generally lower in SD-grown plants and an obvious pattern is not clearly evident. A possible expression peak is seen in the dark period (around ZT17). This is contrary to *Arabidopsis FKF1* which shows distinct peaks around ZT10 in LDs and ZT7 in SDs (Imaizumi *et al*., 2003). One point to note is that *AcFBox* appears to peak in the light period in LDs and in the dark period in SDs. The overall significance of this pattern is unclear and it certainly does not seem that this gene is circadian regulated. The data presented here suggests that *AcFBox* is not the onion *FKF1* homologue. *Arabidopsis FKF1* shows a clear expression pattern and is controlled by the circadian clock (Nelson *et al.*, 2000). Phylogenetic analyses also support the theory that *AcFBox* is not the onion *FKF1* homologue and may be a related gene (discussed in section 6.3.2.3).

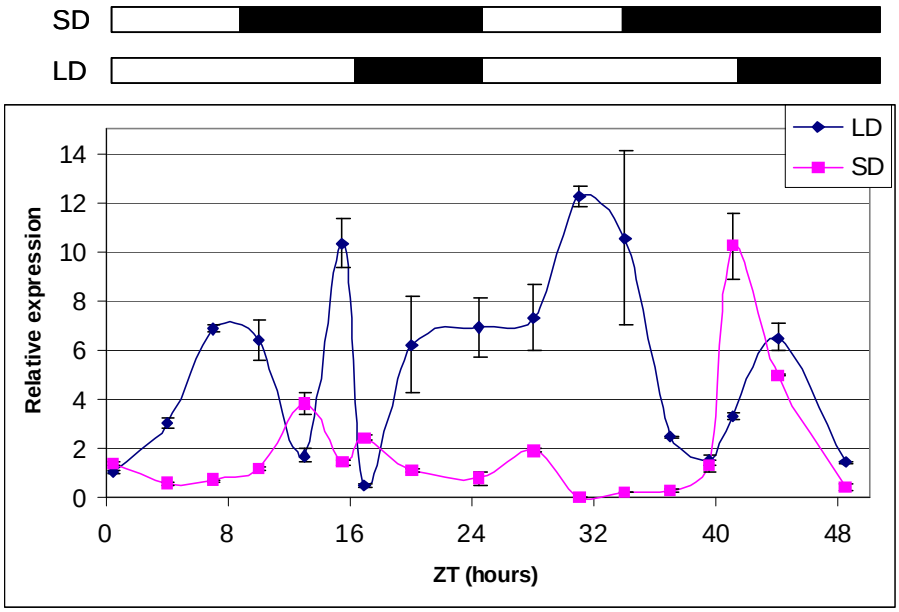
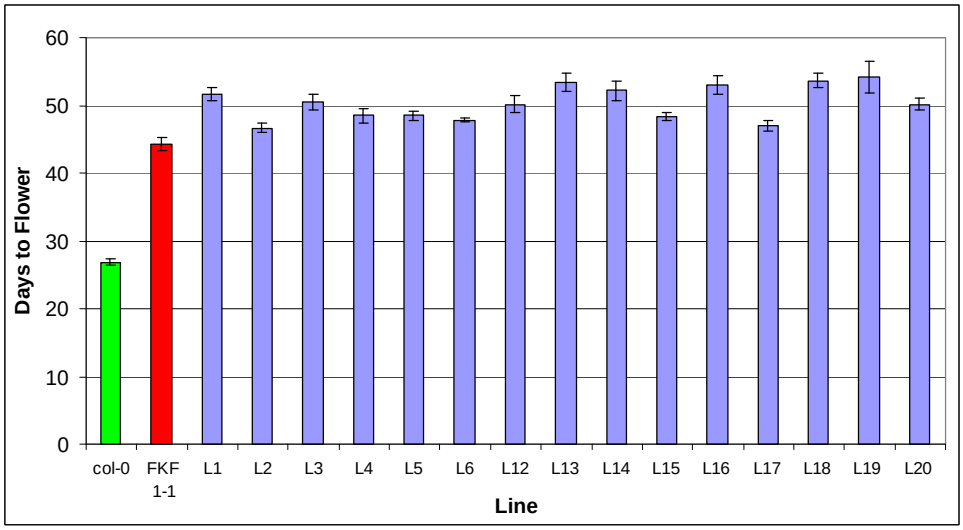


Fig. 6.4: Expression of *AcFBox* in a LD onion variety (Renate F_1) over a 48-hour period, relative to *EF1α*. White and black bars denote light/dark cycles. Error bars represent the SEM.

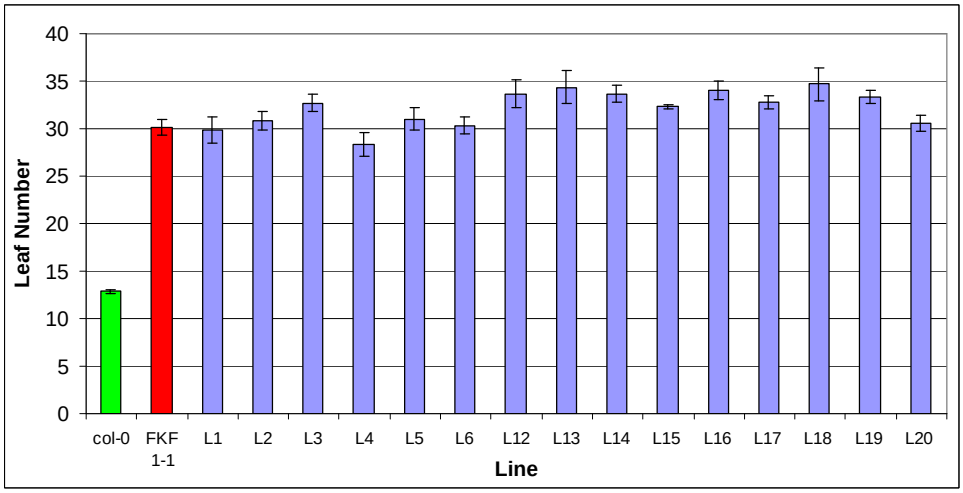
6.3.1.3 Complementation analysis

Transforming *Arabidopsis fkf1-1* mutant plants (late flowering in LDs) to over-express *AcFBox* (section 6.2.1.2) did not lead to earlier flowering than in the *fkf1-1* mutant in LD conditions (Fig. 6.5). This shows that wild-type flowering time was not restored as *AcFBox* did not functionally complement the *Arabidopsis FKF1* gene and suggests that *AcFBox* is not the onion *FKF1* homologue. However, it is possible that the lack of complementation is due to a difference between plant species. Flowering time was seen to be later in many transgenic lines. The significance of this phenotype was tested using one-way ANOVA's. The difference in flowering time was shown to be significant both in terms of days to flower ($p=<0.001$, SED=1.64, degrees of freedom (d.f.)=82) and leaf number ($p=<0.001$, SED=1.57, d.f.=82). This suggests the transgene has some effect on flowering time when overexpressed in wild-type plants. However it is clear that wild-type flowering is not restored in the *fkf1-1* mutant.

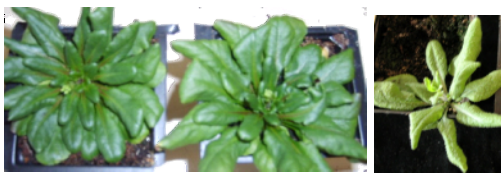
Transforming *Arabidopsis* wild-type Col-0 plants to over-express *AcFBox* (section 6.2.1.2) resulted in a slightly earlier LD flowering phenotype in many of the lines examined (Fig. 6.6). This effect was small but significant both in terms of days to flower and leaf number at flowering. One-way ANOVA's were carried out to assess the significance of the observed differences. It was clear that transgenic lines flowered earlier in terms of days to flower ($p=0.002$, SED=1.04, d.f.= 76) and leaf number ($p=<0.001$, SED=0.62, d.f.=76). The slightly earlier flowering phenotype observed in many of the Col-0 transgenic lines suggests that *AcFBox* may be involved in flowering. However, the exact role of this gene is unclear. It appears that this gene is not an *FKF1* orthologue as the late flowering phenotype of the *fkf1-1* mutant could not be recovered (Fig. 6.5). This data supports the sequence, expression and phylogenetic data. *AcFBox* is an F-box protein belonging to the same gene family as *FKF1*. The F-box is believed to function in targeting proteins for degradation through ubiquitination (Patton *et al.*, 1998; Mizoguchi and Coupland, 2000). The role of this gene (if any) in bulbing is unclear and it was not pursued further as it is clearly not a homologue of *Arabidopsis FKF1*.



(a)

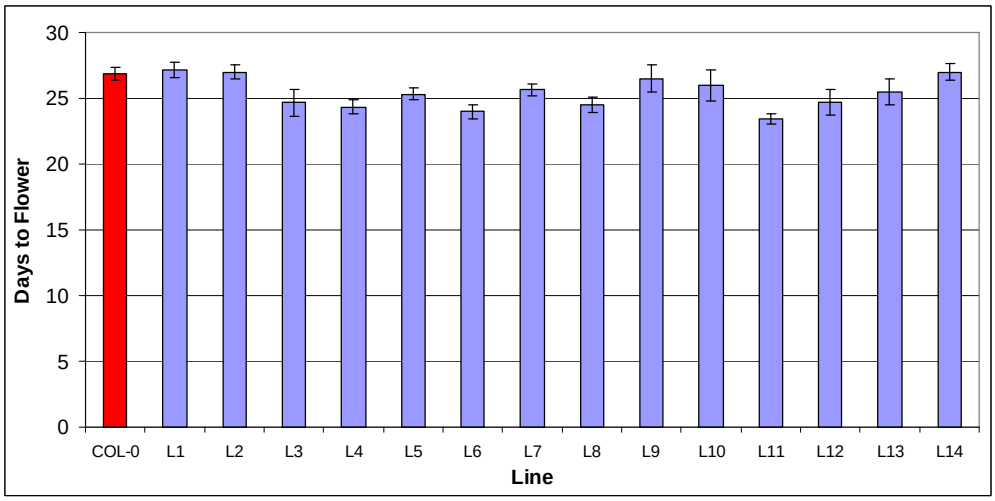


(b)

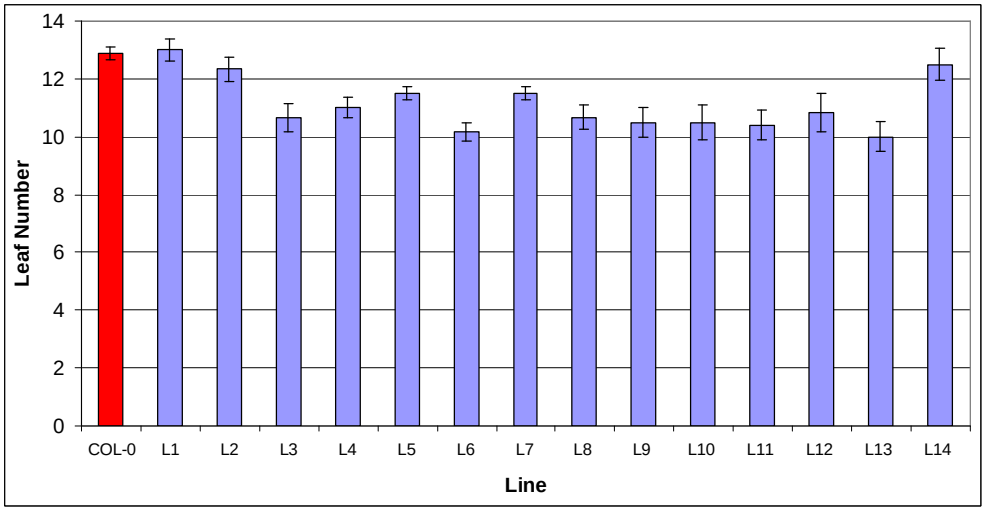


(c)

Fig. 6.5: Mean LD flowering time of 15 independent *Arabidopsis fkf1-1* transgenic lines (T_2 generation), transformed to express onion *AcFBox*. (a) number of days taken to produce bolt; (b) number of leaves upon initiation; (c) comparing a transgenic and non-transgenic plant at the bolting stage. Error bars represent the SEM. All plants were grown under LD conditions. Col-0= Columbia.



(a)



(b)

col-0 Transgenic line (L13)

(c)

Fig. 6.6: Mean LD flowering time of 14 independent *Arabidopsis* Col-0 transgenic lines (T_2 generation), transformed to express onion *AcFBox*. (a) number of days taken to produce bolt; (b) number of leaves upon initiation; (c) comparing a transgenic and non-transgenic plant at the bolting stage. Error bars represent the SEM. All plants were grown under LD conditions. Col-0= Columbia

The expression of the transgene was tested in 5 Col-0 and 5 *fkf1-1* transgenic lines (section 6.2.1.2). The expression of *AcFBox* was observed in all of the transgenic lines tested (Fig. 6.7). This means that any changes in phenotype can be attributed to the presence of the transgene. Therefore, the slightly early flowering time which was observed in many of the Col-0 transgenic lines is due to the expression of *AcFBox*. This indicates a role for this gene in the control of flowering time. The exact role of this gene was not investigated any further at this stage as it is clearly not a homologue/orthologue of *Arabidopsis FKF1*.

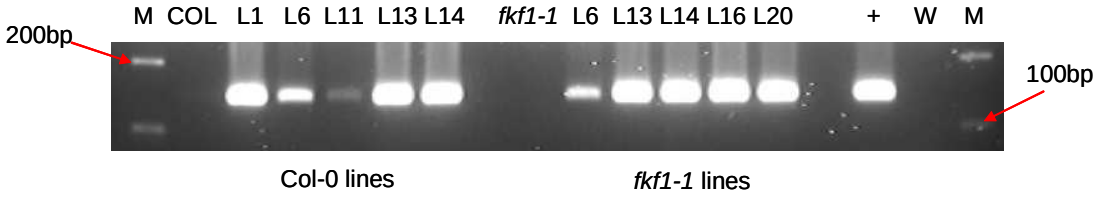


Fig. 6.7: Expression of *AcFBox* in Arabidopsis Col-0 and *fkf1-1* transgenic lines. M=marker, COL=Col-0 wild-type cDNA, *fkf1-1=fkf1-1* mutant cDNA, W=water control, L=transgenic line, positive control is *AcFBox* (cloned into pCMV-SPORT) plasmid DNA.

6.3.1.4 Comparing *AcFBox* with *FKF1* homologues

Alignments of *AcFBox* with related genes allowed sequence comparisons to be made (section 6.2.1.2). It appears that *AcFBox* is more closely related to *Arabidopsis* F-Box and Rice F-Box genes than to *FKF1*/*ZTL* homologues (Table 6.1). This supports the formation of a separate clade within the phylogenetic tree (Fig. 6.13, section 6.3.2.3). Functional analysis of these two genes has not been undertaken and they are simply annotated as F-Box proteins that belong to the same gene family as *FKF1* and *ZTL* (NCBI, 2008). There are many F-box proteins in plants species, controlling a whole range of processes including cell division, gene transcription, signal transduction and development (Patton *et al.*, 1998). This makes functional analysis of *AcFBox* difficult, based on the data obtained here.

Table 6.1: Comparing *AcFBox* with *FKF*/*ZTL* genes from various plant species (in terms of percentage amino acid identity).

Plant Species	Accession Number	Percentage Identity (amino acid)
Arabidopsis FKF1	NM_105475	24.2
Rice *FKF1*	NM_001074600	23.7
Wheat *FKF1*	DQ923399	22.4
M. crystallinum FKF1	AY371291	24.4
Arabidopsis LKP2	NM_179652	24.0
Arabidopsis ZTL	NM_125119	22.1
Arabidopsis F-Box	NM_104033	47.5
Rice F-Box	NM_001067833	51.1

Taken together the expression, phylogenetic and transformation data shows that *AcFBox* is not an *FKF1* homologue. It would appear that this gene is a member of a family of F-box genes which may have a role in flowering, which could also extend to bulbing. To elucidate the precise function of this gene, transformations would have to be carried out on onions. Unfortunately, onion transformation procedures are currently inefficient and are not a feasible option within the time of a PhD project (Eady et al., 2003a; Eady et al., 2003b).

6.3.2 Searching for other onion FKF1-like genes

6.3.2.1 Degenerate PCR

Upon reaching the conclusion that *AcFBox* was not a homologue of *Arabidopsis FKF1*, an experiment was set up to search for the onion FKF1 homologue. The method employed was RT-PCR using a forward degenerate primer and a reverse TAG primer (section 6.2.2.1). This resulted in the amplification of a 1.3 kb product (Fig. 6.8). This product was cloned and sequencing analysis confirmed that it contained various gene sequences, including 2 genes which showed a high homology to genes from the *FKF1*/*ZTL* gene family.

The first gene showed a high level of sequence similarity with *FKF1* so was tentatively annotated *AcFKF1*. The second showed a high level of sequence similarity with *ZTL* so was tentatively annotated *AcZTL*. The PCR method employed yielded information on the 3'end of the 2 genes of interest, including information on

the 3'UTR's. This meant full-length sequence information could be obtained using 5' RACE PCR.

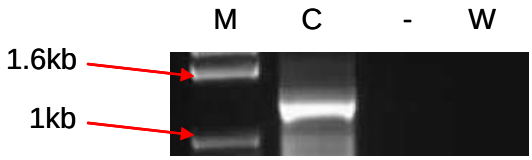


Fig. 6.8: Amplification of a cDNA product using a degenerate primer designed to amplify from *FKF*/*ZTL* family genes. C=tagged cDNA, negative control=untagged cDNA, W=water control.

6.3.2.2 Obtaining full-length sequence information for the putative *FKF1/ZTL* homologues

RACE PCR was carried out in order to obtain full-length gene sequence information for both genes (section 6.2.2.2). This resulted in the amplification of a 1 kb product for both the putative *FKF1* family homologues. Nested PCR resulted in a product for both genes which was then cloned and sequenced (Fig 6.9). This produced sequence information for the 5' end of both of these genes.

Fig. 6.9: Amplification of the 5' end of two putative *FKF1* family genes by nested 5' RACE PCR. $C^f/C2^f$=cDNA template, putative *FKF1* product, $C^z/C2^z$=cDNA template, putative *ZTL* product, W=water control, M=marker. C=1µl of original PCR product used for nested PCR, C2=2µl of original PCR product used.

Contigs were constructed for both genes in order to obtain information on the entire gene sequences of both genes. It was revealed that *AcFKF1* has a coding region spanning 1872 bp (624 amino acids, Fig. 6.10). This is very similar to *Arabidopsis FKF1* which contains a coding region which spans 1860 bp (620 amino acids). It was also revealed that *AcZTL* contains a coding region spanning 1839 bp

(613 amino acids). This is very similar to *Arabidopsis ZTL* which contains a coding region spanning 1830 bp (610 amino acids).

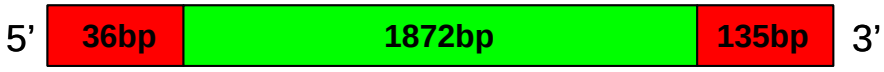


Fig.6.10: Structure of *AcFKF*1 mRNA. The green box represents the coding region; red boxes represent the untranslated regions.

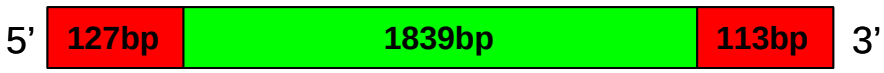


Fig. 6.11: Structure of *AcZTL* mRNA. The green box represents the coding region; red boxes represent the untranslated regions.

Nucleotide and amino acid alignments were carried out which produced percentage identities with *FKF1*/*ZTL* homologues from different plant species (section 6.2.2.2, Tables 6.2/6.3). It is clear that both genes show a very high level of homology with their putative homologues. *AcZTL* shows especially high percentage identities, up to 76 % amino acid identity with the rice *ZTL* homologue. The percentage identities seen in *AcFKF1* can still be considered as very high (up to 70 % amino acid identity with the rice *FKF1* homologue). The data suggests that these genes are homologous to *FKF1* and *ZTL*. Included in both tables is a third member of the *FKF1*/*ZTL* gene family, *LKP2* (*LOV KELCH PROTEIN2*). This gene is proposed to have a role in the circadian clock and is the only other gene reported to contain a LOV domain, an F-Box and six kelch repeats (Schultz et al., 2001). Both genes show a lower percentage identity with *LKP2*, suggesting that neither are homologues of this gene.

Table 6.2: Comparing *AcFKF1* with closely related genes in terms of percentage identity

Plant Species	Accession Number	Percentage Identity (nucleotide)	Percentage Identity (amino acid)
Arabidopsis	NM_105475	57.4	63.4
Rice	NM_001074600	64.0	69.7
Wheat	DQ923399	59.3	61.2
M. crystallinum	AY371291	57.9	59.6
Arabidopsis LKP2	NM_179652	56.5	53.9

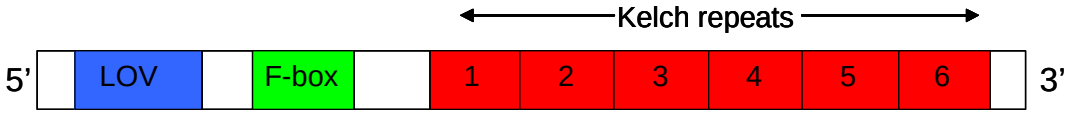
Table 6.3: Comparing *AcZTL* with closely related genes in terms of percentage identity

Plant Species	Accession Number	Percentage Identity (nucleotide)	Percentage Identity (amino acid)
Arabidopsis	NM_125119	68.0	72.1
Rice	NM_001064973	69.1	75.5
P. nil	DQ309278	64.1	72.8
M. crystallinum	AY371290	65.5	72.4
Arabidopsis LKP2	NM_179652	65.0	64.7

It was also shown that both genes contain a LOV domain, an F-box and six kelch repeats (Fig 6.12). This gene structure is characteristic of the LOV family genes and provides further evidence that genes homologous to *Arabidopsis FKF1* and *ZTL* have been isolated from onion (Nelson *et al.*, 2000; Somers *et al.*, 2000). The percentage identity within the conserved domains was then examined, using *Arabidopsis FKF1* and *ZTL* as a standard (Table 6.4). This theory was further tested using a phylogenetic approach.

Table 6.4: Comparing the conserved domains of *AcFKF1* and *AcZTL* with *Arabidopsis FKF1* and *ZTL* in terms of percentage amino acid identity.

Domain	***AcFKF1***	***AcZTL***
LOV	78.4	74.1
F-box	80.4	65.2
Kelch repeats	71.6	84.3



(a)

```
F V V S D A M E P D F P I I Y V N S V F E D S T G Y R A D E V I G R N C R F L Q  AcFKF1
L V V T D A I E I D N P I I Y V N E G F E K G T G Y R A E E V L G R N C R F L Q  AcZTL
F I V S D A L E P D F P L I Y V N R V F E V F T G Y R A D E V L G R N C R F L Q  At_FKF1
F V V T D A V E P D Q P I I Y V N T V F E M V T G Y R A E E V L G G N C R F L Q  At_ZTL
                        h     N           h   G h       E h h           h
                              S

F R D P Q A Q R R H P L V D P T V V S E I R N C L E K G I E F Q G E L L N F R K  AcFKF1
C R G P F A Q R R H P L V D S A V T S E I R K C I E S G L S F Q G D I L N F K K  AcZTL
Y R D P R A Q R R H P L V D P V V V S E I R R C L E E G I E F Q G E L L N F R K  At_FKF1
C R G P F A K R R H P L V D S M V V S E I R K C I D E G I E F Q G E L L N F R K  At_ZTL
                                                                h         K
                                                                          R

D G T P L L N R L C L M P I S D D - G I V T H I I A I Q I F T S                 AcFKF1
D G S P V M N R L Q L S P I F G D D D E V T H Y L G I Q F V T E                 AcZTL
D G T P L V N R L R L A P I R D D D G T I T H V I G I Q V F S E                 At_FKF1
D G S P L M N R L R L T P I Y G D D D T I T H I I G I Q F F I E                 At_ZTL
  G     h
```

(b)

```
W K K F T V G G R V E P S R C N F G A C A V G S R L V L F G  AcFKF1
W K K V T V G G A V E P S R C N F S A C A V G N R V V L F G  AcZTL
W R K F T V G G I V Q P S R C N F S A C A V G N R L V L F G  At_FKF1
W R K L S V G G S V E P S R C N F S A C A V G N R V V L F G  At_ZTL
W                                                   h h h G

G E G I D M R P M D D T F V L D L E S P C P E W H R L D V P  AcFKF1
G E G I N M Q P M N D T F V L D L N A S E P E W R H M K V N  AcZTL
G E G V N M Q P L D D T F V L N L D A E C P E W Q R V R V T  At_FKF1
G E G V N M Q P M N D T F V L D L N S D Y P E W Q H V K V S  At_ZTL
G                                   L             W
```

(c)

```
I L Q L S D E V L A H N V L S R L T P R D V A S I G S V C T  AcFKF1
M F Q L S D E V L S H K I I S K L S P R D I A A V G S S C K  AcZTL
I L Q L S D E V L A H N I L S R L T P R D V A S I G S A C R  At_FKF1
L F Q L S D E V V S M K I L S R L T P R D V A S V S S V C R  At_ZTL

R L H E L T R N E H L R R M V C                              AcFKF1
R L Y Q L T K S E I L W K M V C                              AcZTL
R L R Q L T K N E S V R K M V C                              At_FKF1
R L Y V L T K N E D L W R R V C                              At_ZTL
```

(d)

Fig. 6.12: The conserved domains found in *AcFKF1* and *AcZTL*. (a) overall gene structure; (b) the LOV domain; (c) one of the six kelch repeats; (d) the F-Box (shading represents consensus sequence). Coloured residues are highly conserved in LOV domains and kelch repeats, h=hydrophobic (FILMVWY). At=*Arabidopsis thaliana* (adapted from Nelson *et al*., 2000).

6.3.2.3 Phylogenetic analysis of the *FKF1*/*ZTL* gene family

A phylogenetic analysis of *FKF1* and *ZTL* family proteins was carried out (section 6.2.2.2). A NJ tree was constructed (using amino acid sequences) and rooted through *CDF1* (Fig. 6.13). The first point to note is that *AcFBox* forms a clearly separate clade to the *FKF1* and *ZTL* genes, again providing evidence that this gene is a related F-Box protein but not a homologue for *FKF1* or *ZTL*, as discussed in section 6.3.1.4. It forms a cluster with a rice F-Box gene and an *Arabidopsis* F-box gene. The function of this cluster of genes is currently unknown. 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, proving that this analysis is robust. In both cases, the onion putative homologue clusters with monocot gene sequences. The data here support the data presented in Tables 6.2 and 6.3, providing further evidence that genes homologous to *Arabidopsis* flowering time genes have been isolated. The high level of sequence conservation, mirrored by the relationships seen in the phylogenetic tree, also suggests a level of conservation of function. This theory was tested using quantitative RT-PCR to examine the diurnal expression pattern of these 2 genes.

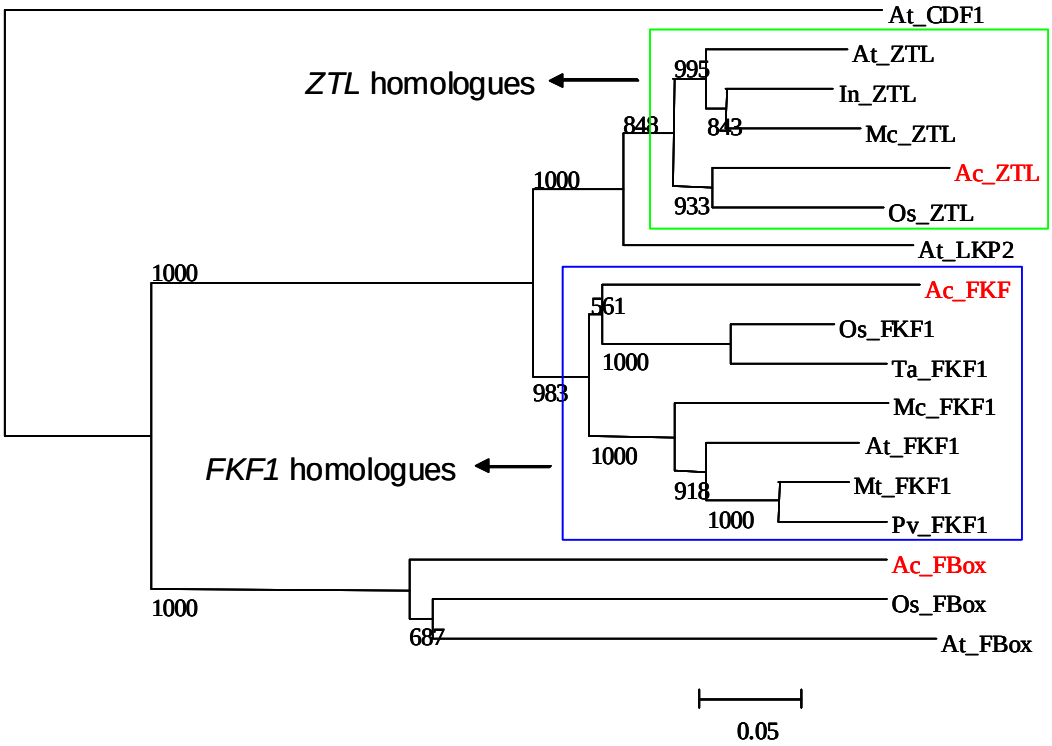


Fig. 6.13: NJ tree showing the evolutionary relationships between members of the *FKF1*/*ZTL* gene family. Numbers represent bootstrap values (1000 replicate). At=*Arabidopsis thaliana*, In=*Impomea nil*, Mc= *Mesembryanthemum crystallinum*, Os=*Oryza sativa*, Ta=*Triticum aestivum*, Mt=*Medicago truncatula*, Pv=*Phaseolus vulgaris*, Ac=*Allium cepa*. Accession numbers can be found in Table A10, Appendix 4.

6.3.3 The expression profiles of AcFKF1 and AcZTL

6.3.3.1 *AcFKF1*

The expression of *AcFKF1* was initially examined using RT-PCR. This gene was shown to be expressed in both the cDNA samples tested (Fig 6.14). Both samples originated from the same RNA stock. The expression level appears to be quite high, but quantitative RT-PCR is required to confirm this. Sequencing confirmed that the primers produced a single PCR product containing only *AcFKF1*.

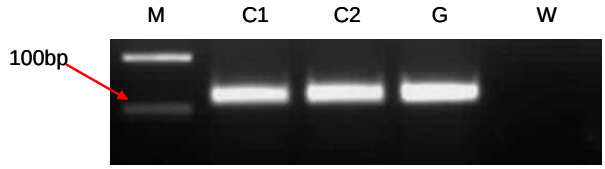


Fig. 6.14: Expression of *AcFKF1* in Renate F_1 onions. M=marker, C1=cDNA sample 1, C2=cDNA sample 2, G=genomic DNA (double haploid), W=water control.

The quantitative expression of *AcFKF1* was initially examined using material from Renate F_1, a LD onion variety (section 6.2.3). The expression was examined at various points over a 24-hour period (Fig. 6.15). It is clear that *AcFKF1* shows a peak of expression at ZT10 in both LD and SD grown plants. This is in slight contrast to *Arabidopsis FKF1* which shows peaks at around ZT10 in LDs and ZT7 in SDs (Imaizumi *et al.*, 2003). It may be that *AcFKF1* shows no difference in expression between SD and LD grown plants. Another possibly explanation is that the peak are occurring at slightly different times in LD and SD grown plants, but the time-points are too far apart to detect these differences. There is a gap of around two and a half hours with no data point between ZT 7.5 and the perceived expression peak at ZT10. It is clearly evident that *AcFKF1* shows a diurnal rhythm of expression, similar to that of *Arabidopsis FKF1*.

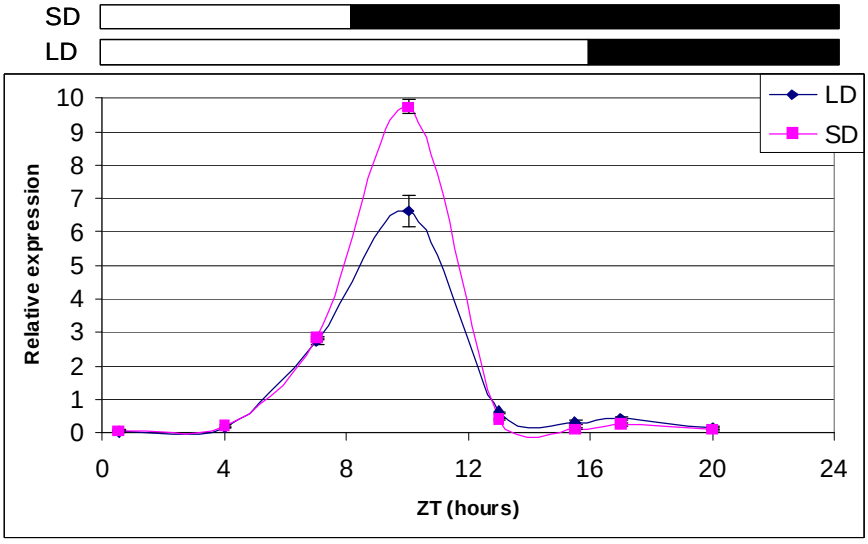


Fig. 6.15: Expression of *AcFKF1* in a LD onion variety (Renate F_1) over a 24-hour period (relative to ß-tubulin). White and black bars denote light/dark cycles. Error bars represent the SEM.

The expression of *AcFKF1* was further investigated, using onion varieties with different daylength responses (section 6.2.3). Under LD conditions, *AcFKF1* is seen to peak around ZT 7-8 in the SD variety (Agrifound Dark, Figs 6.16/6.17) compared with ZT 10 in the LD variety (Renate F1, Fig. 6.15). It would appear that a peak of expression is seen at an intermediate time (around ZT 9) in the ID variety (Candy F_1, Fig. 6.16/6.17). However, the 48-hour data shows a peak which is very similar to the SD variety in the first 24-hour cycle (Fig. 6.16). Therefore, a repeat experiment would be required to confirm the precise timing of the peak in this variety. When calculated as a percentage of the maximum expression, the data from all 3 varieties can be plotted on the same graph (Fig. 6.18). It is clear from this data that there is a distinct difference between the timing of the peak of expression in varieties tested. This shows that the expression of an onion putative photoperiod response gene differs in varieties showing different daylength responses. It is possible that the differential expression of this gene contributes towards the different daylength responses seen in the three varieties tested. The earliest expression peak was seen in the SD variety, a variety which quickly initiates 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 formed.

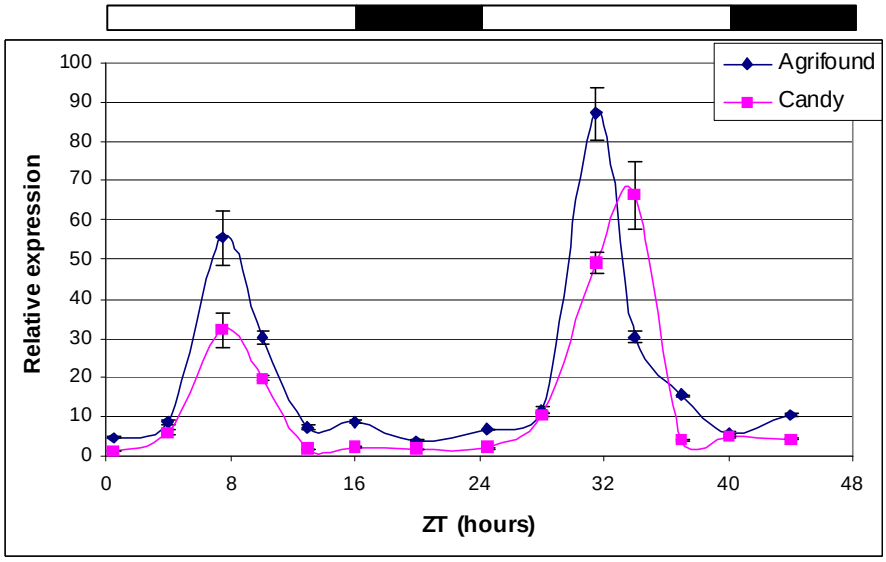


Fig. 6.16: LD expression of *AcFKF1* in an ID (Candy F_1) and a SD (Agrifound Dark) onion variety over a 48-hour period (relative to ß-Tubulin). White and black bars denote light/dark cycles. Error bars represent the SEM.

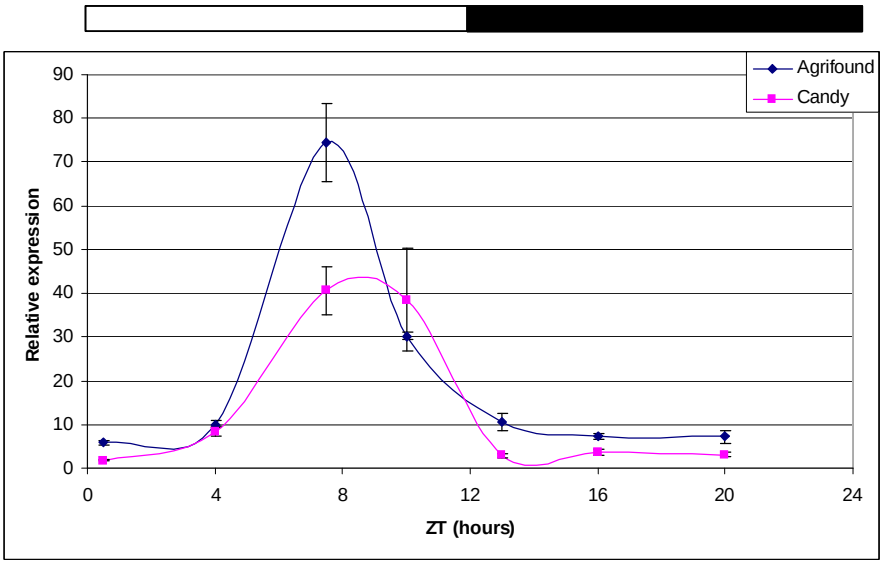


Fig. 6.17: Average LD expression of *AcFKF1* in an ID (Candy F_1) and a SD (Agrifound Dark) onion variety over a 24-hour period (relative to ß-Tubulin). White and black bars denote light/dark cycles.

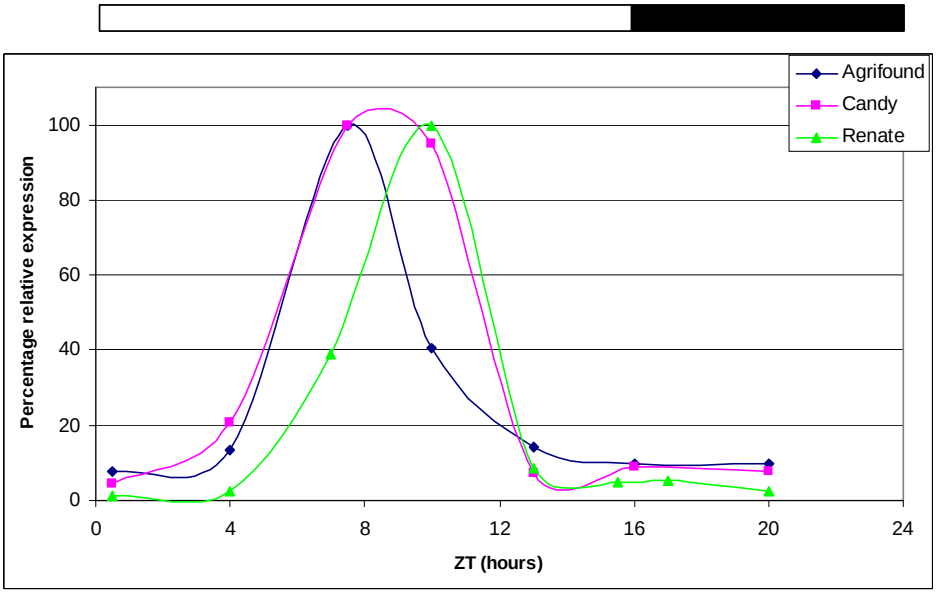


Fig. 6.18: LD expression of *AcFKF1* in a LD (Renate F_1), an ID (Candy F_1) and a SD (Agrifound Dark) onion variety over a 24-hour period (relative to ß-Tubulin). White and black bars denote light/dark cycles

Under SD conditions, the expression of *AcFKF1* is seen to peak around ZT 7-8 in both the SD and ID varieties (Figs. 6.19/6.20). This is in contrast to the peak seen in a LD variety which was around ZT10 (Fig. 6.15). This indicates a difference in expression profiles between varieties, as was observed for LD grown plants. In addition, the peaks of expression of *AcFKF1* in the SD/ID varieties occur during the light period, whereas the peak in the LD variety occurs in the dark period. A peak in the light period may allow *AcFKF1* to function, whereas a peak in the dark period may inhibit function. This would lead to bulb initiation in the SD and ID varieties but not in the LD variety. Crude observations of harvested plants showed that bulbing had been initiated in SD and ID varieties, but not in the LD variety. This is consistent with the expression data, strengthening the hypothesis that *AcFKF1* has a role in bulb initiation.

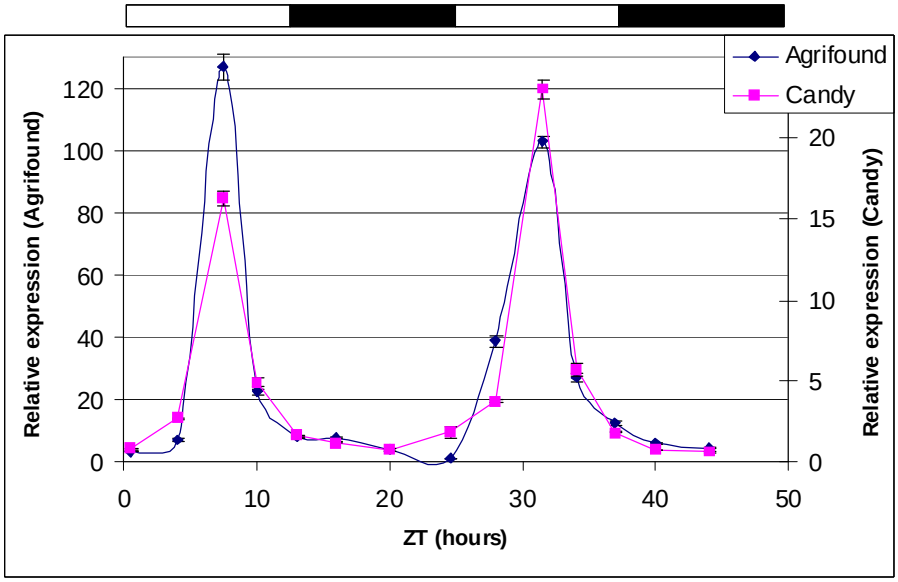


Fig. 6.19: SD expression of *AcFKF1* in an ID (Candy F_1) and a SD (Agrifound Dark) onion variety over a 48-hour period (relative to ß-Tubulin). White and black bars denote light/dark cycles. Error bars represent the SEM.

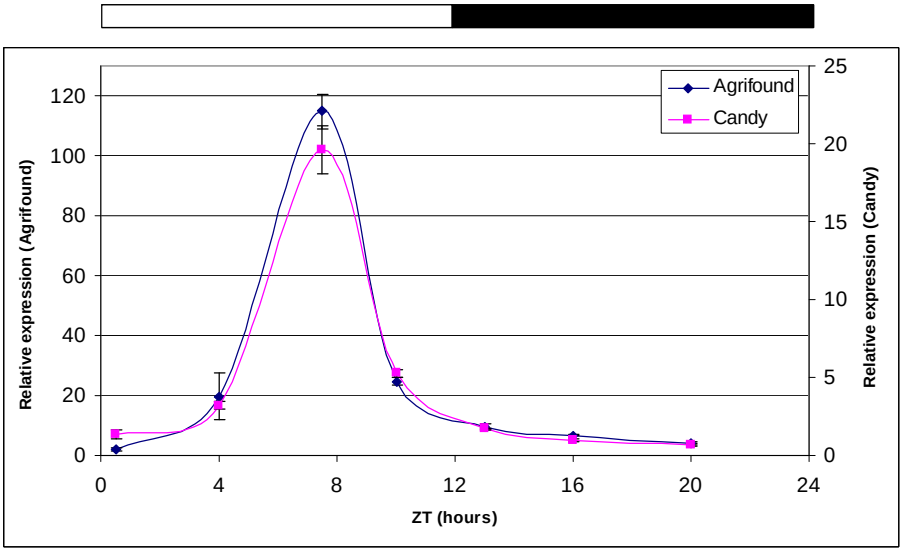


Fig. 6.20: Average SD expression of *AcFKF1* in an ID (Candy F_1) and a SD (Agrifound Dark) onion variety over a 24-hour period (relative to ß-Tubulin). White and black bars denote light/dark cycles. Error bars represent the SEM.

The significance of the differences between the expression of *AcFKF1* in LD and SD conditions as well as between varieties were assessed by fitting a Gaussian curve to the data and carrying out non-linear regression analyses (section 6.2.3). No significant difference between plants grown in LD and SD conditions was observed for Agrifound Dark or Candy F_1 varieties (Table 6.5). A significant difference between LD and SD grown plants was observed for the Renate F_1 variety, despite the fact that the mean values are almost identical (9.27 and 9.29). This is probably due to a larger peak height in SD conditions as the regression analysis takes into account the spread of the curve as well as the mean. In addition, this data originated from only one 24-hour cycle and hence contains less data points which may affect the analysis.

Table 6.5: Significance of the differences between *AcFKF1* expression in SD and LD grown plants and between different onion varieties.

	LD mean	**SD mean**	**P**
Agrifound Dark	7.81	7.04	0.168
Candy F_1	8.80	7.29	0.23
Renate F_1	9.27	9.29	0.012
All varieties LD			0.075
All varieties SD			0.019

The expression differences between varieties were shown to be significant under SD conditions (Table 6.5). The observed differences were not significant under LD conditions (p=0.075). This may reflect the fact that there is a spread of mean values between the three varieties in LDs; whereas in SDs, Agrifound Dark and Candy F_1 plants show a very similar expression level, but very different to that of Renate F_1 plants. More replicates would be required to achieve a significant difference between daylengths. On the whole, the statistical data supports the observations that an onion putative photoperiod response gene is differentially expressed in varieties which show different daylength responses. This leads to the hypothesis that the differential expression of *AcFKF1* contributes towards the different daylength responses seen in the three varieties tested. To assess the wider implications of this hypothesis, a large scale experiment using several different varieties would be of use. This was not possible due to time constraints, but would be a useful future experiment

6.3.3.2 *AcZTL*

The expression of *AcZTL* was initially examined using RT-PCR. Products were obtained from both cDNA and genomic DNA templates (Fig. 6.21). Both products were sequenced to confirm their identity. The expression in cDNA suggests that this is not a pseudogene and is functional in onion. This led into the analysis of *AcZTL* expression by quantitative RT-PCR.

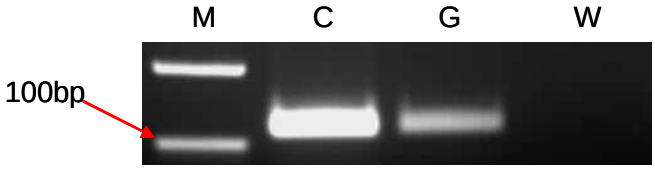


Fig. 6.21: Expression of *AcZTL* in Renate F_1 onions. M=marker, C=cDNA, G=genomic DNA, W=water control.

The expression of *AcZTL* in a SD onion variety (Agrifound Dark) was examined by quantitative real-time PCR (section 6.2.3). It is predicted that the expression of this gene will be similar in all varieties as the mRNA does not show cyclic expression in *Arabidopsis* (Kim *et al.*, 2007). This expression profile shows that *AcZTL* is constitutively expressed in both LD and SD grown plants (Figs.

6.22/6.23). There is no diurnal expression pattern and no obvious expression peaks. The expression is seen to vary slightly in the 48-hour trace (Fig. 6.22). However, when 24-hour averages of expression are calculated, it is clear that there is little fluctuation in expression (Fig. 6.23). This is the case for LD and SD grown plants. This is similar to the expression pattern of *Arabidopsis ZTL* which does not vary greatly through the day. It is in fact the ZTL protein which is seen to oscillate, with a peak at ZT10-13 (Kim *et al.*, 2007). The expression profile, coupled with the phylogenetic data, suggests that *AcZTL* is the onion *ZTL* homologue and a function within the photoperiod pathway is postulated. In *Arabidopsis*, ZTL interacts with GI (Kim *et al.*, 2007). It would be interesting to investigate the possible interaction between AcZTL and AcGI.

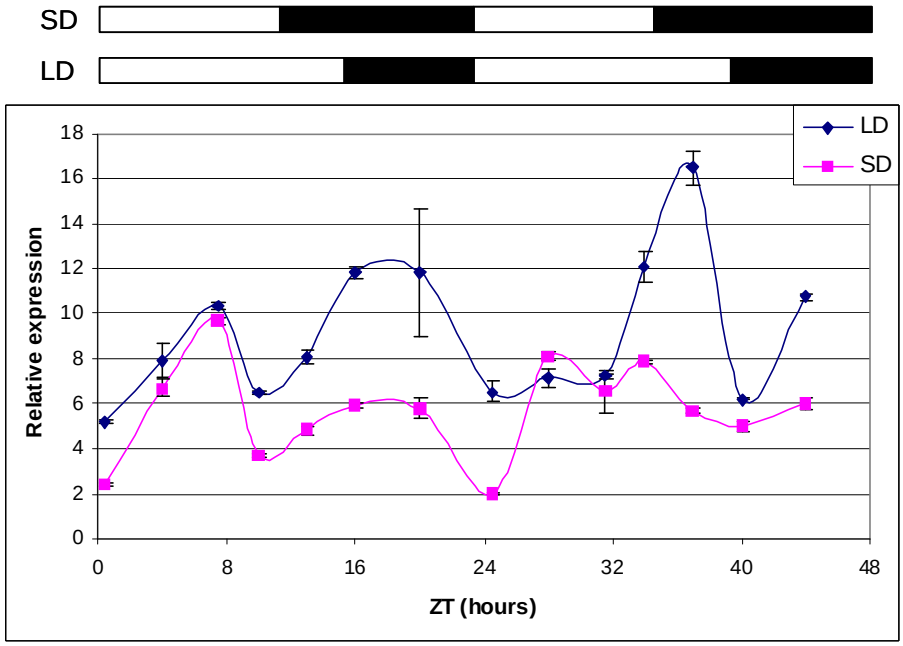


Fig. 6.22: Expression of *AcZTL* in Agrifound Dark onions over a 48-hour period, relative to ß-Tubulin. White and black bars denote light/dark cycles

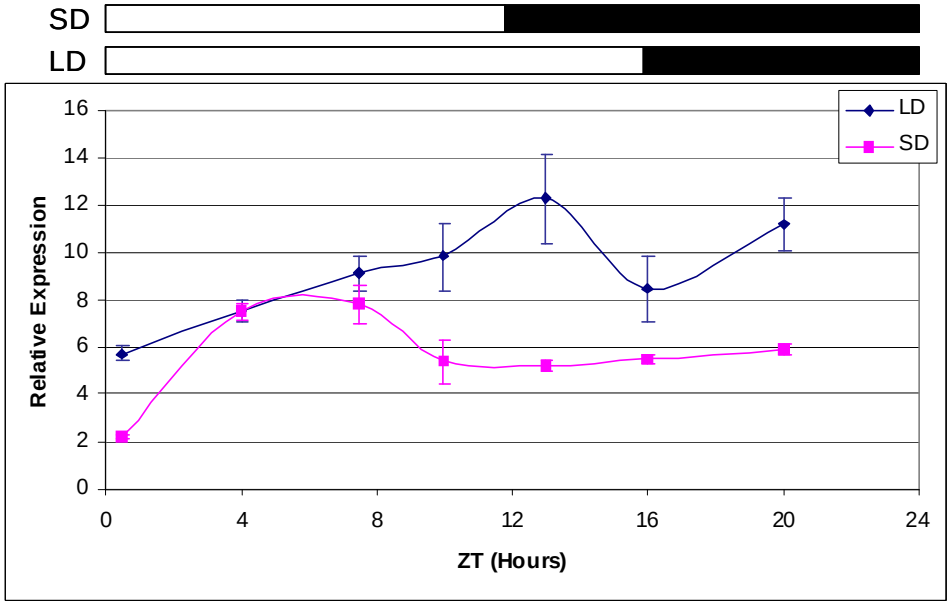


Fig. 6.23: Average expression of *AcZTL* in Agrifound Dark onions over 24 hours, relative to ß-Tubulin. Error bars represent the standard error of the mean (SEM). White and black bars denote light/dark cycles

6.4 Conclusions

The data presented in this chapter indicates that genes homologous to the *Arabidopsis* photoperiod pathway genes *FKF1* and *ZTL* have been isolated from onion. This is the first report detailing the characterisation of an *FKF1* homologue from a species other than *Arabidopsis*. Full-length sequence information was obtained for both genes. Phylogenetic and expression analyses provide further evidence as to the function of these genes and a role in the photoperiodic control of bulb initiation is predicted. Both genes are involved in the photoperiodic regulation of flowering in *Arabidopsis* (Nelson *et al.*, 2000; Mas *et al.*, 2003). Interestingly, both genes form a separate protein complex with GI in order to be functionally active (Mas *et al.*, 2003; Sawa *et al.*, 2007). Thus, it is hypothesised that the system is similar in onion as an onion *GI* homologue has been characterised (described in Chapter 5). An F-Box protein (*AcFBox*) was also characterised. Phylogenetic, expression and transformation data showed that this gene is not an *FKF1* homologue although an influence on flowering time in *Arabidopsis* was observed. The function of this gene is currently unknown.

The expression profile of *AcFKF1* was shown to be different in varieties which show different daylength responses. It is possible that the differences in expression contribute to the different daylength responses observed. However, a large-scale experiment using many more onion varieties and different photoperiods would be required to test this hypothesis. *AcZTL* was shown to be constitutively expressed, a pattern which is similar to *Arabidopsis ZTL* (Kim *et al.*, 2007).

CHAPTER 7: CHARACTERISATION OF OTHER PUTATIVE PHOTOPERIOD RESPONSE GENES

7.1 Introduction

7.1.1 *FLOWERING LOCUS T* (*FT*)

FT promotes flowering in LDs and is described as a floral-pathway integrator gene as it is responsive to signals from several flowering pathways (Jack, 2004; Thomas *et al*., 2006). However, its main function is in the photoperiod pathway where its expression is directly regulated by CO protein (Kobayashi *et al*., 1999). This is discussed in detail in section 1.5.1 and the specific role of *FT* in the photoperiod pathway is illustrated in Fig. 7.1.

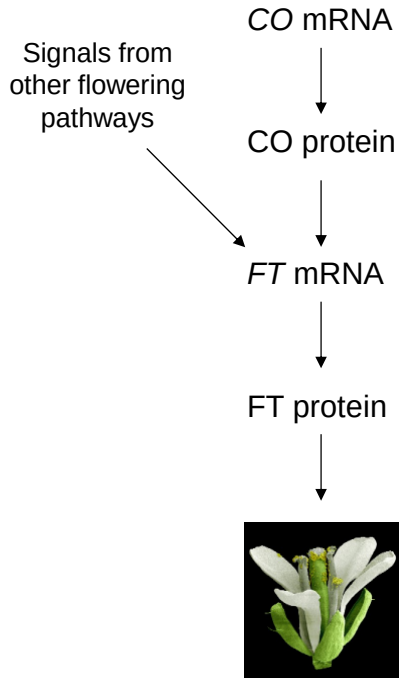


Fig. 7.1: The role of *FT* in the photoperiodic control of flowering in *Arabidopsis* (adapted from Massiah, 2007). *Arabidopsis* flower picture copyright Juergen Berger, Max Planck Institute for Developmental Biology.

The *FT* gene encodes a protein similar to phosphatidylethanolamine-binding protein (PEBP). This is also known as Raf kinase inhibitor protein (RKIP) (Kardailsky *et al*., 1999). There are six RKIP family proteins in *Arabidopsis*; one of

which is *TERMINAL FLOWER 1* (*TFL1*) which is a potent floral repressor, acting in an antagonistic manner to *FT* (Shannon and Meeks-Wagner, 1991; Ahn *et al.*, 2006). Another is *MOTHER OF FT* (*MFT*) which may promote flowering as constitutive expression of this gene has been shown to cause slightly early flowering under long days (Yoo *et al*., 2004). This gene family also contains a gene which shares a high identity with *FT*. This gene is termed *TWIN SISTER OF FT* (*TSF*), and has been shown to act redundantly with *FT* (Yamaguchi *et al*., 2005).

In addition to *Arabidopsis*, the *FT* gene has been implicated in the regulation of flowering in other LD plants such as *Lolium temulentum* (King *et al*., 2006). A more diverse role for *FT* has been shown by the regulation of flowering in SD plants. The rice *FT* orthologue, known as *heading-date 3a* (*Hd3a*), plays an important role in the promotion of flowering in SDs (Kojima *et al*., 2002). A recent study showed that *Hd3a* and *RICE FLOWERING LOCUS T 1* (*RFT1*) are essential for flowering in rice (Komiya *et al*., 2008). Hd3a is the major contributor to flowering in SDs as *RFT1* RNAi mutants show no delay in flowering time. *RFT1*/*Hd3a* double mutants do not flower at all, but *Hd3a* mutants will still flower under SDs (around 30 days later than wild-type). Flowering in the absence of *Hd3a* can be attributed to an increase in *RFT1* expression, indicating that both *Hd3a* and *RFT1* are major floral activators in rice. *FT* has also been implicated in the regulation of flowering in other SD plants such as Pharbitis, Cucurbits and Norway Spruce (Gyllenstrand *et al.*, 2007; Hayama *et al.*, 2007; Lin *et al.*, 2007). Interestingly, the tomato *FT* orthologue (known as *SINGLE-FLOWER TRUSS*) induces flowering in day-neutral tomato and tobacco plants (Lifschitz *et al*., 2006).

There has been some debate recently regarding claims that *FT* may be the elusive 'florigen' which has been discussed since the 1930's. It has been known for some time that a signal is transmitted through the phloem, from leaves to meristem, leading to flowering. A recent study seemed to have elucidated that *FT* mRNA was the mobile signal (Böhlenius *et al*., 2007). However, this paper was retracted and the current theory is that FT protein is the mobile signal. This is supported by data from both *Arabidopsis* and rice (Corbesier *et al*., 2007; Jaeger and Wigge, 2007; Tamaki *et al*., 2007). It has been shown that the export of FT protein from phloem companion cells induces flowering in *Arabidopsis*, further supporting the theory that FT protein is a component of 'florigen' (Mathieu *et al.*, 2007). The theory of florigen requires that a component of the flowering pathway is conserved in all plants

(Zeevaart, 1976). The presence of an active *FT* gene in LD, SD and day-neutral plants, as well as evidence that the signal is transmissible, suggests the FT protein could be the long sought after florigen. One question which remains is whether FT is the only component of florigen or whether more exist. The current evidence suggests that FT is a major component of the illusive florigen (Zeevaart, 2008).

7.1.2 Photoreceptors involved in the photoperiodic control of flowering

In *Arabidopsis*, there are nine photosensory receptors. Among these receptors, the phytochromes (*PHYA-E*) and cryptochromes (*CRY1* & *CRY2*) regulate flowering time (Mockler *et al*., 2003). The role of these genes is illustrated in Fig. 7.2. *PHYA, PHYB, PHYD, PHYE, CRY1* and *CRY2* are all involved in mediating light input to the circadian clock (Somers *et al.*, 1998; Devlin and Kay, 2000). Perception of low irradiance red and blue light is controlled by *PHYA*. Light input to the clock controls the expression of *LHY* and *CCA1* in the early part of the day (Martínez-García *et al.*, 2000; Kim *et al.*, 2003a). *PHYA* is required for the perception of LDs in *Arabidopsis* and acts through the photoperiod pathway as it has no effect on flowering in SDs (Johnson *et al.*, 1994). It has been shown that *CRY2* expression changes in response to photoperiod and *CRY2* mutants show a greatly reduced photoperiod response (El-Assal *et al*., 2003). This indicates that *CRY2* is the major blue light photoreceptor affecting flowering. CRY1, CRY2 and PHYA all act to stabilise the CO protein towards the end of a LD (Valverde *et al.*, 2004). This provides a mechanism for the external coincidence model of daylength regulation in *Arabidopsis* (Thomas, 2006).

A recent study indicates a role for *CRY2* in the regulation of *FT* expression in vascular bundles, probably through stabilisation of the CO protein (Endo *et al*., 2007). It has also been shown that the control of photoperiodic flowering by *CRY2* (and *CRY1*) requires a functional copy of the *TERMINAL FLOWER 1* (*TFL1*) gene (Buchovsky *et al.*, 2008). In *tfl1* mutant plants, the *CRY* genes promoted flowering under SDs as well as LDs (Buchovsky *et al.*, 2008). *TFL1* belongs to the same gene family as *FT*, and acts as a floral repressor (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). *CRY2* has been shown to enhance *FT* expression in LDs (Yanovsky and Kay, 2002). However, *CRY2* also enhances *FT* expression in SDs although this does not lead to floral induction (Buchovsky *et al.*, 2008). It is proposed that *TFL1* activity

sets a threshold of *FT* activity necessary to induce flowering and that this level is not reached under SD conditions.

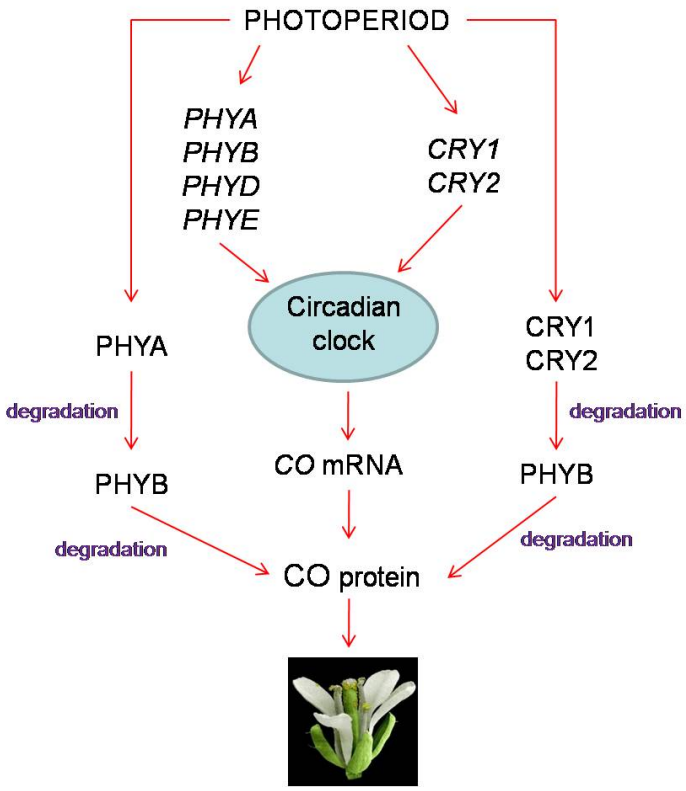


Fig. 7.2: The role of *PHY* and *CRY* genes in the photoperiodic control of flowering in *Arabidopsis* (adapted from Massiah, 2007). *Arabidopsis* flower picture copyright Juergen Berger, Max Planck Institute for Developmental Biology.

7.1.3 Genes Involved in the circadian clock

The circadian clock regulates flowering through an output pathway involving *CO* (discussed in section 1.5.2). Two components of the clock are the partially redundant genes *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*, Niwa *et al*., 2007). The function of these genes is controlled by an upstream activator, *TIMING OF CAB EXPRESSION 1* (*TOC1*). It has been shown recently, through mutant studies, that the circadian clock system is closely linked to the output pathway involving *CO* (Mizoguchi *et al*., 2005). It is suggested that the circadian clock may open an exit for the output pathway involving *CO* during the day time. Thus, it would appear that *LHY* and *CCA1* are important genes in photoperiodic control of flowering time.

This chapter describes some preliminary work carried out on the characterisation of onion genes showing homology with additional genes involved in the photoperiodic control of flowering. The genes described in this chapter have not been characterised to the extent of the other genes mentioned in chapters 4-6. The starting point was EST's showing homology to *FT*, *PHYA*, *CRY2* and *LHY*.

7.2 Materials and Methods

7.2.1 Characterisation of an FT-like gene

7.2.1.1 Expression analysis

The *A.cepa* Gene Index contains an EST which shows sequence similarity with *Arabidopsis FT*. This gene was designated the name *AcFTL* (for *A.cepa FT*-like). The expression of *AcFTL* was analysed by RT-PCR using the primers EE96 RT FOR and EE96 RT REV2 (see Appendix 2 for sequences) with an annealing temperature of 61 ºC and a cycle number of 30. Products were purified and sequenced using the same primers, as described in sections 2.2.6 & 2.2.8. Quantitative real-time PCR was used to assess the expression pattern of *AcFTL* in the leaves of a SD and an ID onion variety (as described in section 2.3.4). Plants were grown in LD (16 hours of light) and SD (12 hours of light) conditions. The primers used were FTL-RT FOR and FTL-RT REV (see Appendix 2 for sequences) at a concentration of 0.2 µM. All cDNA samples were used undiluted and run in triplicate. Data were normalised to the expression of ß-tubulin and standard errors calculated. A selection of PCR products were purified and sequenced using both forward and reverse primers.

The expression of *AcFTL* was also examined in the leaves of plants which had bolted (see section 2.3.5). The primers used were EE96 FOR and EE96 REV1 (see Appendix 2 for sequences), with an annealing temperature of 61 ºC and a cycle number of 30. Products were purified and sequenced using both forward and reverse primers.

7.2.1.2 Phylogenetic analysis

Sequences of *FT* and related genes from plant species were collated in FASTA format within Notepad (NCBI, 2008). Sequences of the genes *TSF*, *MOTHER OF FT (MFT)* and *BROTHER OF FT (BFT)* were included in this analysis. Alignments were carried out and phylogenetic trees constructed as described in section 2.3.7. A NJ-tree was constructed using predicted amino acid sequences. This analysis was based on PEBP domain sequences. The tree was rooted through *MFT*.

7.2.2 Searching for other FT-like genes in onion

Degenerate primers were designed to amplify additional members of the *FT*-like gene family in onion. A forward primer (FT-like FOR) containing 25% degeneracy and a reverse primer (FT-like REV) containing 21% degeneracy were designed (see Appendix 2 for sequences). RT-PCR was carried out with an annealing temperature of 55 ºC and a cycle number of 35. Products of the expected size (185 bp) were purified as described in section 2.2.6. A second round of PCR was carried out to re-amplify the products, using the same conditions and the purified product as a template. Products of this PCR were purified. PCR products (both original and re-amplified) were cloned into a pMOS*Blue* vector, following the manufacturer's guidelines (Amersham Biosciences). Electroporation was the method of transformation used (see section 2.2.9). Colony PCR was carried out on 20 colonies using FT-like FOR & REV primers with an annealing temperature of 55 ºC and a cycle number of 30 (as described in section 2.2.1). Products were purified and sequenced (using T7 and U-19 primers) as described in sections 2.2.6 & 2.2.8. Double-stranded sequence was obtained for all positive PCR products and alignments carried out using the MegAlign package of DNAStar (DNAStar Inc.).

7.2.3 Characterisation of onion homologues of photoreceptor genes

7.2.3.1 *AcPHYA*

An EST in the *A.cepa* Gene Index shows sequence similarity with *Arabidopsis PHYA*. This gene was designated the name *AcPHYA* (*Allium cepa PHYTOCHROME A*). The clone containing *AcPHYA* was obtained (Prof. Mike Havey, USDA, University of Wisconsin) and sequenced using M13 forward and reverse primers, as described in section 2.2.8. A contig was constructed using the SeqMan package of DNAStar (DNAStar Inc.) to cover the entire sequence of the clone. Sequences of *PHYA* homologues (along with *Arabidopsis PHYB-E* genes) were gathered and stored in FASTA format in a Notepad file. Alignments were carried out and phylogenetic trees constructed using amino acid sequences, as described in section 2.3.7. The tree was rooted through a clade containing *PHYB-E* genes.

7.2.3.2 An onion putative *CRY2* homologue

A further gene in the *A.cepa* Gene Index showed sequence similarity with *Arabidopsis CRY2* (accession number ES449821). Sequences of *CRY2* homologues (along with *Arabidopsis CRY1*) were gathered and stored using the EditSeq package of DNAStar (DNAStar Inc.). Alignments based on nucleotide sequences were carried out using the MegAlign package of DNAStar (DNAStar Inc.) and percentage identities calculated.

7.*2.4 Onion Putative LHY Homologues*

A number of EST's in the *A.cepa* Gene Index also show significant sequence similarity with *Arabidopsis LHY* (DFCI, 2008). Alignments were carried and percentage identities calculated out using the MegAlign package of DNAStar (DNAStar Inc.). The EST sequences were aligned with *Arabidopsis LHY* (accession number NM_179237) and *CCA1* (accession number NM_180129).

7.3 Results and Discussion

7.3.1 Characterisation of AcFTL

7.3.1.1 Expression analyses

RT-PCR using *AcFTL*-specific primers (section 7.2.1.1) resulted in a 150 bp product from cDNA (Fig. 7.3). Sequencing confirmed that this product was amplified from the *AcFTL* gene. A weak PCR product was observed with the cDNA sample tested, suggesting that this gene is expressed at quite low levels. The cDNA sample originated from LD onions in which bulbing had been initiated, showing that this gene is expressed at this stage of development. A PCR product was also observed when genomic DNA was used as a template. This product was much larger than the cDNA product and shows the presence of a 250 bp intron in this part of the *AcFTL* gene. This product was also verified by direct sequencing.

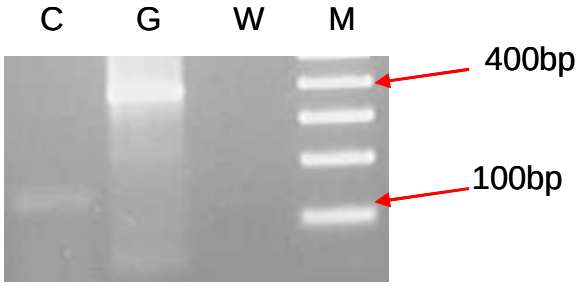


Fig. 7.3: Amplification of PCR products from *AcFTL* using onion cDNA (C) or genomic DNA (G) as a template. W=water control, M=marker.

It was also shown that *AcFTL* is expressed in onions which had bolted (Fig. 7.4). This shows that this gene is active in both flowering and bulbing onions and allows for the possibility of a role in onion flowering. Expression was higher at ZT5 and ZT12.5 than ZT9, suggesting some variation over a 24-hour period.

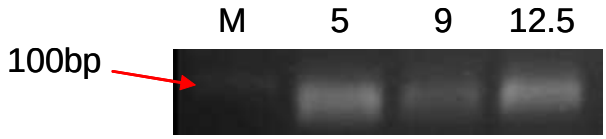


Fig. 7.4: Expression of *AcFTL* in the leaves of onions which have bolted. Numbers represent zeitgeber time, M=marker.

The expression of *AcFTL* was further investigated using quantitative real-time PCR. The expression in a SD onion variety (Agrifound Dark) shows peaks during the light period in both LD and SD grown plants (Fig. 7.5). The precise timing of the peaks was not consistent between the first and second 24 hours suggesting that the gene is not under strict circadian or daylength control. This is in contrast to *Arabidopsis FT*, which shows one distinct peak at the end of a LD and is expressed at very low levels in SDs (Kardailsky *et al*., 1999). This suggests that *AcFTL* is not the onion *FT* homologue. There is a potential daytime peak of expression in both LD and SD grown plants. This is only evident when average twenty-four hour values are calculated and is associated with very large standard errors (Figure A1, Appendix 3).

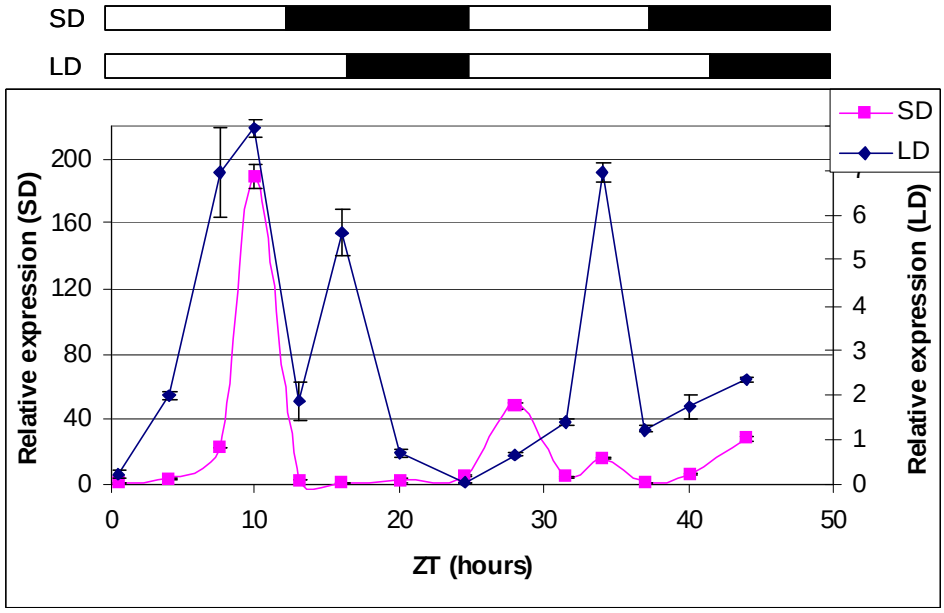


Fig. 7.5: Expression of *AcFTL* in a SD onion variety (Agrifound Dark), relative to ß-tubulin. The expression was calculated as a percentage of the maximum level. White and black bars denote light/dark cycles

The expression of *AcFTL* was also investigated in an ID onion variety (Candy F_1). It was found that this gene shows peaks of expression during the light period in LD- and SD-grown plants, but an additional peak is seen in the dark period for SD-grown plants (Fig. 7.6). Once again, the precise timing of the expression peaks is not consistent between the two days and 24-hour averages are associated with large standard errors (Fig. A6, Appendix 3). The evidence here suggests that *AcFTL* is not the onion *FT* homologue, but is a member of a family of *FT*-like genes such as those that been described in rice and barley (Izawa *et al.*, 2002; Faure *et al.*, 2007).

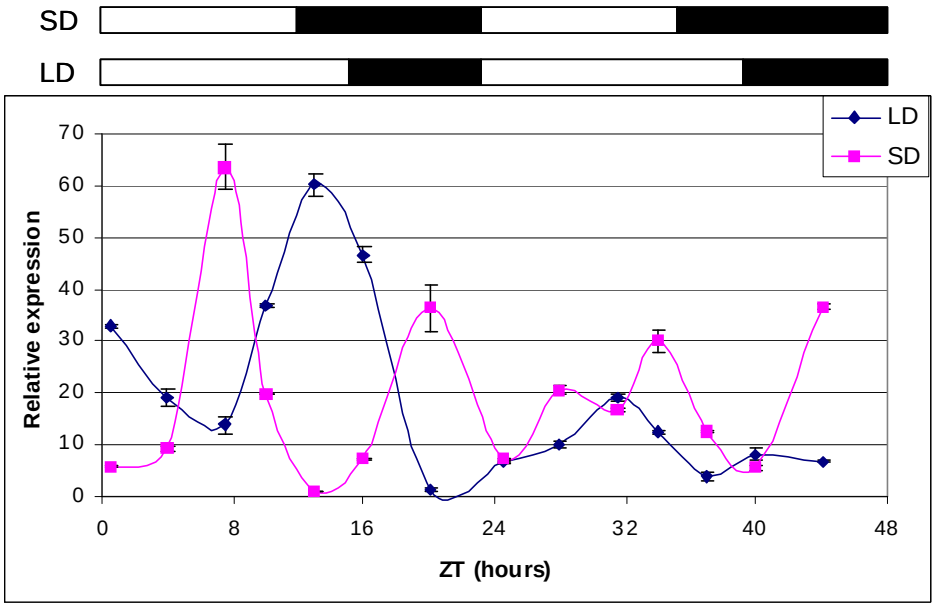


Fig. 7.6: Expression of *AcFTL* in an ID onion variety (Candy F_1), relative to ß-tubulin. White and black bars denote light/dark cycles. Error bars represent the SEM.

7.3.1.2 Phylogenetic analysis of *FT* and *FT*-like genes

In order to further investigate the identity of *AcFTL*, a phylogenetic analysis of *FT* and *FT*-like genes from various plant species was carried out (7.2.1.2). The resulting phylogenetic tree produced three major groups of *FT* and *FT*-like genes (Fig. 7.7). This is in agreement with a previous analysis of this gene family, which was also based around the PEBP domain (Faure *et al.*, 2007). Genes which are orthologous to *Arabidopsis FT* cluster exclusively in Group 1. This includes rice

Hd3a and putative orthologues in lettuce and *Antirrhinum*. *AcFTL* forms part of Group 3 in this analysis, its closest relative being rice *FT*-like 4 (OsFTL4). Data has not been published on the diurnal expression pattern of this gene but it is not expressed in early development when *FT* is seen to be expressed. The function of *AcFTL* is currently unclear, but it is clearly not an orthologue of *FT*.

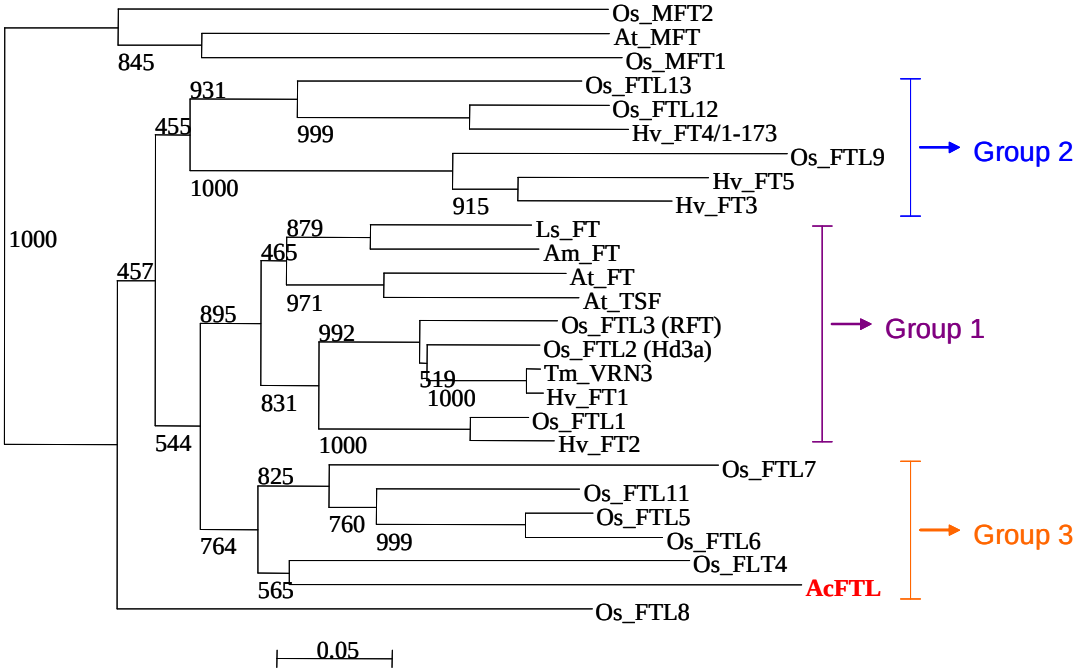


Fig. 7.7: NJ tree showing the evolutionary relationships within the *FT*-like gene family. Numbers represent bootstrap values from 1000 replicates. Os=*Oryza sativa*, At=*Arabidopsis thaliana*, Hv=*Hordeum vulgare*, Ls=*Lactuca sativa*, Am=*Antirrhinum majus*, Tm=*Triticum monoccum*. Accession numbers can be found in Table A11, Appendix 4.

7.3.2 Searching for other FT-like genes

Degenerate PCR (section 7.2.2) resulted in a mixed PCR product which was shown to contain many different onion gene fragments (Fig. 7.8). Analysis of the sequences obtained from 20 different clones revealed 3 different genes of interest (Fig. 7.9). Firstly, *AcFTL* was detected in 2 of the clones. This was expected as the primers were designed to pick up this gene and other family members. A second gene was found in 5 different clones (named *AcFTL2*) and a third gene in six

different clones (named *AcFTL3*). The remaining clones contained genes which were not members of the *FT*-like gene family.

Double-stranded sequence was obtained for *AcFTL2* and *AcFTL3*. Approximately 150 bp of sequence was obtained for each clone. This was used to compare both genes (along with *AcFTL*) with members of the rice *FT*-like gene family. It was found that *AcFTL* showed the highest percentage identity with *OsFTL4* (Table 7.1). This confirms the observations from the phylogenetic analysis (Fig. 7.7). It was shown that *AcFTL2* shows the highest identity with *OsFTL5* and *OsFTL6*. The roles of these genes in rice are currently unknown. According to the grouping of *FT*-like genes, *AcFTL2* is likely to be a Group 3 gene (Faure *et al.*, 2007) & Fig. 7.7). The onion gene *AcFTL3* shows the highest identity with *OsFTL11*. This suggests *AcFTL3* is also a group 3 gene. The role of *OsFTL11* in rice is also unknown at the current time.

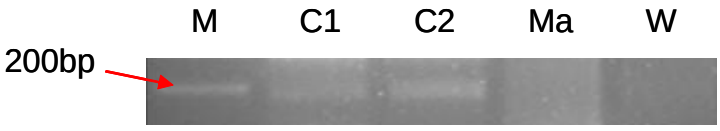


Fig. 7.8: Amplification of PCR products containing *FT*-like gene fragments. M=marker, C1/C2=onion cDNA samples, Ma=maize genomic DNA, W=water control.

Preliminary evidence suggests that there is a large family of *FT*-like genes in onion, as is reported for rice and barley (Izawa *et al*., 2002; Faure *et al*., 2007). As is the case for the *CO*-like genes, very few of these genes have been characterised. This makes it difficult to assign a function to the genes isolated here without detailed characterisation. This would be a useful future experiment and should include the isolation of further members of this gene family in onion. At the current time, an onion *Hd3a* (*FT*) homologue has not been isolated.

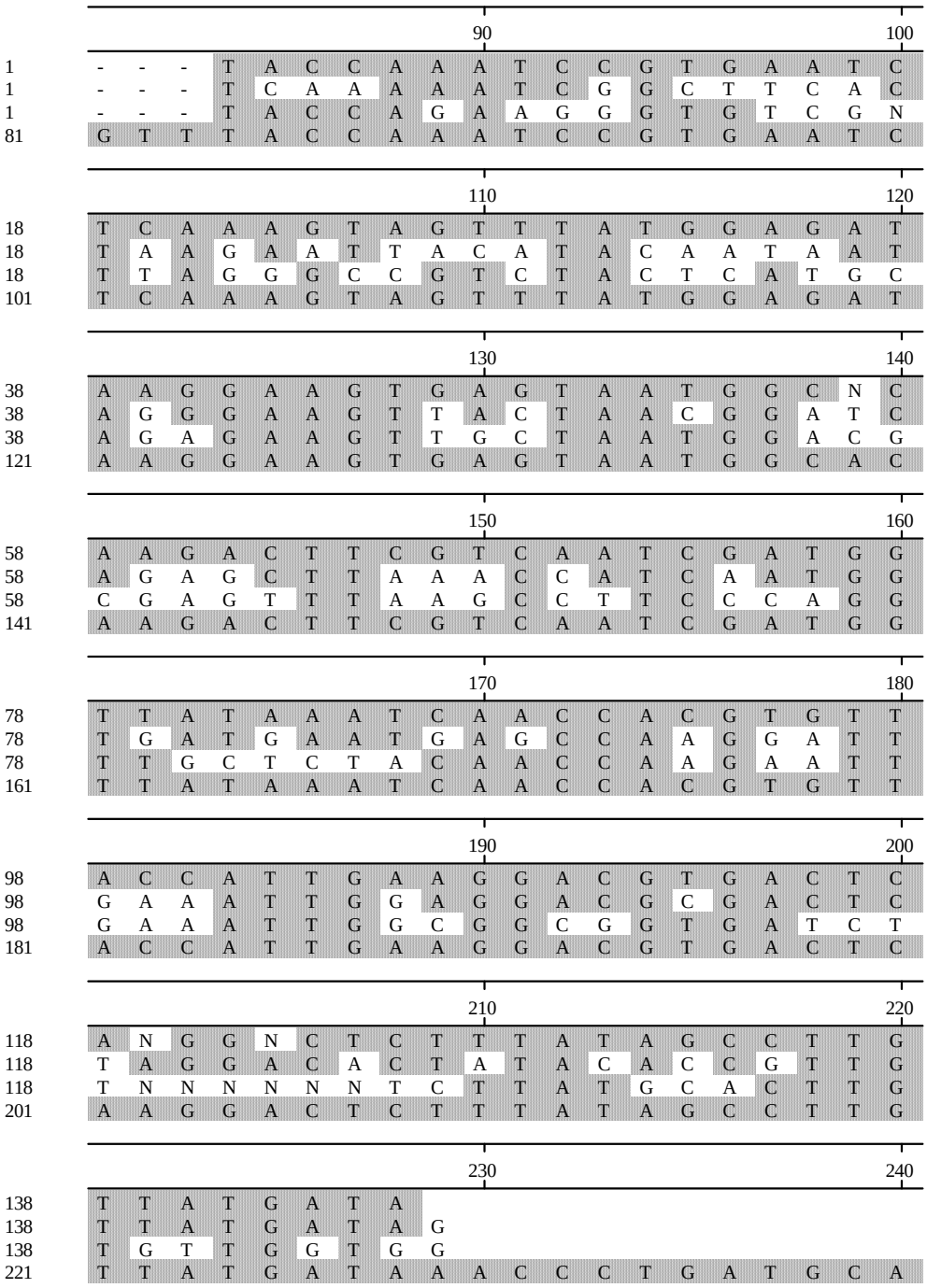


Fig. 7.9: Alignment of *FT*-like genes isolated using degenerate PCR. Shading represents bases which match the consensus sequence. *AcFTL1* was shown to be identical to *AcFTL*. The alignment was carried out using the MegAlign package of DNAStar (DNAStar Inc.).

Table 7.1: Comparing onion and rice *FT*-like genes in terms of percentage nucleotide identity.

Gene	Locus number	*AcFTL*	*AcFTL2*	*AcFTL3*
FTL1	Os01g11940	54.1	41.1	52.7
FTL2 (*Hd3a*)	Os01g06320	56.1	45.9	42.5
FTL3 (*RFT*)	Os06g06300	57.8	45.9	41.8
FTL4	Os08g04780	64.3	59.6	48.6
FTL5	Os0239064	63.5	70.5	47.9
FTL6	Os04g41130	62.2	70.5	54.1
FTL7	Os12g13030	39.8	54.1	31.5
FTL8	Os01g10590	26.3	8.2	8.9
FTL9	Os01g54490	37.0	57.5	25.3
FTL10	Os05g41180	51.7	54.1	36.3
FTL11	Os11g11870	61.3	64.4	54.8
FTL12	Os01g35940	48.9	26.7	28.1
FTL13	Os0213830	54.3	8.9	21.2
MFT1	Os01g02120	25.2	15.8	13.0
MFT2	Os06g30370	45.4	14.4	8.2

7.3.3 Characterisation of onion homologues of photoreceptor genes

7.3.3.1 *AcPHYA*

Sequencing analysis revealed that the clone of *AcPHYA* was not full-length (described in section 7.2.3). The insert covers 373 amino acids of *AcPHYA*, an estimated 33% of the gene length (estimated from *Arabidopsis PHYA*). Percentage identities reveal that *AcPHYA* shares 54-57% amino acid identity and 59-65% nucleotide identity with other *PHYA* homologues (Table 7.2). This high level of identity suggests a conservation of function. The phylogenetic tree, which was constructed using *PHYA* sequences from various plant species, shows a separation between *PHYA* homologues and the *PHYB-E* genes (Fig. 7.10). Within the *PHYA* specific clade there is a clear split between monocot and dicot *PHYA* homologues. It is clear that *AcPHYA* is present in the monocot *PHYA* sub-clade of the tree, providing further evidence that this gene is indeed the onion *PHYA* homologue. It is hypothesised that this gene is involved in light input to the pathway controlling photoperiodic bulb initiation in onion. This is the case in *Arabidopsis* where *PHYA* is thought to be a sensor for light input to the photoperiod pathway (Lin, 2000). In

onion, far-red light is essential for bulb initiation (Lercari, 1982). In *Arabidopsis*, *PHYA* mediates the response to far-red light, suggesting a role for *AcPHYA* in mediating bulb initiation in response to far-red light (Thomas, 2006). Further analysis of *AcPHYA* is required to elucidate the exact function of this gene.

Table 7.2: Comparing *AcPHYA* with other plant *PHYA* homologues. All genes included are *PHYA* homologues with the exception of *Arabidopsis PHYB*.

Gene	**Accession Number**	**Percentage Identity (nucleotide)**	**Percentage Identity (amino acid)**
Arabidopsis (PHYA)	NM_001123784	61.2	55.8
Arabidopsis (PHYB)	AT2G18790	54.5	40.2
S.bicolor	SBU56729	64.5	55.0
S.lycospersicum	AJ001914	62.5	55.0
S.tuberosum	DQ208423	62.5	54.4
O.sativa	AB109891	61.9	56.3
H.vulgare	DQ201158	61.1	57.4
G.max	EU428746	61.2	55.8
P.sativum	AY688953	59.1	54.2

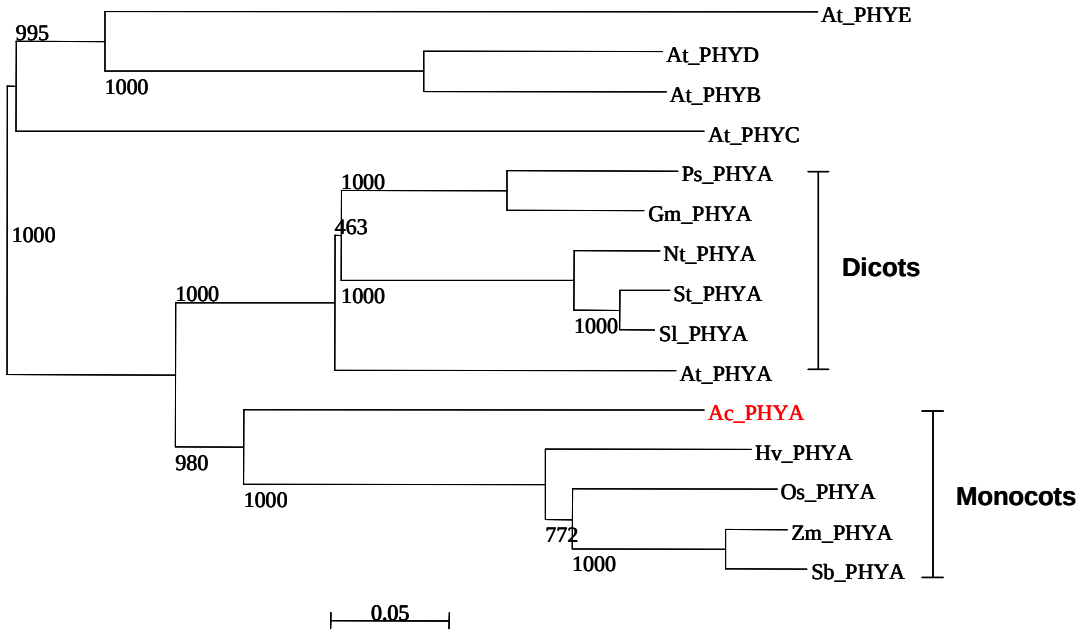


Fig. 7.10: NJ-tree showing the evolutionary relationships between various *PHYA* homologues. The tree is rooted through *PHYB-E* genes. Numbers represent bootstrap values from 1000 bootstrap replicates. At=*Arabidopsis thaliana*, Ps=*Pisum sativum*, Gm=*Glycine max*, Nt=*Nicotiana tabacum*, St=*Solanum tuberosum*, Sl=*Solanum lycopersicum*, Hv=*Hordeum vulgare*, Os=*Oryza sativa*, Zm=*Zea mays*, Sb=*Sorghum bicolor*. Accession numbers can be found in Table A12, Appendix 4.

7.3.3.2 An onion putative *CRY2* homologue

The onion putative *CRY2* (section 7.2.3.2) homologue shares 34-37% nucleotide identity with other *CRY2* genes (Table 7.3). This EST also shares 24% identity with *Arabidopsis CRY1*. Although the percentage identities are lower than was seen for some of the previously mentioned onion EST's, *CRY2* is the closest relative of this gene (NCBI, 2008). Unfortunately, this gene does not translate to a protein. Translation is interrupted by several stop codons. This may be due to a sequencing error causing a frame-shift. This EST only covers an estimated 24% of the gene sequence so further characterisation would be required to thoroughly characterise this gene. The discovery of this gene leads to the hypothesis that a similar light input pathway controls both photoperiodic flowering and bulb initiation. Further work would be required to test this hypothesis.

Table 7.3: Comparing an onion putative *CRY2* homologue with *CRY2* genes from different plant species in terms of percentage nucleotide identity.

Gene	Accession Number	Percentage Identity (nucleotide)
Arabidopsis CRY2	NM_100320	34.9
Arabidopsis CRY1	NM_116961	24.4
B.napus CRY2	AJ889779	35.6
P.sativum CRY2	AY161312	36.7
O.sativa CRY2	AB103094	34.9
T.aestivum CRY2	EF601542	33.6

7.3.4 Genes involved in the circadian clock

The *A.cepa* gene index also contains several EST's which show sequence similarity with *LHY*/*CCA1*. The *Arabidopsis LHY* and *CCA1* genes are highly homologous and partially redundant in function (Niwa *et al.*, 2007). One particular EST (contig number TC4733) shares 25.9% nucleotide identity with both *Arabidopsis LHY* and *CCA1*. This translates to 30.5% and 32.4% amino acid identity with *Arabidopsis LHY* and *CCA1* respectively. This opens up the possibility that the method by which the circadian clock controls photoperiodic bulb initiation is conserved.

It was found that the *A.cepa* Gene Index also contains EST's which show sequence similarity with two recently characterised photoperiod pathway genes, *CONSTITUTIVE PHOTOMORPHOGENIC* (*COP1*) and *EARLY FLOWERING 3* (*ELF3*). The characterisation of these EST's is discussed in section 8.1.

7.4 Conclusions

This chapter details the preliminary characterisation of additional onion putative photoperiod response genes. Three *FT*-like genes were isolated and all appear to be Group 3 *FT*-like genes. The function of these genes is currently unknown. The expression profile of *AcFTL* shows no diurnal or circadian pattern, suggesting it is not an orthologue of *Arabidopsis FT*. Preliminary evidence suggests that there is a large family of *FT*-like genes in onion, as is reported in other monocots

such as rice (Izawa *et al.*, 2002). Potential homologues of photoreceptor genes involved in the photoperiodic control of flowering were also examined. One particular gene (*AcPHYA*) shows high percentage identities with *PHYA* homologues from other plant species, and a role in mediating bulb initiation in response to far-red light is predicted. A putative homologue of *CRY2* was also found. In addition, putative homologues of the *Arabidopsis* circadian clock related genes *LHY*/*CCA1* were examined. It would appear that many of the genes controlling the photoperiod response in *Arabidopsis* are conserved in onion. It remains to be seen whether these genes are involved in bulbing, flowering or both.

CHAPTER 8: GENERAL DISCUSSION

8.1 Conservation of the Photoperiod Pathway

The genetic network controlling photoperiodic flowering in *Arabidopsis* is proposed to be conserved across plant species. Many studies have shown the conservation of several photoperiod pathway genes across species including legumes, *Brassicas,* rice, potato and wheat (Robert *et al.*, 1998; Kojima *et al.*, 2002; Martínez-García *et al.*, 2002; Hecht *et al.*, 2005; Miller *et al.*, 2008). In the case of potato, the photoperiod genes are proposed to control a different end process, namely tuberisation. This provided the hypothesis that the same genes also control bulb initiation in onion. This would appear to be the case as homologues of *GI*, *FKF1* and *ZTL* were isolated in this project, as well as genes closely related to other photoperiod pathway genes.

This theory is supported by the expression pattern of *AcGI* which is seen to be different in inductive (LD) conditions and non-inductive (SD) conditions, leading to bulb initiation only in LDs (Figs. 5.3 and 5.4, section 5.3.1.2). This is similar to the expression pattern seen in *Arabidopsis GI*, a pattern which allows for *CO* expression to peak towards the end of a LD (Fowler *et al.*, 1999). However, *AcGI* shows the same expression pattern in ID and SD onion varieties. This provided the theory that if the difference in daylength response is due to a change in the photoperiod pathway, this change occurs downstream of *AcGI.* The expression of *AcFKF1* was seen to vary in varieties showing different daylength responses. This suggests that a change in *AcFKF1* expression leads to a different daylength response.

An interesting point to note is that 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 5.2, section 5.3.2.2/Table 6.2, section 6.3.3.2). Therefore it is feasible that the same interactions occur in onion. In a LD onion variety (Renate F_1) it was shown that the expression profiles of *AcGI* and *AcFKF1* are very similar in LD conditions (Fig. 8.1). The expression of both genes is seen to peak around ZT10. This suggests that the two genes 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 (Fig. 8.2). 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* 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 explains, at least partly, why this variety does not initiate bulbing under SD conditions.

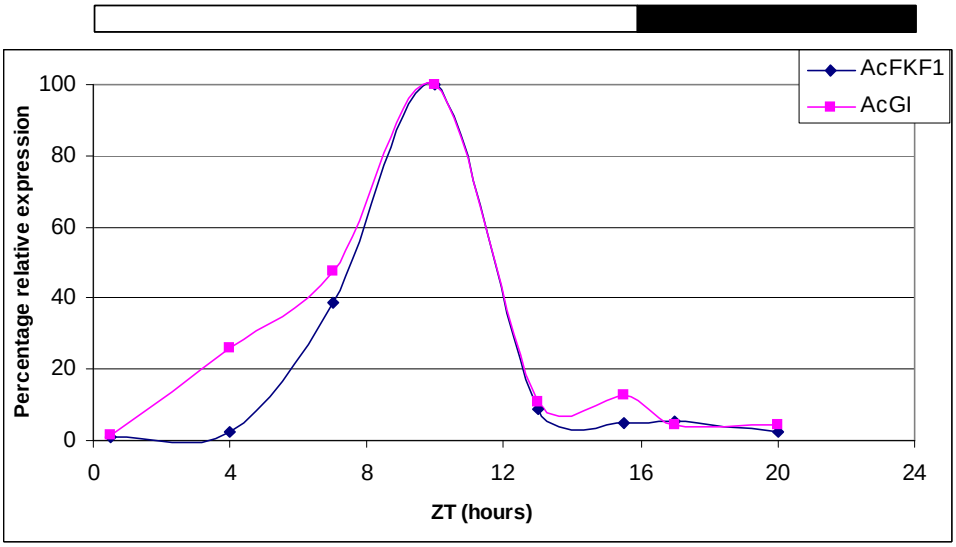


Fig. 8.1: Relative expression of *AcGI* and *AcFKF1* in a LD onion variety (Renate F_1) grown in LD conditions. White and black bars denote light/dark cycles.

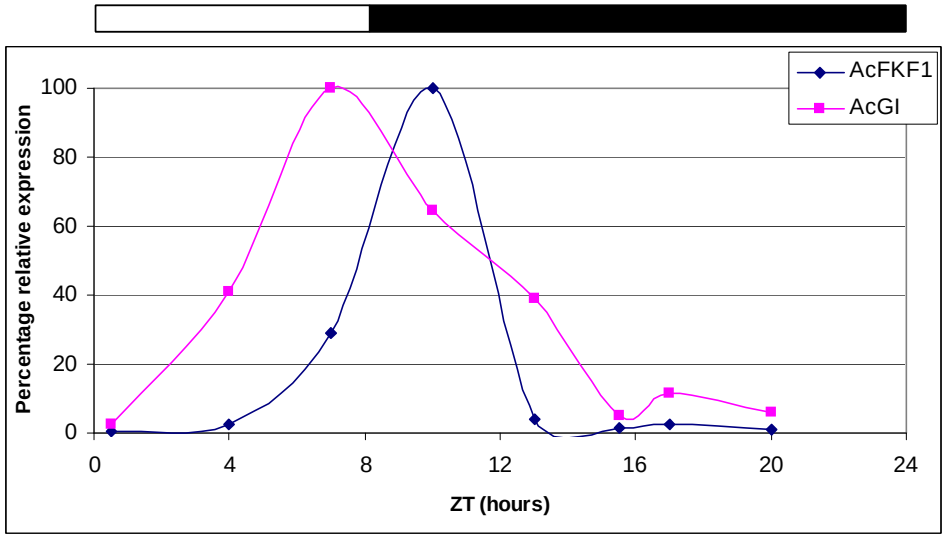


Fig. 8.2: Relative expression of *AcGI* and *AcFKF1* in a LD onion variety (Renate F_1) grown in SD conditions. White and black bars denote light/dark cycles.

The expression of *AcGI* and *AcFKF1* were also examined in an intermediate day (ID) onion variety. 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'. The expression profiles of *AcFKF1* and *AcGI* were seen to be very similar under LDs, peaking around ZT 9-10 (Fig. 8.3). This would allow for the daytime complex to form and bulbing to be initiated. The expression profiles of *AcFKF1* and *AcGI* were also very similar under SD conditions, both genes peaking around ZT7-8 (Fig. 8.4). 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 (Fig 8.5). 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 (Fig. 8.6). 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.

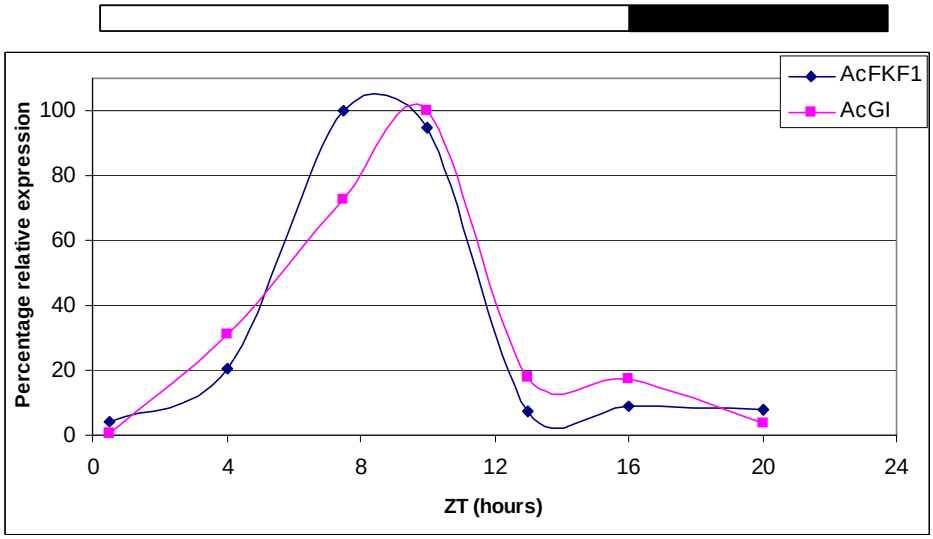


Fig. 8.3: Relative expression of *AcGI* and *AcFKF1* in an ID onion variety (Candy F_1) grown in LD conditions. White and black bars denote light/dark cycles.

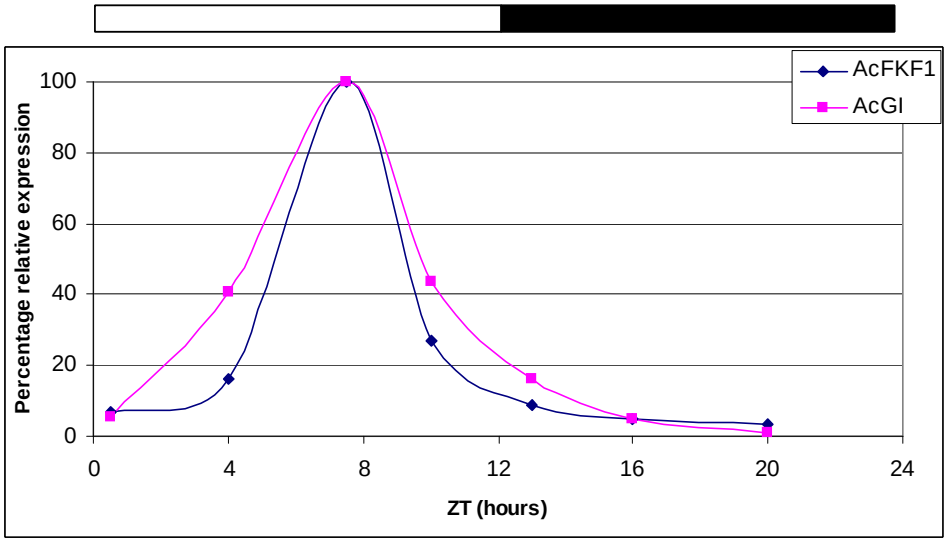


Fig. 8.4: Relative expression of *AcGI* and *AcFKF1* in an ID onion variety (Candy F_1) grown in SD conditions. White and black bars denote light/dark cycles.

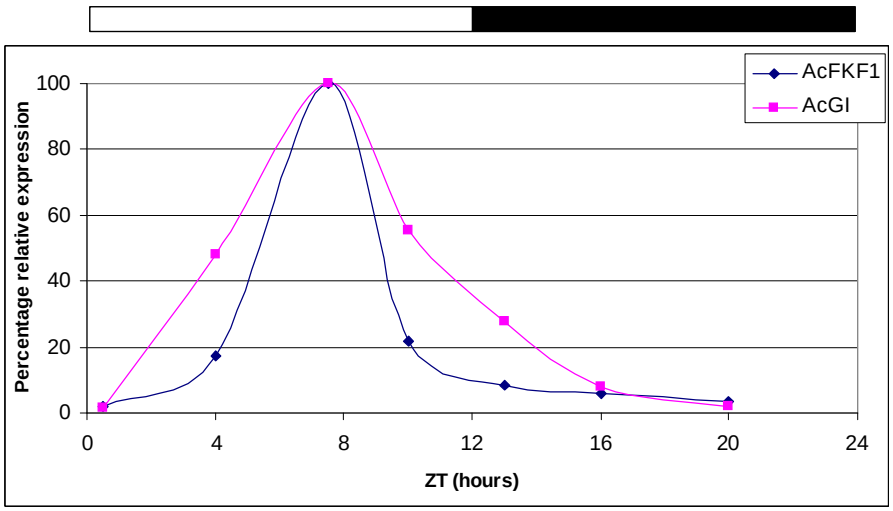


Fig. 8.5: Relative expression of *AcGI* and *AcFKF1* in a SD onion variety (Agrifound Dark) grown in SD conditions. White and black bars denote light/dark cycles.

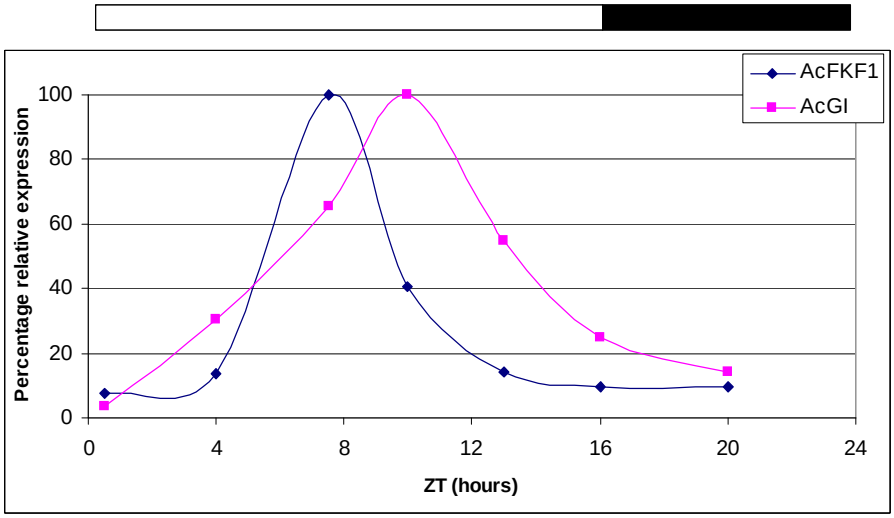


Fig. 8.6: Relative expression of *AcGI* and *AcFKF1* in a SD onion variety (Agrifound Dark) grown in LD conditions. White and black bars denote light/dark cycles.

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 (described in section 6.1.2). It has been shown that GI is required to maintain the oscillation of the ZTL protein. An onion *ZTL* homologue (*AcZTL*) showing 72.1% amino acid identity with *Arabidopsis ZTL* was identified (Chapter 6). The high level of sequence similarity suggests 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 *TOC1*).

Recent advancements have improved the knowledge of the seasonal control of flowering in *Arabidopsis*. It was shown that *COP1* and the clock-associated protein *EARLY FLOWERING 3* (*ELF3*) mediate signalling from *CRY2* to *GI* in the photoperiod pathway (Yu *et al.*, 2008). This is achieved through the destabilisation of GI, specifically at night. It was shown that CRY2 inhibits COP1, an inhibition which is proposed to stabilise GI during the daytime. COP1 has also been shown to mediate the degradation of CO at night, especially in SDs (Jang *et al.*, 2008). Hence COP1 controls CO at transcriptional (through GI) and posttranslational levels. A closer examination of the *A.cepa* Gene Index reveals that there is an EST which shows significant sequence similarity with *Arabidopsis COP1*. This EST is 938bp long, the coding region covering an estimated 35 % of the full coding sequence. Alignments were carried out and percentage identities calculated using the MegAlign package of DNAStar. The onion putative *COP1* homologue shows very high percentage identities with other *COP1* homologues, up to 82% amino acid identity with rice COP1 (Table 8.1). A conservation of function could be extrapolated from this, leading to the hypothesis that this gene acts to degrade an onion *CO* homologue.

Table 8.1: Comparing an onion putative *COP1* homologue (Accession number CF451443) with *COP1* genes from other plant species.

Plant species	Accession Number	Percentage nucleotide identity	Percentage amino acid identity
Arabidopsis	NM_128855.3	69.0	79.4
Rice	AB040053	72.7	81.9
Maize	EU975702	72.0	81.1
Tomato	AF029984	69.7	81.5
Pea	Y09579	68.7	76.9
Japanese morning glory	AF315714	69.1	81.5

The *A.cepa* Gene Index also contains three sequences which show similarity with *Arabidopsis ELF3*. All three EST's are around 1 kb in size and contain coding regions spanning an estimated 27-40% of the full coding sequence. The EST sequences were compared with *ELF3* homologues as described above. Although the percentage identities are much lower than those observed for *COP1*, *ELF3* is the closest relative of all the genes examined (Table 8.2). This provides further evidence for the hypothesis that the genes controlling the photoperiod response are conserved in onion.

Table 8.2: Comparing three onion EST's with *ELF3* homologues from other plant species in terms of percentage amino acid identities.

Plant species	**Accession number**	**TC7227**	**CF446779**	**CF452640**
Arabidopsis	NM_128153	27.4	30.4	32.3
Duckweed	AB210847	24.3	28.3	32.8
Iceplant	AY371292	25.9	35.1	30.1
Wheat	DQ923398	25.5	39.5	23.1

The onion EST database contains 12,245 unique sequences (DFCI, 2008). This represents an estimated 25-40% of the genes in the entire onion genome. The presence of many putative photoperiod response genes in this pool of genes is promising and suggests a large degree of gene conservation. In addition, it was possible to isolate *FKF1* and *ZTL* homologues using a PCR-based method. This strengthens the hypothesis that there is a large degree of conservation. All the information gathered on onion putative photoperiod response genes was collated and a potential model for the genetic control of bulb initiation in response to daylength created (Fig. 8.7). This model takes into account the functions of *Arabidopsis* photoperiod pathway genes and predicts a similar role in onion. There is evidence that all the genes in this model exist in onion although further work would be required to characterise the specific roles of these genes.

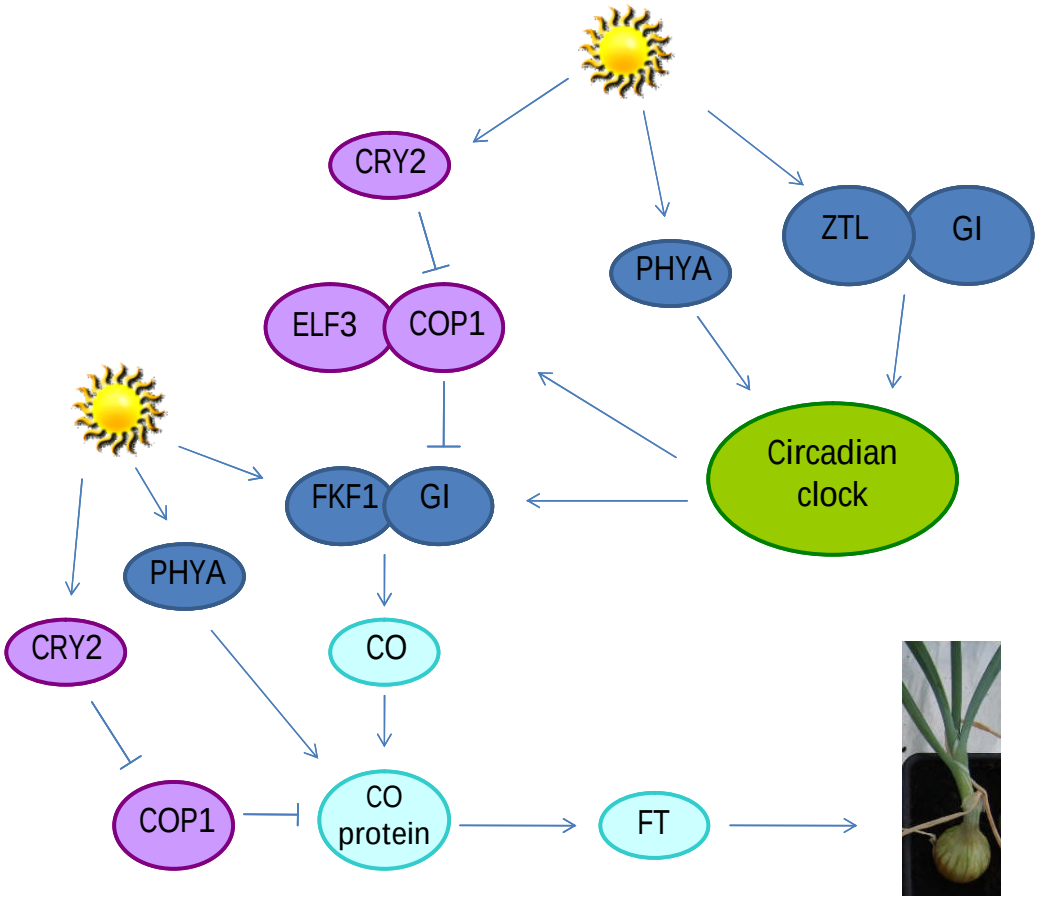


Fig. 8.7: Modelling the genetic control of photoperiodic bulb initiation in onion. Interactions between genes are predicted from work on the model species *Arabidopsis*. Blue boxes indicate that there is strong evidence of an onion orthologue; lilac boxes indicate that preliminary evidence suggests an orthologue exists, turquoise boxes indicate that the existence of an onion orthologue is predicted from the characterisation of genes from the same family.

Although the genes controlling daylength response are predicted to be conserved across species, it is possible that their roles differ slightly. This was shown to be the case in soybean where a homologue of *Arabidopsis CRY1* appears to be the major blue light photoreceptor controlling flowering (Zhang *et al.*, 2008). This is in contrast to *Arabidopsis*, where *CRY2* is the major blue light photoreceptor controlling photoperiodic flowering (El-Assal *et al.*, 2003). It was also shown that soybean *CRY1*, and not *CRY2*, was circadian regulated (Zhang *et al.*, 2008). Soybean is a SD plant and it was shown that an increased level of CRY1 protein correlates with a decrease in flowering time in LDs. This allows for soybean plants

to flower in northern latitudes and accounts for the latitudinal distribution of the crop. It was previously shown that an *Arabidopsis* accession from the Cape Verde Islands (near the equator) contains a *CRY2* variant which shows increased light stability, leading to flowering in a shorter daylength, suggesting a latitudinal cline in flowering time (El-Assal *et al.*, 2001). However, a study on many accessions showed no such latitudinal cline (Lempe *et al.*, 2005). It is possible a latitudinal cline in bulb initiation has allowed the development of onions for growth at various latitudes around the world. If this is the case, it is possible that such pattern is due to changes in onion PHY/CRY proteins and/or AcFKF1 protein. A study of allelic variation of these genes in an onion diversity set would therefore be a useful future experiment.

8.2 The Reversibility of the Bulbing Response

Bulb initiation in onion is controlled by photoperiod. In temperate onions, bulbing is stimulated by LDs (Lancaster *et al.*, 1996). It was shown that bulbing is reversible in a LD variety (Chapter 3). Plants transferred from LD to SD conditions resumed vegetative growth and those transferred from SD to LD conditions initiated bulbing. However, a delay in response of at least two weeks was noted when plants were transferred from SD to LD conditions, especially when transfers were carried out at a later developmental stage. It was hypothesised that this delay could be due to the accumulation of an inhibitor of bulb initiation in SDs. In *Arabidopsis*, the *CO* gene is central to the photoperiodic control of flowering (Jackson, 2008). There are many known inhibitors of *CO* transcription including CDF1, COP1 and SPA1 (Imaizumi *et al.*, 2005; Laubinger *et al.*, 2006; Jang *et al.*, 2008). It is possible that an onion orthologue of COP1, SPA1 or CDF1 could accumulate in onions remaining in SD conditions. The level of such a protein would remain high for some time after transfer to inductive LDs and the onion *CO* orthologue (presuming such a gene exists) would remain repressed. It is also possible that a repressor of any one of the photoperiod pathway genes is responsible for the inhibition of bulbing. However, SPA1 and COP1 inhibit *CO* (and *GI* in the case of COP1) at night and are degraded during the daytime in *Arabidopsis* (Laubinger *et al.*, 2006; Yu *et al.*, 2008). In addition, CDF1 represses CO in the early part of the day and is itself repressed in the

afternoon (Sawa *et al.*, 2007). An alternative explanation may be the accumulation of gibberellic acid (GA) in plants left in SD conditions. GA has been proposed to have an inhibitory role in bulb initiation, so an accumulation in SDs would delay bulbing (Mita and Shibaoka, 1984). GA promotes flowering in *Arabidopsis*, but inhibits flowering in other plant species (Thompson and Guttridge, 1959; Blazquez *et al.*, 1998). A continued repression following transfer from non-inductive to inductive conditions is not evident in the case of photoperiodic flowering (Jack, 2004). Therefore, such a repression in onion may have been acquired over the domestication of the species and could act to delay bulb initiation until conditions are optimum.

8.3 Bulbing vs. Flowering

An added dimension to the problem is the fact that onions will also flower, usually following a period of vernalisation (Brewster, 1997). This raises the question of which genes are involved in flowering and which are involved in bulbing. It is clear that bulb initiation is photoperiodically controlled (Chapter 3/Lancaster *et al.*, 1996). Under inductive daylength conditions, onions will initiate bulbing and flowering will be inhibited (Brewster, 2008). This illustrates that bulb initiation and not floral initiation is the photoperiod response in onion, and hence it was hypothesised that the genes controlling photoperiodic flowering in *Arabidopsis* control photoperiodic bulbing in onion.

A further complication in onion is the production of bulbils (small bulbs) in place of normal flowers and seed capsules (Brewster, 2008). Bulbil formation is a result of a change in meristem identity and occurs following a heat treatment. In *Arabidopsis*, floral meristem identity genes such as *APETALA1* (*AP1*) control the change from vegetative to reproductive growth (Abe *et al.*, 2005). The expression of *AP1* is promoted by FT and the bZIP protein FD at the shoot apex. It could be predicted that the same meristem identity genes exist in onion. This draws into question the potential role of an onion *FT* orthologue. It is possible that there is some overlap here between the control of floral meristem initiation and the control of bulb initiation (leaf or bulb scale production). Is there a completely different set of genes which control floral meristem identity and bulb initiation or is there a level of

overlapping function? Because FT is the mobile signal controlling flowering in *Arabidopsis* (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007) and bulb initiation is very similar to floral initiation on a physiological level, it would follow that this is the mobile signal controlling bulb initiation in onion. If this is the case, then what are the targets of FT in the basal plate? It may be that *FT* has dual roles in onion or it may be that other members of the same gene family have distinct roles in either flowering or bulbing. It is possible that a further member of the *FT*-like gene family is responsible for triggering the transformation from flower production to bulbil production. The *FT*-like gene family is large in rice and barley (Izawa *et al.*, 2002; Faure *et al.*, 2007) and preliminary work suggests that a similar family exists in onion.

In *Arabidopsis*, flowering occurs through 6 major pathways as described in section 1.5.1 (Jack, 2004). One such pathway is vernalisation which acts to repress *FLOWERING LOCUS C* (*FLC*), thus allowing the expression of floral integrator genes such as *FT* and *SOC1* leading to flowering. In onion, flowering normally occurs following a period of vernalisation (Brewster, 2008). It can be predicted that this occurs through the repression of a homologue of *FLC*. If this is the case, then it is possible that an onion *FLC* homologue inhibits a member of the *FT*-like gene family to control flowering in onion. This again suggests either dual roles for *FT* or a role for other members of the *FT*-like gene family in onion.

Overexpression of an *FT* homologue, Poplar *FT2*, has been shown to shorten the juvenile phase in this species (Hsu *et al.*, 2006). This suggests that expression of *FT* occurs at the end of the juvenile phase, leading to floral initiation. The juvenile phase in onion applies to both bulbing and flowering, since neither response can be initiated during this period (Sobeih and Wright, 1986; Brewster, 2008). Following the juvenile phase there is a thermophase during which flowering can be initiated by vernalisation. However, flowering is inhibited if conditions favour bulb development (i.e. LDs in a LD variety). This suggests an interaction between the regulatory processes controlling bulbing and flowering. There may be interaction between different onion *FT*-like genes which determines whether a plant will produce a bulb or an inflorescence meristem. In *Arabidopsis* a gene which is closely related to *FT*, *TERMINAL FLOWER 1* (*TFL1*), has an inhibitory role on flowering (Ahn *et al.*, 2006). This gene shares 56% amino acid identity with *FT*. It is possible that an onion homologue of *TFL1* inhibits bulbing or flowering. Many onion

varieties are selected by growers for their 'bolting resistance'. This phenotype may be due to an increased expression of genes which induce bulbing but inhibit (either directly or indirectly) flowering. Members of the *FT*-like gene family are candidates for the control of this response.

An additional floral induction pathway in *Arabidopsis* involves GA (Jack, 2004). GA promotes flowering through the floral integrator genes, especially *LFY* (Blazquez *et al.*, 1998). It has been proposed that GA has an inhibitory role on bulbing and a stimulatory role on flowering in onion (Mita and Shibaoka, 1984; Rabinowitch, 1990). This again indicates a crossover between the genetic control of bulbing and flowering in onion. The role of GA in onion has not been fully characterised, so this is only speculative at this stage.

A major assumption made during this project was that a homologue of *Arabidopsis CO* is central to the photoperiodic control of bulb initiation. This is supported by an observed role for *CO* in the photoperiodic control of tuberisation in potato (Martínez-García *et al.*, 2002). A new study has provided evidence for a broader role for *CO* in potato. Potato plants over-expressing *Arabidopsis CO* were shown to flower later irrespective of daylength (González-Schain and Suárez-López, 2008). Flowering is considered to be daylength independent in potato. Therefore, it appears that *CO* delays flowering in potato in a daylength independent manner. This study contradicts previous work which showed that over-expressing *Arabidopsis CO* in potato did not affect flowering time (Martínez-García *et al.*, 2002). This group showed that *Arabidopsis CO*, when over-expressed, delays tuberisation in inductive SDs. It is suggested that *CO* has multiple roles in potato. If *CO* does control flowering in potato in a daylength independent manner, then it may have a role in onion flowering which is also daylength independent. It is possible that different *CO*-like genes are involved in the control of bulbing and flowering in onion. It has been shown that certain *CO*-like genes can affect flowering time in *Arabidopsis*, adding weight to this argument (Cheng and Wang, 2005; Datta *et al.*, 2006). This suggests that the characterisation of different onion *CO*-like genes, as opposed to exclusively a *CO* homologue, would be useful.

An onion *CO*-like gene (*AcCOL*) was characterised during this project (Chapter 4). This gene could not complement *Arabidopsis CO* mutants but had an effect on flowering time. Phylogenetic analyses showed that the closest relative of *AcCOL* in *Arabidopsis* is *COL4* (section 4.3.2). Yeast two-hybrid studies have

shown that COL4 interacts with LOV KELCH PROTEIN 2 (LKP2), a gene which is involved in the circadian clock (Schultz *et al.*, 2001; Stark *et al.*, 2006). This suggests a role for *COL4* in the photoperiodic control of flowering in *Arabidopsis* and poses the question of whether *AcCOL* is involved in bulbing, flowering or both.

8.4 Recommendations for Future Experimental Work

This is the first report on the genetic control of bulb initiation in onion. Therefore, there are many further experiments which would be useful to build on this work. Firstly, it would be useful to set up experiments involving the transfer of plants into constant light conditions. The expression of *AcGI* and *AcFKF1* could then be examined in these plants. If the expression of *AcGI* and *AcFKF1* was seen to continue to cycle with the same phase, it could then be concluded that both genes are circadian regulated (Fowler *et al.*, 1999). It would also be extremely useful to analyse the expression of *AcFKF1* in many more onion varieties, showing different daylength responses. This would be useful to confirm the differential expression of *AcFKF1* in varieties showing different daylength responses. It would also be useful to examine the expression of *AcFKF1* in plants grown in several different daylengths (for example, 8,10,12,14 and 16 hours of light). This would allow any small differences in expression between daylength to be detected, providing more information on the function of this gene.

A further experiment could be set up to examine the expression of *AcGI* and *AcFKF1* over the course of development. This would involve harvesting weekly from the first true leaf stage right through to the mature bulb stage. It would be expected that *AcGI* would be expressed throughout development as this is the case for *Arabidopsis GI* (Fowler *et al.*, 1999). The expression of *AcFKF1* may be expected to increase prior to an increase in bulbing ratio thus allowing the FKF1/GI complex to form and bulbing to be initiated. It would also be useful to examine the expression of *AcGI*, *AcFKF1* and any other photoperiod pathway genes in onion sets where bulb formation has been arrested. A lower expression level of certain genes may be expected in these plants. In order to further functionally characterise *AcGI* and *AcFKF1*, complementation studies using *Arabidopsis* mutants would be advantageous. This experiment was partially completed for *AcGI* (section 5.3.5) and

it would be useful to complete this study. It would also be useful to transform *Arabidopsis fkf1* mutant and wild-type lines to express *AcFKF1*. A positive result (functional complementation) would show an involvement of this gene in the photoperiod response.

The potential interactions between GI and FKF1/ZTL were discussed in section 8.2. It would be useful to investigate these potential interactions, possibly using a Yeast Two-Hybrid system (Gietz *et al.*, 1997) or a co-immunoprecipitation assay (Lee, 2008). The outcome of this would be the further functional characterisation of onion putative photoperiod response genes. If interactions were observed, it could be hypothesised that these parts of the photoperiod pathway are very similar in onion and *Arabidopsis*. Mapping of *AcGI* is a project which is currently in progress in collaboration with Dr. John McCallum (Crop & Food Research, New Zealand). The aim is to see if *AcGI* maps to the same chromosomal region as *Arabidopsis* and rice *GI*. It would also be advantageous to map *AcFKF1*/*AcZTL* as well as any other photoperiod genes which are isolated in the future. Mapping of a gene involved in onion bulb fructans content has been previously reported and mapping of onion putative photoperiod response genes will follow a similar protocol (McCallum *et al.*, 2006). In addition to this, the isolation and sequencing of full-length genomic clones of these genes would be of interest. An experiment designed to isolate and sequence *AcGI*/*AcFKF1* from onion varieties with different daylength response would also be useful. It would then be possible to investigate whether onion varieties which respond to different daylengths have slightly different copies of putative photoperiod response genes.

Previous work suggests that bulbing can be initiated in blue light conditions (Terabun, 1965). It would therefore be advantageous to test this theory, especially as the complexes that form between GI and FKF1/ZTL are formed specifically under blue light conditions in *Arabidopsis* (Kim *et al.*, 2007; Sawa *et al.*, 2007). An experiment could be set up comparing the bulbing ratios of plants grown under blue, red, far-red and white light conditions. This would allow for further characterisation of the bulbing process and help contribute towards linking a physiological response to a genetic pathway. It was shown that *AcZTL* is constitutively expressed (section 6.3.3.2). A further experiment could be set up to investigate whether or not the AcZTL protein levels oscillate, as is reported for *Arabidopsis* ZTL (Kim *et al.*,

2007). This would require protein extraction and immunoblotting procedures, as described for the analysis of the *Arabidopsis* ZTL protein (Kim *et al.*, 2003b).

The characterisation of onion *FT*-like genes is described in Chapter 7. It would be useful to isolate full-length copies of *AcFTL*, *AcFTL2* and *AcFTL3*. It would also be useful to isolate and characterise other members of this gene family. A potential method for this would be the screening of a cDNA library. If other members of this gene family could be isolated, it would then be useful to examine their expression in LD and SD grown plants. It would also be interesting to investigate the expression of *FT*-like genes in onions which have been induced to bolt. The FT protein has recently been shown to be the mobile signal controlling floral initiation in both rice and *Arabidopsis* (Corbesier *et al*., 2007; Jaeger and Wigge, 2007; Tamaki *et al*., 2007). It would be interesting to investigate which members of the onion *FT* gene family are involved in flowering and/or bulbing. Further characterisation of the other genes discussed in Chapter 7 would also be advantageous. The presence of onion putative homologues of *ELF3* and *COP1* was discussed in section 8.2. Further work would involve obtaining full-length clones of these genes by cDNA library screening and/or RACE PCR. These genes could then be functionally characterised using similar methods to those described for other putative photoperiod response genes.

It is debatable whether further resources should be invested in a search for an onion *CO* homologue. Without a thorough genome sequencing project, this may prove a difficult task. In order to further characterise *AcCOL*, it would be useful to source an *Arabidopsis col4* mutant line. Phylogenetic analyses suggest that this gene is the closest relative of *AcCOL*. The mutant line could be transformed to over-express *AcCOL* and the phenotype assessed. In addition, the SD flowering time of *Arabidopsis* plants expressing *AcCOL* could be investigated.

Due to the relatively small amount of work carried out on sequencing the onion genome, the *A.cepa* Gene Index (DFCI, 2008) was exhausted for the purpose of this study. There are no further putative homologues of *Arabidopsis* photoperiod response genes present in this database. In order to fully characterise the photoperiod response in onion, additional genes would need to be isolated and characterised. The suggested method for this would be degenerate PCR using tagged cDNA (described in section 4.2.5) as this was successful for the isolation of *AcFKF1* and *AcZTL*. This would be followed by a library screen or RACE PCR in order to

isolate full-length clones. Genes of interest would include *CDF1*, *TOC1*, *FD*, *TSF* and *TFL1* as well as additional phytochrome and cryptochrome homologues.

A small scale experiment was carried out to investigate the reversibility of the bulbing process in onion (Chapter 3). It would be of use to set up a large scale experiment, involving the transfer of plants throughout development. This would help test the hypothesis that an inhibitor of bulb initiation builds up when plants are left in SDs. In addition, it would be interesting to examine the expression of genes such as *AcFKF1* and *AcGI* (and any onion *CO* homologues) following transfer. If a lower level expression of any of these genes was observed, when compared to expression in plants grown in constant LDs, it could then be hypothesised that an inhibitor is active, preventing expression of these genes and hence bulbing.

The most informative experiment would involve knocking out each of the onion putative photoperiod response genes characterised during this project and assessing the effect on bulb initiation in both LD and SD conditions. This is possible in onion due to advancements in RNAi technology, but is still very time consuming (Eady *et al.*, 2008). Another method which could be investigated is the use of virus induced gene silencing (Lu *et al.*, 2003).

8.5 PROJECT CONCLUSIONS

The project conclusions are discussed in terms of the set of aims outlined in section 1.11. The overall aim of this project was to test the hypothesis that the genes controlling daylength response are conserved between the model plant *Arabidopsis* and onion and hence between the different end-processes bulbing and flowering. This hypothesis was rigorously tested throughout the project, resulting in the isolation of several putative photoperiod response genes. The results presented suggest that many of the photoperiod pathway genes are conserved in onion. The expression patterns observed for onion *GI*, *FKF1* and *ZTL* homologues also suggest a conservation of function. Phylogenetic analyses support this theory.

A more specific aim of this project was to isolate and clone onion *CO*/*CO*-like genes (with particular emphasis on the isolation of a *CO* orthologue). It was not possible to isolate an onion *CO* orthologue during this project. However, many methods were attempted including those which have been shown to be successful for

other species, including monocots (e.g. Martin *et al.*, 2004). It is possible that a direct *CO* orthologue does not exist in onion. However, it is more likely that an orthologue does exist but is expressed at a very low level, as is observed for *Arabidopsis CO* (Putterill *et al.*, 1995). In addition, the onion genome is very large (around 107 times the size of the *Arabidopsis* genome), making the isolation of low expressed genes challenging (Kuhl *et al.*, 2004). It may require a full onion genome sequencing project in order to isolate genes such as *CO*, a project which has become possible with the advent of next generation sequencing technologies (reviewed by Mardis, 2008).

A full-length clone of an onion *CO*-like gene (*AcCOL*) was obtained during this project. This shows that at least one member of this gene family exists in onion and suggests that further members of this gene family also exist. A further aim of this project was to determine the expression pattern of onion *CO*/*CO*-like genes in SD and LD-grown plants. The expression of *AcCOL* did not show any discernable diurnal pattern, suggesting that this gene is not a major part of the photoperiod pathway as the major genes in this pathway are circadian regulated and show distinctive expression patterns (Jackson, 2008). In addition, this gene does not functionally complement *Arabidopsis CO*, suggesting it is not a *CO* orthologue. However, *AcCOL* does have a small effect on flowering time in *Arabidopsis*. It would appear that the closest relative of *AcCOL* is *Arabidopsis COL4*.

A further aim of this project was to characterise the physiological aspects of bulb development and establish a system for studying the expression of putative photoperiod response genes. This was achieved with an effective system developed for the LD onion variety Renate F_1 (Chapter 3). This variety showed a clear photoperiod response and illustrated the reversibility of the bulbing process. This allowed for effective gene expression studies and should allow further studies to continue in the future. Renate F_1 plants transferred from LD to SD conditions respond faster than those transferred from SD to LD conditions, suggesting that an inhibitor of bulb initiation accumulates under SD conditions. It is possible that this is an inhibitor of an onion photoperiod pathway gene, but further work would be required to test this hypothesis.

The final aim of this project was to isolate and characterise other candidate genes involved in the photoperiodic flowering pathway which may function in the photoperiodic regulation of bulbing in onion. This was the area in which the most

progress was made. Homologues of the photoperiodic flowering genes *GI*, *FKF1* and *ZTL* were isolated and studied in detail. Full-length copies of these genes were obtained. The expression profiles of these genes were similar to their *Arabidopsis* counterparts, suggesting a conservation of function. The expression of *AcGI* and *AcFKF1* were in onion varieties which exhibit different daylength responses. It was found that the expression profile of *AcGI* is very similar across varieties. It would appear that *AcFKF1* is differentially expressed in onion varieties which exhibit different daylength responses, suggesting this gene may contribute towards the different responses. Members of the onion *FT*-like gene family were isolated, suggesting that an onion *FT* homologue exists. Phylogenetic analyses suggested that the genes isolated were homologues of rice *FTL4*, *FTL5*/*6* and *FTL11* respectively. The functions of these genes are currently unknown. Putative orthologues of *COP1* and *ELF3* as well as the photoreceptor genes *PHYA* and *CRY2* were also studied and preliminary work suggests that these genes are also homologous to their *Arabidopsis* counterparts.

The rationale behind this project was that gaining a greater knowledge of the daylength response in onion is important for adapting new varieties for growth at different latitudes. This extends to the choice of which current varieties will perform best at different latitudes. This piece of work is the first of its kind in respect of studying onion photoperiod response genes. It will provide a base for future work towards a thorough characterisation of the photoperiodic control of bulb initiation. Future work should be centred on *AcFKF1* as this gene appears to show differential expression in onion varieties showing different daylength responses. It would be particularly useful to compare sequences of *AcFKF1* from many different onion varieties in search of allelic variation. Following this, it may be possible to perform genetic screens on onion varieties in order to predict which varieties will perform the best at different latitudes. This information would be extremely useful in accelerating onion breeding programmes.

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Appendix 1- Media recipes

YEB media (1 litre):

1 g yeast extract

5 g peptone

5 g beef extract

5 g sucrose

0.492 g $MgSO_4.7H_20$

Made up to 1 litre with sterile distilled water and autoclaved

pH 7.6

Freezer media (1 litre):

1.8 g KH2PO4

10 g Tryptone

5 g Yeast Extract

10 g NaCl

6.26 g K2HPO4

0.5 g Trisodium citrate

0.99 g Magnesium sulphate

0.898 g Ammonium sulphate

44 ml Glycerol

Made up to 1 litre with sterile distilled water and autoclaved.

SOC medium:

0.5 % w/v Yeast Extract

2 % w/v Tryptone

10 mM NaCl

2.5 mM KCl

10 mM $MgCL_2$

10 mM $MgSO_4$

20 mM Glucose

Appendix 2- Primer sequences (all displayed 5’ to 3’)

CHAPTER 2:

EST and general Sequencing primers:

M13 F: GTAAAACGACGGCCAG

M13 R: CAGGAAACAGCTATGAC

SP6 Promoter Primer: ATTTAGGTGACACTATAG

T7 Promoter Primer: TAATACGACTCACTATAGGG

T3 Primer: TAATACGACTCACTATAGGG

U-19 Primer: GTTTTCCCAGTCACGACGT

For sequencing AcCOL:

CO1: CGCACGTAACGCACAGCGTCGCC

CO2: CCCGTACAGCAGACCTTTGCC

For sequencing AcFBox:

FKF1: CCCTAATCCACGAAATACAGC

FKF2: GGCTACGTTGAAACTGTTATGG

FKF3: ATGACACATGGATCGCACTCGC

FKF4: CCAAATACATACAAACATCGTCC

Primers for housekeeping genes (EF1α/ß-TUBULIN):

EF1-RT F3: TGGCATCCAACTCTAAGGACGAT

EF1-RT R3: AATGTGAGATGTGTGGCAATCCA

TUB FOR1: GTCTTCAGAGGCAAGATGAGCAC

TUB REV1: TCAGTCCAGTAGGAGGAATGTCG

CHAPTER 4

Expression of AcCOL:

CO-RT F1: AGAGAAGCGAAAGAATAGAAAGTT

CO-RT R1: ATCCGCATAAGAATCGTTGTC

BR20-RT F2: CGTATTTGGATTTGGATGGAATGG

BR20-RT R2: ATCGTCCCTTTCTCCTGCCTAT

TC2898 FOR: GAACCGACGCCACCTACCTCTGC

TC2898 REV: CATACCCCATCCCCTTTCCCAACA

CO degenerate primers:

B-box F1: CGCTGTGCGTKRCGTGCGACG

B-box F2: CGWGCGCGTGYGGSTGTGCGAGG

CCT R1: CTTGRCGAARCGKCCCTTSA

CCT R2: BCTTSYKCTTCTCCYKGTA

preCCT: ATGGACAGRGARGCMAGRGT

CCT new: CGTATKGTCTTCTCAAACTT

Towards a library screen:

MONCO F: TCAGGTCCTYCRCTTCAGATGCC

MONCO R1: CCCTTGATCCKYGGYCGTGCTTC

MONCO R2: GAGAACATCTGGTCCACTTC

BR20 CCT F: ATGCGATACAGAGAGAAGCGAAA

BR20 CCT R: GGCGAACCTTCCTTTAATCCTGG

Degenerate PCR using tagged cDNA:

B-box 1 DEG: TACCAGGCATTCGCTTCATGCRYSGTGTACTGCCRYGCBGA

B-box 2 DEG:

TACCAGGCATTCGCTTCATAGRTBCACTCYGCVAACCCRCTYGC

CCT DEG 1:

TACCAGGCATTCGCTTCATCTSAGRTACARRGAGAAGARGAAG

CCT DEG 1:

TACCAGGCATTCGCTTCATGTTTGAGGAARACMATAMGKTAYG

PolyT Adapter:

GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTVN

TAG Primer: GCGAGCACAGAATTAATACGAC

CHAPTER 5

Expression of AcGI:

GI-RT F: CACAGATGGATTGCTTGTTGATG

GI-RT R: ATTGGCTACGAGATGAACTGCTC

Library screen:

GI FOR2: CAGGCCGAGAAGGATTTACAAC

GI REV: CAAAACTCCGGTTCTGACAGTG

RACE PCR:

GI GSP: GGCACGAAGAAGAAGATCCGAGGCACTA

GI GSP NEST: CAACATCACAAAGCGCATCCACTACCT

Amplifying/sequencing full-length AcGI gene:

GI 5'UTR 3: GCCTTCTTCACGAAAAATCGCAGTG

GI 5' UTR 4: GTGCATAATTGAAGACGGTTTTG

GI 3' UTR 1: CCAAGACGATTACAAGGATGATAGA

GI 3' UTR 2: AATCCTGGCCACCGAGCAAAAACC

GI SEQ 1: GCTCAGCTTTGAATATTGGACGA

GI SEQ 2: CCTGCTCTATTACTTCCTCCTCC

GI SEQ 3: GTGCTGCAATCAATTTGTGCCAC

GI SEQ 4: TGCTCACACATACGCTTGGTATA

GI SEQ 5: CAGTTAACACGACGAATAGGAGTC

GI SEQ 6: GCAGTTGCAAACACATCTCAGGA

GI SEQ 7: CCATGGATCTTGCATCTAGACTCC

GI SEQ 8: GTTAGGTTTGCAGTTTCATAGCGAG

GI SEQ 9: GCACAGCAGCATCAAGCGCATCT

Gateway primers:

GI 5' UTR 4 att:

GGGGACAAGTTTGTACAAAAAAGCAGGCTGTGCATAATTGAAGACG GTTTTG

GI 3' UTR 1 att:

GGGGACCACTTTGTACAAGAAAGCTGGGTCCAAGACGATTACAAGG ATGATAGA

CHAPTER 6

Expression of AcFBox:

FKF-RT F1: ATGGTCCTGTTCGGTGGTC

FKF-RT R1: ATTCTCGTATTTTATCTCTGTCC

AV44-RT2 FOR: GTGGGTCATTCCGCTAATGTTAT

AV44-RT2 REV: GTTTCAACGTAGCCTTGTTATGG

Searching for other LOV family genes:

FKF DEG 1: ATGGTHTGTCARAAYGCDTGGGG

RACE PCR:

FKF GSP: CCACCCGCTTCCAAGTGGGCTGGTTTG

FKF GSP NEST: CGAGGGTGGTGAGTTCGCGGGAGAGT

ZTL GSP: GCCCTTGCCTACCACATCCACCAAAT

ZTL GSP NEST: CCTGCTGCTGGCATGGTTTCTAACGC

Expression of AcFKF1 and AcZTL:

pFKF1-RT FOR 3: CCGGTGCAGTTGTTTATGTTGGAT

pFKF1-RT REV 3: TCCCACCCACCACACAGGTACTAT

ZTL RT FOR1: GTTTGGTGGTCTGGCTAAGAGTG

ZTL RT REV: CTCCAGGCATACTGCTACCTGTT

CHAPTER 7

Expression of AcFTL:

EE96-RT FOR: CGTGTTACCATTGAAGGACGTGA

EE96-RT REV1: AGTTGGATTAGTTGGGCTTGGTG

EE96-RT REV2: AACTTGCATCGACGTTTTCTGGT

FT-like FOR: GGWGAYGTKBTGGACCCRTT

FT-like REV: GGRCTTGGWGCATCWGGRT

FTL-RT FOR: GTGTTACCATTGAAGGACGTGACT

FTL-RT REV: TAACTTGCATCGACTGTTTCTGGT

Appendix 3- Additional data

CHAPTER 3

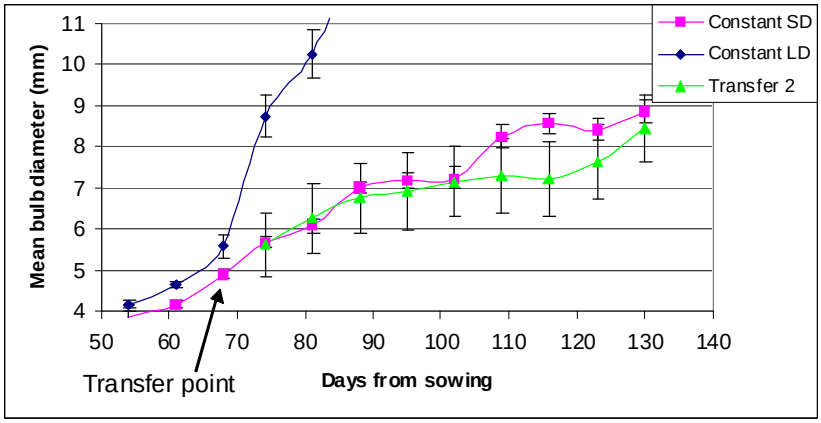

Fig. A1: Bulb diameters of Renate F_1 plants transferred from SD to LD conditions (transfer 2). Error bars represent the SEM. The mean bulb diameter of plants grown in LDs was 17.7 mm after 130 days.

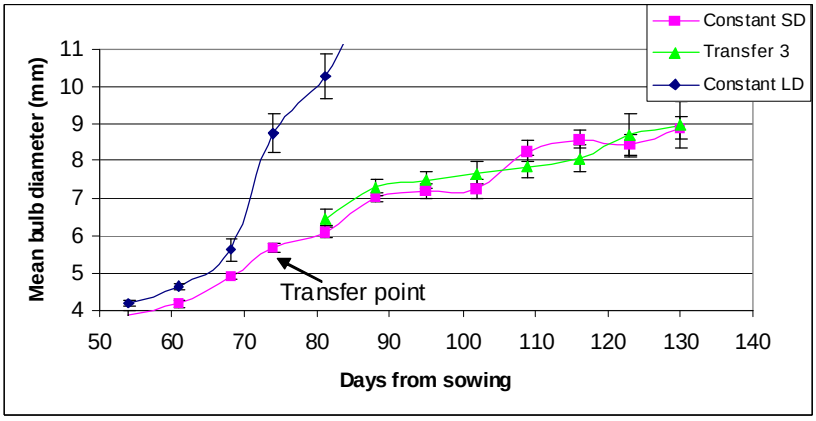

Fig. A2: Bulb diameters of Renate F_1 plants transferred from SD to LD conditions (transfer 3). Error bars represent the SEM. The mean bulb diameter of plants grown in LDs was 17.7 mm after 130 days.

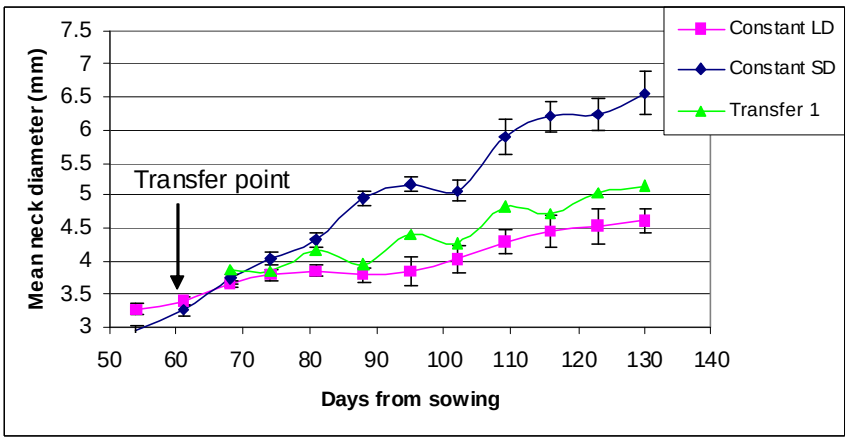

Fig. A3: Neck diameters of Renate F_1 plants transferred from SD to LD conditions (transfer 1). Error bars represent the SEM

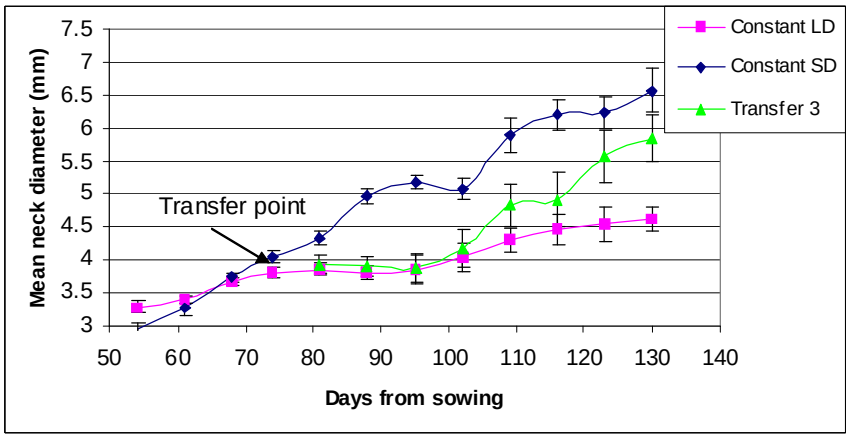

Fig. A4: Neck diameters of Renate F_1 plants transferred from SD to LD conditions (transfer 3). Error bars represent the SEM

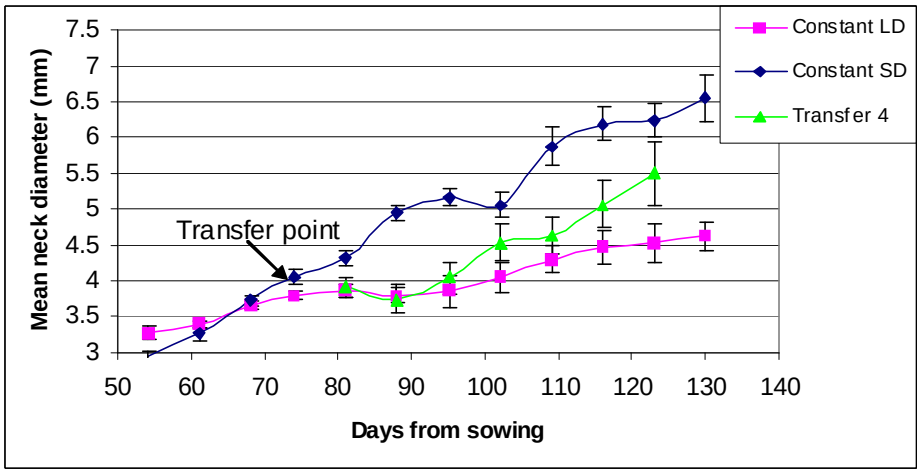

Fig. A5: Neck diameters of Renate F_1 plants transferred from SD to LD conditions (transfer 4). Error bars represent the SEM

Table A1: Log transformed bulbing ratio data for LD to SD transferred plants. TP=transfer point

Days from sowing	Constant LD	Constant SD	T1	T2	T3	T4
54	0.24	0.28				
61	0.32	0.24	TP			
68	0.43	0.28	0.35	TP		
76	0.87	0.34	0.48	0.57	TP	
83	0.97	0.34	0.54	0.55	0.93	TP
90	1.23	0.38	0.59	0.62	0.89	1.12
97	1.29	0.33	0.51	0.57	1.09	1.19
104	1.37	0.36	0.52	0.58	1.04	1.22
111	1.35	0.34	0.39	0.49	0.90	1.13
118	1.33	0.33	0.39	0.47	0.84	1.12
125	1.35	0.30	0.35	0.44	0.79	0.97
132	1.33	0.31	0.33	0.43	0.75	0.90
139	1.28	0.27	0.31	0.32	0.71	0.86

Table A2: Log transformed bulbing ratio data for SD to LD transferred plants. Numbers in red indicate the transfer point. TP=Transfer point.

Days from sowing	Constant LD	Constant SD	T1	T2	T3	T4
54	0.24	0.28				
61	0.32	0.24	TP			
68	0.43	0.28	0.33	TP		
76	0.87	0.34	0.43	0.34	TP	
83	0.97	0.34	0.53	0.40	0.34	TP
90	1.23	0.38	0.62	0.47	0.45	0.34
97	1.29	0.33	0.63	0.45	0.44	0.37
104	1.37	0.36	0.66	0.47	0.44	0.39
111	1.35	0.34	0.70	0.53	0.48	0.38
118	1.33	0.33	0.70	0.53	0.51	0.43
125	1.35	0.30	0.81	0.61	0.53	0.45
132	1.33	0.31	0.85	0.66	0.60	0.50
139	1.28	0.27	0.87	0.71	0.65	0.53

Table A3: Log transformed Bulb diameter data for SD to LD transferred plants. TP=Transfer point.

Days from Sowing	Constant LD	Constant SD	T1	T2	T3	T4
54	1.43	1.35				
61	1.57	1.38	TP			
68	1.75	1.60	1.68	TP		
76	2.22	1.71	1.79	1.73	TP	
83	2.32	1.78	1.94	1.83	1.86	TP
90	2.57	1.96	2.02	1.91	1.98	1.93
97	2.62	1.97	2.07	1.93	2.01	2.01
104	2.76	1.98	2.08	1.97	2.03	2.02
111	2.80	2.11	2.19	1.98	2.06	2.05
118	2.81	2.15	2.18	1.97	2.08	2.06
125	2.85	2.13	2.19	2.03	2.15	2.09
132	2.85	2.18	2.23	2.13	2.18	2.12
139	2.93	2.23	2.27	2.18	2.20	2.16

Table A4: Log transformed Neck diameter data for LD to SD transferred plants. TP=Transfer point.

Days from Sowing	Constant LD	Constant SD	T1	T2	T3	T4
54	1.18	1.07				
61	1.25	1.13	TP			
68	1.32	1.32	1.35	TP		
76	1.35	1.37	1.35	1.33	TP	
83	1.35	1.45	1.42	1.44	1.36	TP
90	1.33	1.58	1.37	1.48	1.35	1.36
97	1.34	1.64	1.48	1.55	1.34	1.31
104	1.39	1.62	1.45	1.56	1.41	1.39
111	1.45	1.76	1.57	1.68	1.56	1.50
118	1.49	1.82	1.54	1.69	1.57	1.52
125	1.50	1.83	1.61	1.72	1.70	1.61
132	1.52	1.87	1.62	1.73	1.75	1.69
139	1.65	1.97	1.63	1.82	1.79	1.71

Table A5: Assessing the significance of the differences in bulb diameter between treatments. The controls refer to differences between plants grown in constant LD and constant SD conditions. p=probability, MS=mean square (green colour indicates strongest effect).

Transfer	Source of Variation	P	MS	Degrees of Freedom
Control	Days from sowing	<0.001	2.70	12
Control	Daylength	<0.001	14.86	1
Control	Days from sowing x daylength	<0.001	0.22	12
2	Days from sowing	<0.001	1.59	10
2	Daylength	<0.001	7.92	3
2	Days from sowing x daylength	<0.001	0.09	30
3	Days from sowing	<0.001	0.90	9
3	Daylength	<0.001	7.88	3
3	Days from sowing x daylength	0.553	0.03	27

Table A6: Assessing the significance of the differences in neck diameter between treatments. The controls refer to differences between plants grown in constant LD and constant SD conditions. p=probability, MS=mean square (green colour indicates strongest effect).

Transfer	Source of Variation	P	MS	Degrees of Freedom
Control	Days from sowing	<0.001	0.65	12
Control	Daylength	<0.001	1.65	1
Control	Days from sowing x daylength	<0.001	0.12	12
1	Days from sowing	<0.001	0.57	11
1	Daylength	<0.001	0.97	3
1	Days from sowing x daylength	<0.001	0.08	33
2	Days from sowing	<0.001	0.54	10
2	Daylength	<0.001	1.04	3
2	Days from sowing x daylength	<0.001	0.06	30
3	Days from sowing	<0.001	0.49	9
3	Daylength	<0.001	0.91	3
3	Days from sowing x daylength	<0.001	0.06	27
4	Days from sowing	<0.001	0.38	8
4	Daylength	<0.001	1.13	3
4	Days from sowing x daylength	0.002	0.04	24

CHAPTER 7

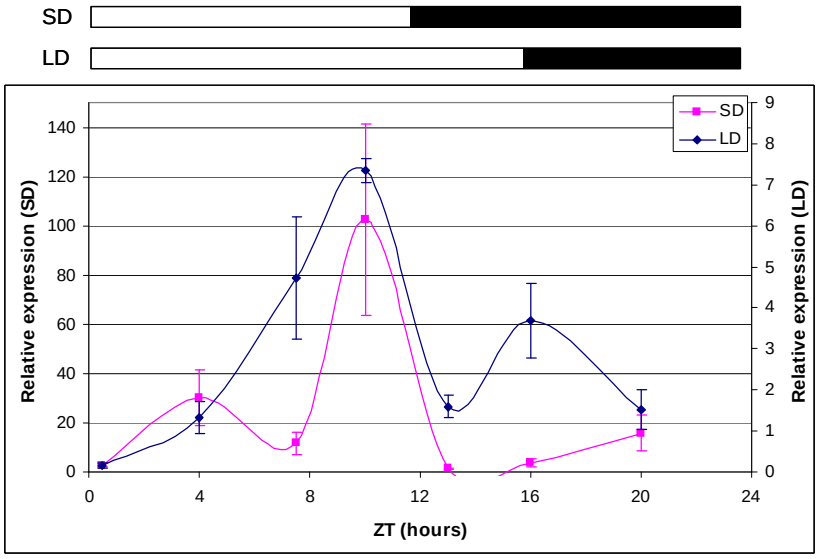

Fig. A6: Average 24-hour expression of *AcFTL* in a SD onion variety (Agrifound Dark), relative to ß-Tubulin. The expression was calculated as a percentage of the maximum level. Bars show the light/dark growing conditions plants were subjected to.

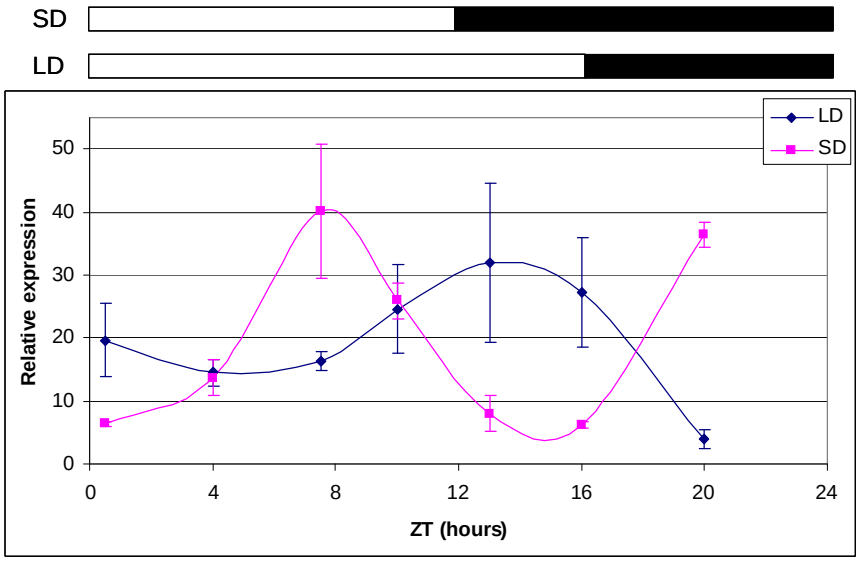

Fig. A7: Average 24-hour expression of *AcFTL* in an ID onion variety (Candy F_1), relative to ß-Tubulin. Bars show the light/dark growing conditions plants were subjected to.

Appendix 4- Accession numbers

Table A7: Databases used to access gene sequences for phylogenetic analyses

Database	Full Name	URL
NCBI	National Centre for Biotechnology Information	http://www.ncbi.nlm.nih.gov/
PGN	Plant Genome Network	http://pgn.cornell.edu/index.pl
DFCI	Dana-Farber Cancer Institute	http://compbio.dfci.harvard.edu/tgi/
CGP	Compositae Genome Project (v2)	http://compgenomics.ucdavis.edu/
RGA	Rice Genome Annotation	http://rice.plantbiology.msu.edu/
TAIR	The Arabidopsis Information resource	http://www.arabidopsis.org/

CHAPTER 4

Table A8: Accession numbers for phylogenetic analysis of *CO* and *CO*-like genes

Plant Species	Common Name	Name Assigned	Identifier	Database
A.trichopoda	Amborella	Atr_COL	Unigene 196552	PGN
A.formosa x A.pubescens	Aquilegia	Af_COLa	TC9320	DFCI
A.formosa x A.pubescens	Aquilegia	Af_COLb	TC15214	DFCI
A.formosa x A.pubescens	Aquilegia	Af_COLc	TC10456	DFCI
A.formosa x A.pubescens	Aquilegia	Af_COLd	TC12057	DFCI
A.formosa x A.pubescens	Aquilegia	Af_COLe	TC15917	DFCI
A.formosa x A.pubescens	Aquilegia	Af_COLf	TC17147	DFCI
A.thaliana	Thale cress	At_CO	X94937	NCBI
A.thaliana	Thale cress	At_COL1	NM_121590	NCBI
A.thaliana	Thale cress	At_COL2	L81120	NCBI
A.thaliana	Thale cress	At_COL3	NM_128038	NCBI
A.thaliana	Thale cress	At_COL4	NM_122402	NCBI
A.thaliana	Thale cress	At_COL5	AY114006	NCBI
A.thaliana	Thale cress	At_COL6	AY081541	NCBI

A.thaliana	Thale cress	At_COL7	NM_106047	NCBI
A.thaliana	Thale cress	At_COL8	NM_103803	NCBI
A.thaliana	Thale cress	At_COL9	NM_111644	NCBI
A.thaliana	Thale cress	At_COL10	AB023039	NCBI
A.thaliana	Thale cress	At_COL11	NM_117613	NCBI
A.thaliana	Thale cress	At_COL12	NM_113084	NCBI
A.thaliana	Thale cress	At_COL13	NM_130356	NCBI
A.thaliana	Thale cress	At_COL14	NM_128910	NCBI
A.thaliana	Thale cress	At_COL15	NM_102570	NCBI
A.thaliana	Thale cress	At_COL16	NM_102355	NCBI
A.officinalis	Asparagus	Ao_COLa	Unigene 377970	PGN
A.officinalis	Asparagus	Ao_COLb	Aof01-5ms1-b04	PGN
A.officinalis	Asparagus	Ao_COLc	Aof01-10ms1-b05	PGN
B.napus	Oilseed rape	Bn_CO	AY280868	NCBI
B.napus	Oilseed rape	Bn1CON19	AF016010	NCBI
B.napus	Oilseed rape	Bn9CON10	AF016011	NCBI
B.napus	Oilseed rape	Bn_COLa	AY280868	NCBI
B.napus	Oilseed rape	CONST-BN-1	AF230668	NCBI
B.napus	Oilseed rape	Bn_COLb	TC6929	DFCI
B.napus	Oilseed rape	Bn_COLc	CB686270	NCBI
B.napus	Oilseed rape	Bn_COLe	NP1239731	NCBI
B.napus	Oilseed rape	Bn_COLf	TC15426	DFCI
B.napus	Oilseed rape	Bn_COLg	CN831196	NCBI
B.nigra	Black mustard	Bni_COL2	AF269129	NCBI
B.nigra	Black mustard	Bni_COLa	AF269127	NCBI
B.nigra	Black mustard	Bni_COLb	AF269126	NCBI
B.oleracea	Cabbage/wild mustard	Bo_CONST-BO-1	AF230671	NCBI
B.oleracea	Cabbage/wild mustard	Bo_COL1	AM295783	NCBI
B.oleracea	Cabbage/wild mustard	Bo_CO	AM295784	NCBI
B.rapa	Turnip/field mustard	Br_COL2	AY356370	NCBI
B.rapa	Turnip/field mustard	Br_COLa	AY379532	NCBI
B.rapa	Turnip/field mustard	Br_COLb	AY379531	NCBI
B.rapa	Turnip/field mustard	Br_CO	AF230670	NCBI
musa_x_paradisica	Banana	Mp_COL	DQ153049	NCBI

H.vulgare	Barley	Hv_CO1	AF490468	NCBI
H.vulgare	Barley	Hv_CO2	AF490469	NCBI
H.vulgare	Barley	Hv_CO3	AF490473	NCBI
H.vulgare	Barley	Hv_CO4	AF490474	NCBI
H.vulgare	Barley	Hv_CO5	AY082958	NCBI
H.vulgare	Barley	Hv_CO6	AY082961	NCBI
H.vulgare	Barley	Hv_CO7	AY082963	NCBI
H.vulgare	Barley	Hv_CO8	AY082964	NCBI
H.vulgare	Barley	Hv_CO9	AY082965	NCBI
B.vulgaris	Beet	Bv_COLa	TC163	DFCI
B.vulgaris	Beet	Bv_COLb	TC2903	DFCI
B.vulgaris	Beet	Bv_COLc	BQ582975	NCBI
B.juncea	Indian mustard	Bj_COL1	DQ534066	NCBI
C.reinhardtii	Algae	Cr_COL	TC44431	DFCI
G.hirsutum	Cotton	Gh_COLa	TC74594	DFCI
G.hirsutum	Cotton	Gh_COLb	TC77456	DFCI
G.hirsutum	Cotton	Gh_COLc	TC62622	DFCI
G.hirsutum	Cotton	Gh_COLd	TC67724	DFCI
G.hirsutum	Cotton	Gh_COLe	TC63375	DFCI
G.hirsutum	Cotton	Gh_COLf	TC74580	DFCI
G.hirsutum	Cotton	Gh_COLg	TC69728	DFCI
G.hirsutum	Cotton	Gh_COLh	TC70279	DFCI
F.pratensis	Meadow fescue	Fp_COL	AJ833018	NCBI
G.hybrida	Asteraceae	Ghy_COL a	Unigene 323609	PGN
G.hybrida	Asteraceae	Ghy_COL b	Ghy02-14f1-d05	PGN
G.hybrida	Asteraceae	Ghy_COL c	Unigene 322270	PGN
G.hybrida	Asteraceae	Ghy_COL d	Unigene 323595	PGN
G.max	Soybean	Gm_COLa	BE440901	NCBI
G.max	Soybean	Gm_COLb	TC203701	DFCI
G.max	Soybean	Gm_COLc	TC203807	DFCI
G.max	Soybean	Gm_COLd	TC203446	DFCI
G.max	Soybean	Gm_COLe	TC204287	DFCI
G.max	Soybean	Gm_COLf	TC228911	DFCI
G.max	Soybean	Gm_COLg	TC207413	DFCI
G.max	Soybean	Gm_COLh	TC204284	DFCI
G.soja	Wild soybean	Gs_COL	BM521298	NCBI
V.vinifera	Grapevine	Vv_COLa	TC54036	DFCI
V.vinifera	Grapevine	Vv_COLb	TC56447	DFCI
V.vinifera	Grapevine	Vv_COLc	TC67200	DFCI
V.vinifera	Grapevine	Vv_COLd	TC59037	DFCI
V.vinifera	Grapevine	Vv_COLe	TC68605	DFCI
M.crystallinum	Ice plant	Mc_COL	TC5300	DFCI

I.nil	Japanese morning glory	In_CO	AF300700	NCBI
I.batatas	Sweet potato	Ib_COL	CB330801	NCBI
L.sativa	Lettuce	Ls_COLa	QG_CA_CONTIG_5120	CGP
L.sativa	Lettuce	Ls_COLb	TC10014	DFCI
L.sativa	Lettuce	Ls_COLc	TC9842	DFCI
L.sativa	Lettuce	Ls_COLd	BQ844678	DFCI
L.sativa	Lettuce	Ls_COLe	TC8632	DFCI
L.sativa	Lettuce	Ls_COLf	BU008340	DFCI
L.perenne	Perennial ryegrass	Lp_CO	AY600919	NCBI
L.perenne	Perennial ryegrass	Lp_COL1	DQ534011	NCBI
L.temulentum	Darnel ryegrass	Lt_COL	AY553297	NCBI
L.japonicus	Wild legume	Lj_COLa	TC15164	DFCI
L.japonicus	Wild legume	Lj_COLb	TC14920	DFCI
L.japonicus	Wild legume	Lj_COLc	BP044699	DFCI
M.domestica	Apple	Md_COL1	AF052584	NCBI
M.domestica	Apple	Md_COL2	AF052585	NCBI
M.domestica	Apple	Md_COLa	DR996733	NCBI
M.domestica	Apple	Md_COLb	DR994522	NCBI
Z.mays	Maize	Zm_COLa	TC358916	DFCI
Z.mays	Maize	Zm_COLb	TC351450	DFCI
Z.mays	Maize	Zm_COLc	TC353614	DFCI
Z.mays	Maize	Zm_COLd	TC341592	DFCI
Z.mays	Maize	Zm_COLe	AY105193	DFCI
M.truncatula	Barrel medic	Mt_COLa	AC127169	NCBI
M.truncatula	Barrel medic	Mt_COLb	TC101055	DFCI
M.truncatula	Barrel medic	Mt_COLc	TC101481	DFCI
M.truncatula	Barrel medic	Mt_COLd	TC101494	DFCI
M.guttatus	Monkey flower	Mg_COL	Unigene 397001	PGN
N.benthamiana	n/a	Nb_COLa	TC8501	DFCI
N.benthamiana	n/a	Nb_COLb	TC7390	DFCI
N.benthamiana	n/a	Nb_COLc	TC7626	DFCI
A.cepa	Onion	Ac_COLa	CF435233	DFCI
A.cepa	Onion	Ac_COLb	CF444358	DFCI
P.americana	Avocado	Pa_COL	Unigene 210499	PGN
P.hybrida	Petunia	Ph_COLa	TC1039	DFCI
P.hybrida	Petunia	Ph_COLb	CV292862	DFCI
P.vulgaris	Common bean	Pv_COLa	TC341	DFCI
P.vulgaris	Common bean	Pv_COLb	TC2819	DFCI
P.patens	Moss	Pp_COL2	AJ890107	NCBI
P.patens	Moss	Pp_COL3	AJ890108	NCBI

P.patens	Moss	Pp_COL1	AB185925	NCBI
P.abies	Norway spruce	Pa_COL1	AM267544	NCBI
P.sitchensis	Sitka spruce	Ps_COL	TC41907	DFCI
P.taeda	Loblolly pine	Pt_COLa	TC74212	DFCI
P.taeda	Loblolly pine	Pt_COLb	TC68015	DFCI
P.radiata	Monterey pine	Pr_CO	AF001136	NCBI
P.sativum	Umbrella pine	Psa_COLa	AY830921	NCBI
P.sativum	Umbrella pine	Psa_COLb	AY805328	NCBI
P.deltoides	Eastern cottonwood	Pd_CO1	AY515150	NCBI
P.deltoides	Eastern cottonwood	Pd_CO2	AY515151	NCBI
P.deltoides	Eastern cottonwood	Pd_COLa	CX169784	NCBI
Populus	Poplar	Pop_COLa	TC44127	DFCI
Poplar	Poplar	Pop_COLb	TC58179	DFCI
Poplar	Poplar	Pop_COLc	TC63903	DFCI
Poplar	Poplar	Pop_COLd	TC62371	DFCI
Poplar	Poplar	Pop_COLe	TC52728	DFCI
R.sativus	Radish	Rs_COL1	AF052690	NCBI
R.americanum	American blackcurrent	Ra_COLa	Ram01-1ms4-b02	PGN
R.americanum	American blackcurrent	Ra_COLb	Ram01-7ms1-m21	PGN
R.chinensis	China rose	Rc_COL	BI977339	NCBI
S.henryi	n/a	Sh_COL	Unigene 307605	PGN
S.pratensis	Meadow clary	Sp_Hd1	AJ833018	NCBI
S.demissum	Nightshade	Sd_CO	DQ499755	NCBI
S.tuberosum	Potato	St_COLa	TC133338	DFCI
S.tuberosum	Potato	St_COLb	TC137765	DFCI
S.tuberosum	Potato	St_COLc	TC139328	DFCI
S.tuberosum	Potato	St_CO	DQ499754	NCBI
S.bicolor	Sorghum	Sb_COLa	BH245471	NCBI
S.bicolor	Sorghum	Sb_COLb	TC104612	DFCI
S.bicolor	Sorghum	Sb_COLc	TC103306	DFCI
S.bicolor	Sorghum	Sb_COLd	TC96578	DFCI
S.bicolor	Sorghum	Sb_COLe	TC104084	DFCI
Frageria_x_ananassa	Strawberry	Fa_CO	AB211134	NCBI
S.officinarum	Sugarcane	So_COLa	TC57917	DFCI
S.officinarum	Sugarcane	So_COLb	CA282239	DFCI
S.officinarum	Sugarcane	So_COLc	CA301318	DFCI
S.officinarum	Sugarcane	So_COLd	CA246422	DFCI

S.officinarum	Sugarcane	So_COLe	TC61108	DFCI
S.officinarum	Sugarcane	So_COLf	TC57918	DFCI
S.officinarum	Sugarcane	So_COLg	CO373363	DFCI
S.officinarum	Sugarcane	So_COLh	TC52963	DFCI
H.annuus	Sunflower	Ha_COLa	TC17464	DFCI
H.annuus	Sunflower	Ha_COLb	QHM23M12.YG.AB1	CGP
H.annuus	Sunflower	Ha_COLc	TC24334	DFCI
H.annuus	Sunflower	Ha_COLd	DY908026	DFCI
H.annuus	Sunflower	Ha_COLe	CD846649	DFCI
N.tabacum	Tobacco	Nt_COLa	TC5337	DFCI
N.tabacum	Tobacco	Nt_COLb	TC6832	DFCI
S.lycopersicum	Tomato	Sl_CO1	AY490251	NCBI
S.lycopersicum	Tomato	Sl_CO3	AY490253	NCBI
S.lycopersicum	Tomato	Sl_COLa	TC174945	DFCI
S.lycopersicum	Tomato	Sl_COLb	TC179854	DFCI
W.mirabilis	n/a	Wm_COL	Wmi03-10ms3-h02	PGN
T.aestivum	Wheat	Ta_COLa	BE443693	DFCI
T.aestivum	Wheat	Ta_COLb	TC255259	DFCI
T.aestivum	Wheat	Ta_COLc	TC248314	DFCI
T.aestivum	Wheat	Ta_COLd	TC262516	DFCI
T.aestivum	Wheat	Ta_COLe	TC247314	DFCI
T.aestivum	Wheat	Ta_COLf	CA679347	DFCI
T.aestivum	Wheat	Ta_COLg	AL821541	DFCI
T.aestivum	Wheat	Ta_COLh	TC250974	DFCI
T.aestivum	Wheat	Ta_COLi	TC247073	DFCI
T.aestivum	Wheat	Ta_COLj	AL819614	DFCI
T.aestivum	Wheat	Ta_Hd1-1	AB094490	NCBI
Y.filamentosa	Yucca	Yf_COL	Yfi01-3ms4-g04	PGN
O.sativa	Rice	Os_CO1	NM_001054557	NCBI
O.sativa	Rice	Os_CO5	NM_001053934	NCBI
O.sativa	Rice	Os_CO6	NM_001064782	NCBI
O.sativa	Rice	Os_CO9	NM_001055451	NCBI
O.sativa	Rice	Os_Hd1	AB041840	NCBI
O.sativa	Rice	Os_COLa	NM_001069178	NCBI
O.sativa	Rice	Os_COLb	NM_001067109	NCBI
O.sativa	Rice	Os_COLc	NM_001063976	NCBI
O.sativa	Rice	Os_COLd	NM_001063868	NCBI
O.sativa	Rice	Os_COLe	NM_001054510	NCBI
O.sativa	Rice	Os_COLf	NM_001059743	NCBI
O.sativa	Rice	Os_COLg	NM_001057588	NCBI
O.sativa	Rice	Os_COLh	NM_001052609	NCBI
O.sativa	Rice	Os_COLi	NM_001056649	NCBI
O.sativa	Rice	Os_COLj	AP005439	NCBI
O.sativa	Rice	Os_COLk	AAAA01008321	NCBI
O.sativa	Rice	Os_COLl	AC079874	NCBI

A.thaliana	Thale cress	At_TOC1	NP200946	NCBI
O.sativa	Rice	Os_TOC1	AAU20772	NCBI
A.thaliana	Thale cress	At_ZIM	BAA97678	NCBI
A.thaliana	Thale cress	At_ZML1	NP566676	NCBI
A.thaliana	Thale cress	At_ZML2	NP564593	NCBI
O.sativa	Rice	Os_ZMLa	ABF98241	NCBI
O.sativa	Rice	Os_ZMLb	NP001045892	NCBI
O.sativa	Rice	Os_ZMLc	NP001050916	NCBI
O.sativa	Rice	Os_ZMLd	NP001058468	NCBI
O.sativa	Rice	Os_ZMLe	NP001051183	NCBI
A.thaliana	Thale cress	At_APRR5	NP568446	NCBI
A.thaliana	Thale cress	At_APRR9	NP566085	NCBI
A.thaliana	Thale cress	At_APRR3	NP568919	NCBI
A.thaliana	Thale cress	At_APRR7	NP568107	NCBI
O.sativa	Rice	Os_PRRa	ABA91559	NCBI
O.sativa	Rice	Os_PRRb	NP001063760	NCBI
O.sativa	Rice	Os_PRR37	BAD38855	NCBI
O.sativa	Rice	Os_PRRc	AAN64489	NCBI
O.sativa	Rice	Os_PRR73	NP001049760	NCBI
A.thaliana	Thale cress	At_PRR	CAB86035	NCBI
A.thaliana	Thale cress	At_CIL	NP849445	NCBI
A.thaliana	Thale cress	At_CIA2	NP568852	NCBI
H.vulgare	Barley	Hv_CIL	TC134030	DFCI
G.hirsutum	Cotton	Gh_CIL	TC61609	DFCI
C.reinhardtii	algae	Cr_CIL	AV394694	DFCI
Populus	Poplar	Pop_CIL	TC70773	DFCI
S.bicolor	Sorghum	Sb_CIL	BF656873	DFCI
T.aestivum	Wheat	Ta_CIL	TC258233	DFCI
S.tuberosum	Potato	St_CIL	TC146341	DFCI
H.vulgare	Barley	Hv_PPD-H1	AAY42111	NCBI
A.thaliana	Thale cress	At_ASML2	NM_112125	NCBI
H.vulgare	Barley	Hv_ZCCT	DQ492699	NCBI
Triticum monococcum	Wheat	Tm_ZCCT2	AY485968	NCBI
Triticum monococcum	Wheat	Tm_ZCCT1	AY485976	NCBI

CHAPTER 5

Table A9: Accession numbers for phylogenetic analysis of GI genes.

Plant Species	Common Name	Name used	Accession Number	Database
A.formosa_x_A.pubescens	Aquilegia	AfxAp	TC20877	DFCI
Pinus taeda	Pine	Pt	TC105492	DFCI
A. thaliana	Thale cress	At	NM_102124	NCBI
P. sativum	Pea	Ps	EF185297	NCBI
O. sativa	Rice	Os	NM_001048755	NCBI
Z. mays	Maize	Zm GI1A	BK006299	NCBI
Z. mays	Maize	Zm GI1B	BK006298	NCBI
T. aestivum	Wheat	Ta	AF543844	NCBI
L. paucicostata	Duckweed	Lpa	AB210843	NCBI
H. vulgare	Barley	Hv	AY740523	NCBI
L. gibba	Swollen Duckweed	Lg	AB210848	NCBI
G. max	Soybean	Gm	TC260450	DFCI
L. perenne	Perennial ryegrass	Lp	DQ534010	NCBI
P. vulgaris	Common bean	Pv	EF643235	NCBI
L. sativa	Lettuce	Ls	TC27622	DFCI
N. tabacum	Tobacco	Nt	DV162002	NCBI
V. vinifera	Grape	Vv	TC75660	DFCI
Populus	Poplar	Pop	TC124305	DFCI
S. bicolor	Sorghum	Sb	TC113678	DFCI
C. annuum	Pepper	Ca	TC11758	DFCI
S. tuberosum	Potato	St	TC184182	DFCI
S. lycopersicum	Tomato	Sl	TC200218	DFCI
G. hirsutum	Cotton	Gh	TC102876	DFCI
H. annuus	Sunflower	Ha	TC38332	DFCI

CHAPTER 6

Table A10: Accession numbers for phylogenetic analysis of *FKF1*/*ZTL* gene families. All sequences were acquired from NCBI with the exception of Mc_FKF which was acquired from DFCI.

Plant Species	Common name	Abbreviation used	Accession/locus number
A. thaliana	Thale Cress	At_FKF1	NM_105475
T. aestivum	Wheat	Ta_FKF1	DQ923399
O. sativa	Rice	Os_FKF1	NM_001074600
M. crystallinum	Common iceplant	Mc_FKF1	AY371291
P. vulgaris	Common bean	Pv_FKF1	EF643234
M. truncatula	Barrel Medic	Mt_FKF1	TC130448
A. thaliana	Thale Cress	At_ZTL	NM_125119
I. nil	Japanese morning glory	In_ZTL	DQ309278
M. crystallinum	Common iceplant	Mc_ZTL	AY371290
O. sativa	Rice	Os_ZTL	NM_001064973
A. thaliana	Thale Cress	At_LKP2	NM_179652
A. thaliana	Thale Cress	At_CDF1	NM_125637
A. thaliana	Thale Cress	At_FBox	NM_104033
O. sativa	Rice	Os_FBox	NM_001067833

CHAPTER 7

Table A11: Accession numbers for phylogenetic analysis of *FT*/*FT*-like gene family.

Plant Species	Common Name	Abbreviation used	Accession Number/Locus	Database
O. sativa	Rice	Os_FTL8	Os01g10590	RGA
O. sativa	Rice	Os_FTL1	Os01g11940	RGA
O. sativa	Rice	Os_FTL9	Os01g54490	RGA
O. sativa	Rice	Os_FTL5	Os0239064	RGA
O. sativa	Rice	Os_FTL13	Os0213830	RGA
O. sativa	Rice	Os_FTL6	Os04g41130	RGA
O. sativa	Rice	Os_FTL10	Os05g41180	RGA
O. sativa	Rice	Os_FTL3 (RFT)	Os06g06300	RGA
O. sativa	Rice	Os_FTL2 (Hd3a)	Os01g06320	RGA
O. sativa	Rice	Os_FTL12	Os01g35940	RGA

O. sativa	Rice	Os_FTL4	Os09g33850	RGA
O. sativa	Rice	Os_FTL11	Os11g11870	RGA
O. sativa	Rice	Os_FTL7	Os12g13030	RGA
O. sativa	Rice	Os_MFT1	Os01g02120	RGA
O. sativa	Rice	Os_MFT2	Os06g30370	RGA
H. vulgare	Barley	Hv_FT1	DQ100327	NCBI
H. vulgare	Barley	Hv_FT2	DQ297407	NCBI
H. vulgare	Barley	Hv_FT3	DQ411319	NCBI
H. vulgare	Barley	Hv_FT4	DQ411320	NCBI
H. vulgare	Barley	Hv_FT5	EF012202	NCBI
A. thaliana	Thale cress	At_FT	NM_105222	NCBI
A. thaliana	Thale cress	At_TSF	AB027506	NCBI
A. thaliana	Thale cress	At_MFT	NM_101672	NCBI
A. thaliana	Thale cress	At-BFT	NM_125597	NCBI
L. sativa	Lettuce	Ls_FT	CLSS2320	CGP
A. majus	Snapdragon	Am_FT	AJ803471	NCBI
T. monoccum	Wheat	Tm_VRN3	DQ890163	NCBI

Table A12: Accession numbers for phylogenetic analysis of PHYA genes. All sequences were acquired from NCBI with the exception of At_PHYB, At_PHYC, At_PHYD and At_PHYE which were acquired from TAIR.

Plant Species	**Common Name**	**Abbreviation used**	**Accession Number**
A. thaliana	Thale cress	At_PHYA	NM_001123784
A. thaliana	Thale cress	At_PHYB	At2g18790
A. thaliana	Thale cress	At_PHYC	At5g35840
A. thaliana	Thale cress	At_PHYD	At4g16250
A. thaliana	Thale cress	At_PHYE	At4g18130
P. sativum	Pea	Ps_PHYA	AY688953
G. max	Soybean	Gm_PHYA	EU428746
N. tabacum	Tobacco	Nt_PHYA	X66784
S. tuberosum	Potato	St_PHYA	DQ208423
S. lycopersicum	Tomato	Sl_PHYA	AJ001914
H. vulgare	Barley	Hv_PHYA	DQ201158
O. sativa	Rice	Os_PHYA	AB109891
Z. mays	Maize	Zm_PHYA	AY234826
S. bicolor	Sorghum	Sb_PHYA	SBU56729

Appendix 5: Onion gene sequences

AcCOL (green=B-box 1, red=B-box 2, pink=CCT domain):

CATAATACATAACAAAAAAAAATGGCGGTAAACTACTGGGGGTTAACAGCAAAGCACTG
CGCCAATTGCGTCTCATCTCCTGCCGTCATGTACTGTAGAACCGACGCCACCTACCTCTGC
AGCACGTGCGAAGCGCGATCCCACTCGTCGCACGTGCGCGTGTGGCTGTGCGAGGTCTGC
GAGCAGGCGCCGGCGGCTGTGACGTGCAAGGCCGACGCGGCGACGCTGTGCGTTACGTG
CGACGCGGACATCCACGCTGCGAACCCACTGGCTAGGAGGCACGAACGGGTGCCAGTGG
TACCGGTGGGGAATCCGACGGTGCAGGTTAAGGAGGATCTGTTTGGCGAAGATGGAGAA
GGTGACACGTGGAAGGGGATGATGGTGGATTTGAACTGCTTTGGTGGATTCAGCAACGA
ATTAGTGGATCCGTATTTGGATTTGGATGGTAATGGGGATGGATTGGTGCCTGTCCAGGA
GAAGCATGTTTATGGGTACGGGTATAGGCAGGAGAAAGGGACGATGATGCCAAAAGGG
ACGGTGGATATTGATTTTGGAGCTGTTGGGAAAGGGGATGGGTATGGTTGTGGCCATGGT
GGATATACAGTTGGAGTTCAGTCCATGAGCCATAGTACTACTGTATCTTCATCAGAAGCT
GGAGTCGTGCCAGATAATAGCAGCTCCATGGCAGTAGCTGATGTATCCAACCCGTACAG
CAGACCTTTGCCAAATCCAATGGATGCAATGGACAGAGAAGCAAGAGTTATGCGATACA
GAGAGAAGCGAAAGAATAGAAAGTTTGAGAAGACCATACGCTACGCTTCCAGAAAGGC
CTACGCAGAGACTCGGCCCAGGATTAAAGGAAGGTTCGCCAAACGGGTCGACAACGATT
CTTATGCGGATCCAATGCACTCAGTGATTAATGCCTCCACTGCTTTCATGAATGACAGCG
GGTATGGGGTTGTCCCATCCTTTTGATGCAGGATCCCACATGGTGTATTATTTTGTAAAAT
GTAAGATTTTATTTTGTTTTCTGCATTTCGGTGTATATGGGAAAAGTAAACTTGCTTTTAA
AATTTGCAAGATAATTATAATTTCAGTCCTTCAGTTTT

AcGIa:

GTGCATAATTGAAGACGGTTTTGAAGATGTCTGTTGTTTGTGAGAAATGGATCGATGGGT
TACAGTATTCTTCATTGTTGTGGCCCCACCTCAGGATGAGCATCAGAGACAGGCACAAAT
CTTGGCTTATGTTGAGTATTTTGGCCAGTTCACCTCTGAACAATTTCGGAAGATGTGGCAC
AGTTGATTCAGAACCATTATCCATCTAAAGAGCAGCGTCTACTGGACGAAGTATTGGCAA
CTTTTGTTCTTCATCATCCAGAGCATGGGCATGCTATTGTCCATCCTATACTTTCTTGTATA
ATTGATGGAACATTGGTGTATGATAAACATGATCCTCCTTTCAGCTCTTTCATATCTCTAT
TTAACCAAAATTCTGAGAAAGAGTACTCTGAACAATGGGCCCTGGCATGCGGAGAAATT
TTGCGAGTACTCACGCACTACAATCGTCCAATATTCAAAGCTGAGCATCAAAATAAGATT
GAAAGGCTCAGCAGCTGTGATCAAGCAACCACAAGTGACCCTAAAGAGGAGAAAGTAC
ATCATTCATCCATGCCTGAAAATGACAGGAAGCCCGTGAGAGCTTTATCTCCTTGGATCG
CAGATATATTAATAACCTCACCTTTGGGGATCAGAAGTGATTACTTCCGATGGTGTGGTG
GGGTCATGGGAAAGTATGCTGCTGGCGGCGAACTGAAGCCTCCAATAACTAGTAGCAGC
CGAGGATCTGGAAAACATCCTCAGCTAATGCAATCGACACCAAGATGGGCTGTGGCAAA
TGGAGCAGGTGTGATATTAAGTGTTTGTGATGAAGAAGTAGCTCGCTATGAAACTGCAA
ACCTAACAGCTGCGGCTGTTCCTGCTCTATTACTTCCTCCTCCTACAAATGAACACCTTGT

AGCAGGCTTACCCGCCCTTGAACCATATGCTCGGTTATTTCACAGATATTATGCTATTGCA
ACTCCAAGTGCTACCCAGAGGCTGCTTCTTGGACTTCTTGAAGCACCACCATCATGGGCA
CCAGATGCGCTTGATGCTGCTGTGCAACTTGTTGAACTACTTAGAGCAGCTGAAGATTAT
GCATCAGGCATGAAGCTTCCAAGAAACTGGTTACATCTACATTTCTTGCGAGCAATTGGA
ACTGCAATGTCGATGAGAGCTGGAATAGCAGCAGATGCGGCTGCAGCTTTACTCTTTCGT
ATTCTTTCTCAACCTACTTTGCTCTTTCCTCCTATAAGGTTTGCTGAGGGAGTCGAAGTGC
ACCATGAACCTCTGGGTGGTTACATATCTTCCTATAAAAAACAGTTAGAAGTGCCTGCTG
CAGAAGCAACTATTGAAGCAACTGCACAAGGCATTGCTTCAATGCTCTGTGCTCATGGTC
CTGAAGTAGAGTGGCGAATATGCACTATCTGGGAAGCTGCCTACGGTCTTCTCCCTCTAA
CTTCATCTGCAGTCGACTTACCCGAAATAGTAATTTCAACTCCTTTACAACCACCTGCTCT
TTCATGGAGTTTGTACCTTCCTTTACTTAAAGTTCTTGAATATTTACCTAGAGGAAGCCCA
TCCGAAGCATGCCTAATGAGAATATTTGTAGCTACGGTCGAAGCAATTCTAAGTAGAACA
TTTCCACCCGAAAACACAGTAGAGCAATCAAAAAGGACAAGAAGTCAAAGTGGCACATG
GTCTTCCACTAAAAATTTAGCTGTAGCTGAACTTCGTACAATGATACACACTCTTTTTATT
GAATCATGTGCTTCCATGGATCTTGCATCTAGACTCCTATTCGTCGTGTTAACTGTTTGTG
TTAGTCATGAAGCTTCACCTAATGGTAGTAAAAGGCCTACTGGTAATGAAACCGAACCTC
ATATGGGTAATGGTAAAGTAACTATGAAAAGGAAGAAGAAAAGACAGGGACCAGTAGC
TACTTTTGATTCTTATGTGCTGGCTGCTGCTTGTGCTCTTTCTTTTGAGTTACAATTATTCC
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Appendix 6: Statistics tables

Key: d.f.=degrees of freedom, s.s.=sum of squares, m.s.=mean squares, v.r.=variance ratio, F pr.=F probability

CHAPTER 3

Tables A13-A17: ANOVA tables showing the significance of the differences in bulbing ratio between treatments.

Table A13: Controls (constant LD/constant SD)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days_from_sowing	12	55.8840	4.6570	24.10	<.001
Treatment	1	147.1466	147.1466	761.44	<.001
Days_from_sowing.Treatment	12	49.8040	4.1503	21.48	<.001
Residual	182	35.1711	0.1932		
Total	207	288.0057			

Table A14: Transfer 1

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days_from_sowing	11	8.07675	0.73425	36.83	<.001
Treatment	3	33.90188	11.30063	566.83	<.001
Days_from_sowing.Treatment	33	8.50842	0.25783	12.93	<.001
Residual	336	6.69871	0.01994		
Total	383	57.18577			

Table A15: Transfer 2

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days_from_sowing	10	3.76601	0.37660	12.01	<.001
Treatment	3	36.07488	12.02496	383.83	<.001
Days_from_sowing.Treatment	33	8.50842	0.25783	12.93	<.001
Residual	292	9.14815	0.03133		
Total	335	54.34827			

Table A16: Transfer 3

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days_from_sowing	9	1.20431	0.14492	3.22	<.001
Treatment	3	40.09390	13.36463	296.77	<.001
Days_from_sowing.Treatment	27	2.69278	0.09973	2.21	<.001
Residual	275	12.38424	0.04503		
Total	314	56.47524			

Table A17: Transfer 4

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days_from_sowing	8	0.64336	0.08042	2.35	0.019
Treatment	3	46.89356	15.63119	456.44	<.001
Days_from_sowing.Treatment	24	1.66453	0.06936	2.03	0.004
Residual	246	8.42458	0.03425		
Total	281	57.62603			

Tables A18-A22: ANOVA table showing the significance of the differences in bulb diameter between treatments.

Table A18: Controls (constant LD/constant SD).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days_from_sowing	12	32.41195	2.70100	127.17	<.001
Treatment	1	14.86010	14.86010	699.67	<.001
Days_from_sowing.Treatment	12	2.69718	0.22477	10.58	<.001
Residual	182	3.86546	0.02124		
Total	207	53.83468			

Table A19: Transfer 1

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days_from_sowing	11	26.07932	2.37085	103.63	<.001
Treatment	3	23.39769	7.79923	340.89	<.001
Days_from_sowing.Treatment	33	5.21511	0.15803	6.91	<.001
Residual	336	7.68726	0.02288		
Total	383	62.37938			

Table A20: Transfer 2

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days_from_sowing	10	15.88159	1.58816	57.42	<.001
Treatment	3	23.75720	7.91907	286.30	<.001
Days_from_sowing.Treatment	30	2.57543	0.08585	3.10	<.001
Residual	292	8.07663	0.02766		
Total	335	50.29085			

Table A21: Transfer 3

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days_from_sowing	9	8.08150	0.89794	26.95	<.001
Treatment	3	23.62866	7.87622	236.43	<.001
Days_from_sowing.Treatment	27	0.84631	0.03134	0.94	0.553
Residual	275	9.16115	0.03331		
Total	314	41.71762			

Table A22: Transfer 4

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days_from_sowing	8	4.26085	0.53261	17.78	<.001
Treatment	3	26.17614	8.72538	291.21	<.001
Days_from_sowing.Treatment	24	0.57007	0.02375	0.79	0.745
Residual	246	7.37087	0.02996		
Total	281	38.37793			

Tables A23-A27: ANOVA tables showing the significance of the differences in neck diameter ratio between treatments.

Table A23: Controls (constant LD/constant SD)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days_from_sowing	12	7.83510	0.65292	51.84	<.001
Treatment	1	1.64845	1.64845	130.89	<.001
Days_from_sowing.Treatment	12	1.49833	0.12486	9.91	<.001
Residual	182	2.29219	0.01259		
Total	207	13.27407			

Table A24: Transfer 1

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days_from_sowing	11	6.25397	0.56854	37.74	<.001
Treatment	3	2.91218	0.97073	64.44	<.001
Days_from_sowing.Treatment	33	2.50899	0.07603	5.05	<.001
Residual	336	5.06128	0.01506		
Total	383	16.73643			

Table A25: Transfer 2

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days_from_sowing	10	5.36371	0.53637	41.41	<.001
Treatment	3	3.11340	1.03780	80.12	<.001
Days_from_sowing.Treatment	30	1.75685	0.05856	4.52	<.001
Residual	292	3.78247	0.01295		
Total	335	14.01642			

Table A26: Transfer 3

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days_from_sowing	9	4.40908	0.48990	31.86	<.001
Treatment	3	2.72346	0.90782	59.03	<.001
Days_from_sowing.Treatment	27	1.56552	0.05798	3.77	<.001
Residual	275	4.22922	0.01538		
Total	314	12.92728			

Table A27: Transfer 4

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days_from_sowing	8	3.07713	0.38464	20.41	<.001
Treatment	3	3.39957	1.13319	60.12	<.001
Days_from_sowing.Treatment	24	0.98489	0.04104	2.18	0.002
Residual	246	4.63687	0.01885		
Total	281	12.09845			

CHAPTER 4

Tables A28-A31: ANOVA tables showing the significance of the differences in flowering time between plants transformed to over-express *AcCOL* and non-transformed controls.

Table A28: *co-2* transgenic lines (days to flower)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Line	15	122.956	8.197	2.20	0.013
Residual	79	294.033	3.722		
Total	94	416.989			

Table A29: *co-2* transgenic lines (leaf number)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Line	15	113.888	7.593	1.78	0.052
Residual	79	336.533	4.260		
Total	94	450.421			

Table A30: L*er* transgenic lines (days to flower)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Line	14	188.827	13.488	9.06	<.001
Residual	80	119.131	1.489		
Total	94	307.958			

Table A31: L*er* transgenic lines (leaf number)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Line	14	87.2236	6.2303	6.36	<.001
Residual	80	78.3974	0.9800		
Total	94	165.6211			

CHAPTER 5

Tables A32-A36: Regression analyses showing the significance of the differences in *AcGI* expression between LD and SD grown plants and between varieties.

Table A32: Agrifound Dark

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	7	335.90	47.986	16.51	<.001
Residual	20	58.13	2.906		
Total	27	394.02	14.593		
Change	-2	-28.66	14.332	4.93	0.018

Table A33: Candy F_1

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	7	998.2	142.599	20.97	<.001
Residual	20	136.0	6.901		
Total	27	1134.2	42.008		
Change	-2	-84.2	42.081	6.19	0.018

Table A34: Renate F_1

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	7	70.28	10.0395	15.59	<.001
Residual	26	16.74	0.6438		
Total	33	87.01	2.6368		
Change	-2	-5.48	2.7409	4.26	0.025

Table A35: Between varieties, LDs

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	11	607.8	55.258	10.92	<.001
Residual	33	167.1	5.062		
Total	44	774.9	17.611		
Change	-4	-14.3	3.564	0.70	0.595

Table A36: Between varieties, SDs

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	11	1112.28	101.116	76.15	<.001
Residual	33	43.82	1.328		
Total	44	1156.10	26.275		
Change	-4	-6.19	1.547	1.16	0.344

CHAPTER 6

Tables A37-A40: ANOVA tables showing the significance of the differences in flowering time between plants transformed to over-express *AcFBox* and non-transformed controls.

Table A37: *fkf1-1* transgenic lines (days to flower)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Line	15	807.697	53.846	6.56	<.001
Residual	82	672.833	8.205		
Total	97	1480.531			

Table A38: *fkf1-1* transgenic lines (leaf number)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Line	15	332.115	22.141	2.94	<.001
Residual	82	616.875	7.523		
Total	97	948.990			

Table A39: Col transgenic lines (days to flower)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Line	14	127.619	9.116	2.78	0.002
Residual	76	248.908	3.275		
Total	90	376.527			

Table A40: Col transgenic lines (leaf number)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Line	14	88.890	6.349	5.41	<.001
Residual	76	89.242	1.174		
Total	90	178.132			

Tables A41-A45: Regression analyses showing the significance of the differences in *AcFKF* expression between LD and SD grown plants and between varieties.

Table A41: Agrifound Dark

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	7	26272	3753.12	52.11	<.001
Residual	20	1441	72.03		
Total	27	27712	1026.38		
Change	-2	-281	140.52	1.95	0.168

Table A42: Candy F_1

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	7	5377	768.16	11.59	<.001
Residual	20	1326	66.30		
Total	27	6703	248.27		
Change	-2	-208	103.96	1.57	0.233

Table A43: Renate F_1

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	7	121.5845	17.36921	1337.02	<.001
Residual	8	0.1039	0.01299		
Total	15	121.6884	8.11256		
Change	-2	-0.2103	0.10516	8.09	0.012

Table A44: Between varieties, LDs

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	11	12875	1170.46	15.09	<.001
Residual	24	1861	77.55		
Total	35	14736	421.04		
Change	-4	-754	188.57	2.43	0.075

Table A45: Between varieties, SDs

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	11	24237.2	2203.38	58.41	<.001
Residual	24	905.4	37.72		
Total	35	25142.5	718.36		
Change	-4	-544.4	136.11	3.61	0.019

Appendix 7: Published papers

Functional Genomics of Photoperiodic Bulb Initiation in Onion (*Allium cepa)*

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Keywords: *CONSTANS*, *GIGANTEA*, *FKF1*, bulbing, flowering, daylength.

Abstract

Bulb initiation is a process which is photoperiodically driven in temperate onions, drawing parallels with flowering. Flowering is well characterised at molecular and genetic levels and in photoperiodic floral induction occurs when photoreceptors interact with the circadian clock, regulating the expression of the *CONSTANS* (*CO*) gene, which itself regulates the expression of the floral pathway integrating genes *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) leading to floral initiation. The *CO* gene has been characterised in both short-day and long-day plants and has also been postulated to be involved in potato tuberisation; hence a role in bulb initiation is hypothesised. The onion genome is very large with a high level of duplication, presenting challenges for any molecular-based study. The aim of this study is to test the hypothesis that genes controlling daylength response are conserved between model plants and onion and between different end-processes (comparing bulbing with flowering). Bulbing ratios have been used to measure the differential response of onion 'Renate F1' plants to long and short-days. Diurnal expression patterns of genes homologous to *Arabidopsis thaliana* flowering time genes have been examined using quantitative RT-PCR. Phylogenetic analyses have been conducted to validate the identity of the homologues. Molecular and phylogenetic data suggests that an onion *GIGANTEA* (*GI*) homolog has been found. Peaks of expression of ZT10 in long-days and ZT7 in short-days mirror the expression of *Arabidopsis GI*. The data also suggests that an onion *CO*-like gene and a gene related to *FLAVIN-BINDING, KELCH REPEAT, F-BOX 1* (*FKF1*) have also been found. A cDNA library screen is underway in order to find other members of the *CO/CO*-like gene family in onion.

INTRODUCTION

Although much is known about the physiological aspects of onion development, little work has been done on the genetics. The onion is a biennial plant, the bulb being an overwintering stage of the life cycle (Lancaster et al., 1996). The physiology of bulb initiation has been studied extensively and is photoperiodically driven in temperate onions, a process which has parallels with flowering (Mettananda and Fordham, 1997). Flowering has been well characterised at molecular and genetic levels. The flowering time genes in *Arabidopsis* mainly function through six different pathways: autonomous; vernalization; gibberellin; temperature; light quality and photoperiod (Jack, 2004). It is the photoperiod pathway which is of interest for this study (Fig.1). In photoperiodic flowering, light

interacts with the circadian clock (through *PHYTOCHROME* and *CRYPTOCHROME* genes) and the timing of the clock is controlled by feedback loops. *CO* expression is high at the end of long-days and the CO protein is degraded at night. *CO* regulates the expression of the floral integrating genes *FT* and *SOC1* leading to floral initiation. 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 many species including both short-day (SD) and long-day (LD) plants. In addition to flowering, *CO* has also been postulated to be involved in potato tuberisation, a different photoperiod response. Hence, a role in bulb initiation is hypothesised. Other important genes in the photoperiodic pathway are *FKF1* and *GI*. Both genes are circadian regulated and regulate flowering by regulating *CO* expression (Imaizumi et al., 2003; Fowler et al., 1999).

The onion genome is very large, about 36 and 107 times larger than rice and *Arabidopsis* respectively (Ohri et al., 1998). The level of duplication in the onion genome is reported to be greater than for other diploids such as *Arabidopsis*, barley, tomato and rice (King et al., 1998). However, it is reported to be less than the duplication in maize, soybean and some *Brassica* species. The relatively high level of duplication may suggest that the onion is of polyploid origin. However, there is no evidence for this and it has been proposed that duplication is due to tandem, transpositional or RNA-mediated retropositional duplication. The high level of duplication presents challenges for molecular-based studies such as this one. However, an onion EST database has been set up which contains potential homologues of *CO*, *FKF1* and *GI* (DFCI, 2003).

The major aim of this study is to test the hypothesis that: the genes controlling daylength response are conserved between the model plant *Arabidopsis* and onion and hence between the different end-processes (bulbing and flowering). Gaining a greater knowledge of the daylength response in onion is important for adapting new cultivars for growth at different latitudes as well as aiding germplasm screening for choice of current cultivars.

MATERIALS AND METHODS

In order to measure the response to daylength, 'Renate F1' onions were grown in LD (16 hours of light) and SD (12 hours of light) conditions within a glasshouse. Plants in both daylengths were subjected to 8 hours of natural light plus an extension using low level incandescent light in photoperiod chambers. Bulb and neck diameter measurements were taken using callipers and 'Bulbing ratios' calculated using the following equation (Clark and Heath, 1962):

$$\frac{\text{Maximum bulb diameter}}{\text{Neck diameter}}$$

Bulb diameters were measured twice (in two different planes) and an average taken. This is to allow for the fact the not all bulbs are round. Plants are classed as bulbing when the bulbing ratio reaches a value greater than 2. Means, standard deviations and standard errors were calculated.

Initially, EST clones of onion putative photoperiod response genes were sequenced using gene-specific primers (Table 1). To measure the differential expression of these genes, 'Renate F1' onions were grown in LD (16 hours of light) and SD (8 hours of light) conditions. Leaf samples were taken approximately every

3 hours over a 48-hour period. Material was pooled from 3 different plants and this was carried out in duplicate. Harvest points were tailored to the predicted expression peaks of specific genes. Total RNA was extracted and DNase treated. First strand cDNA was then synthesised from 2µg of total RNA. Quantitative real-time PCR was carried out using a BIO-RAD I-cycler. The PCR amplification was carried out using 50 cycles and annealing/extension temperatures deemed appropriate for the primer pair. Fluorescence was detected using SYBR green. Data was normalised to the expression of *elongation factor 1α* (*ef1α*).

Neighbour-joining trees (NJ-trees) were constructed in order to support the expression data. All trees were based on amino acid sequences and constructed using Clustal X (Thompson et al., 1997). Tree Explorer was used to view and edit phylogenetic trees (Tamura, 2005).

RESULTS AND DISCUSSION

The bulbing response of onion is controlled by photoperiod (Fig. 2). A daylength of 16 hours is sufficient to stimulate bulbing. This is similar to the situation observed in flowering plants such as *Arabidopsis* so it can be hypothesised that the daylength sensing mechanism, involving *CO* and its regulatory genes, is also conserved.

The expression of onion gene DQ45 is seen to peak around ZT10 in LD's compared with ZT7 in SD's (Fig. 3a). The expression of *Arabidopsis GI* has been shown to peak at ZT10 in long-days and ZT8 in short-days (Fowler et al., 1999). This data suggests that DQ45 is the onion *GI* homolog. Phylogenetic analyses support this theory as the gene falls within a clade containing other monocot *GI* genes (Fig 3b). *GI* is involved in the regulation of *CO* expression within the photoperiodic flowering pathway (Fowler et al., 1999) and an involvement in bulbing can thus be predicted. Future work will involve transforming an *Arabidopsis gi* mutant with onion DQ45 to see whether the late flowering phenotype of the mutant can be complemented.

The expression profile of onion gene AV44 shows various peaks in LD and SD without an obvious pattern (Fig 4a). This is contrary to *Arabidopsis FKF1* which shows distinct peaks around ZT10 in LD's and ZT7 in SD's (Imaizumi et al., 2003). This suggests that AV44 is not an *FKF1* homolog. Phylogenetic analyses support this theory and suggest that this gene may be an F-box gene related to *FKF1* (Fig 4b).

The expression profile for onion gene BR20 also shows no obvious trend; various peaks are seen in LD and SD (data not shown). *Arabidopsis CO* shows clear expression peaks around ZT16 in LD's and ZT20 in SD's (Suárez-López et al., 2001). *CO* belongs to a gene family consisting of 17 genes in *Arabidopsis* and rice (Griffiths et al., 2003). Phylogenetic analyses suggest onion BR20 may be a homolog of *CO*-like 4 (data not shown). This gene may still be of interest as *CO*-like genes have been shown to affect flowering time (Cheng and Wang, 2005). In addition to this, different members of this gene family may be involved in bulbing and/or flowering in onion. Onion BR20 has been transformed into *Arabidopsis co-2* mutant and wild-type plants to assess any function in the regulation of flowering time. Current work is focussed on searching for further members of the onion *CO/CO*-like gene family. A cDNA library is currently being screened and any positive clones will be sequenced and analysed using both molecular and phylogenetic approaches.

It is hypothesised that the genes which control the photoperiodic control of flowering also control bulbing. Various candidate genes have been characterised and one specific gene, onion putative *GI*, is expressed in a similar manner to *Arabidopsis GI*. The function of an onion *CO*-like gene is being investigated further. Further work is required to characterise other genes in the photoperiod pathway. Future work will also include assessing the expression of potential photoperiod response genes in SD and day-neutral onion cultivars.

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Tables

Table 1. Onion EST sequencing. An asterisk indicates that the clone is a full-length gene.

EST Identifier	*Arabidopsis* Gene	Percentage Identity (amino acid)
BR20*	*CO/CO*-like 4	35.5/48.1
AV44*	*FKF1*	47.3
DQ45	*GI*	65.4

Figures

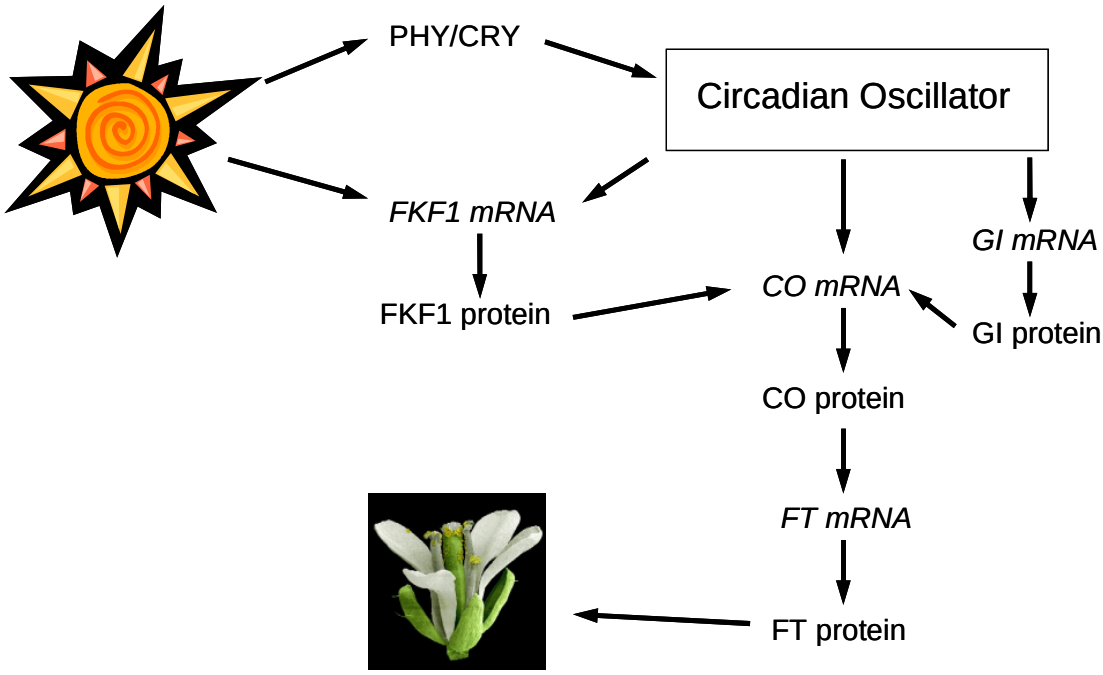


Fig.1. Photoperiodic control of flowering in *Arabidopsis*.

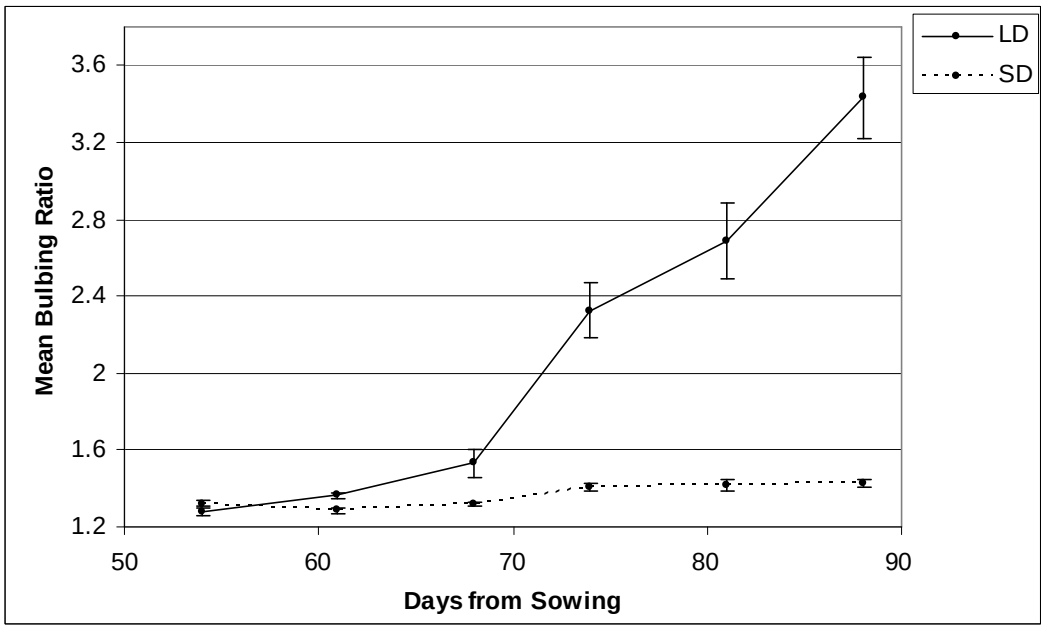
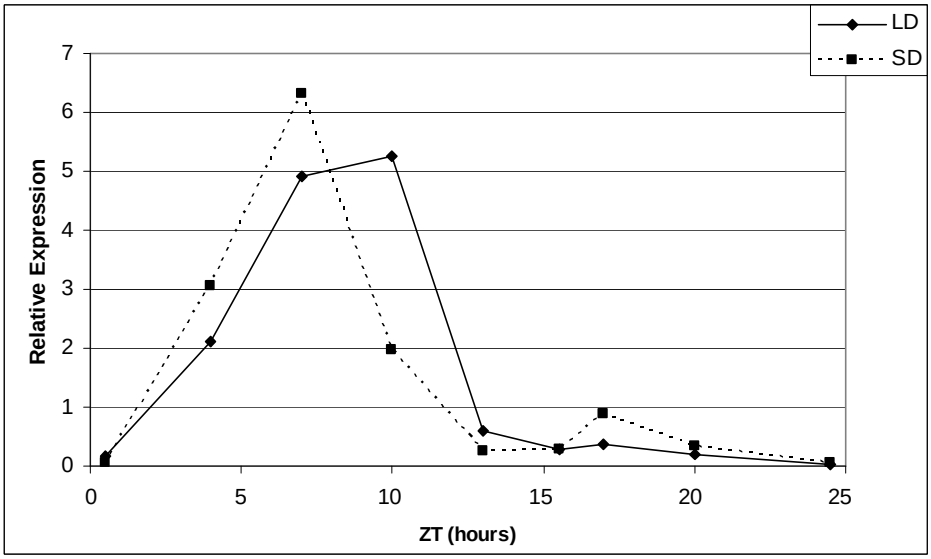
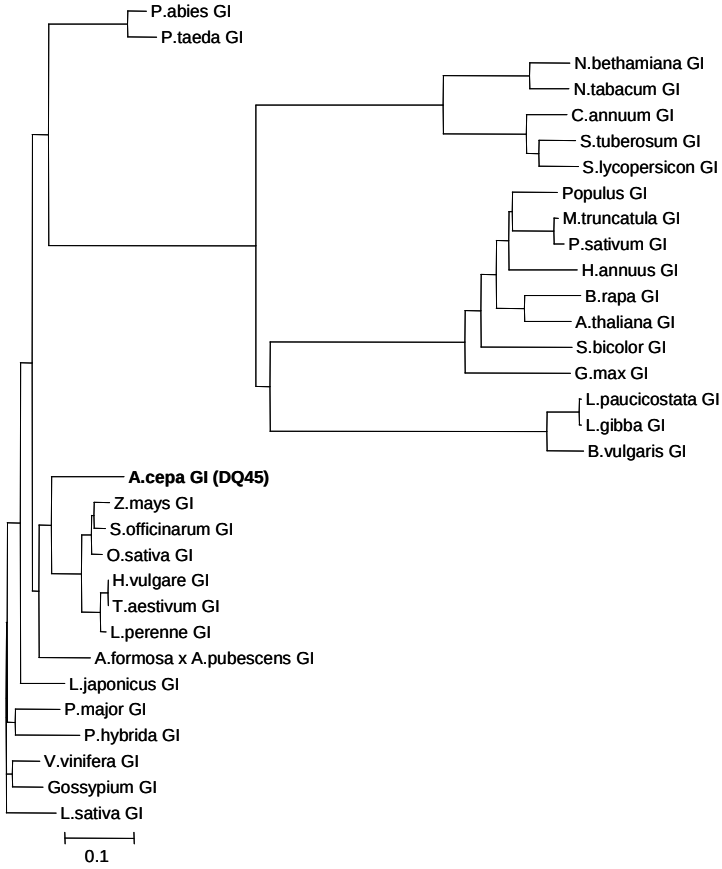


Fig. 2. The response of onion 'Renate F1' plants to different daylengths. Plants were grown in LD's (16 hours of light) and SD's, 12 hours of light) conditions. Error bars represent the standard error of the mean.

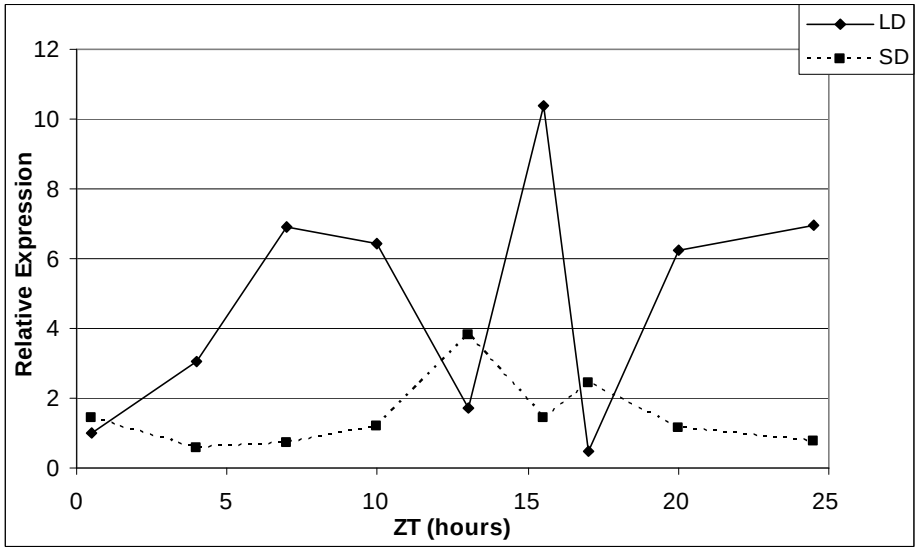


(a)

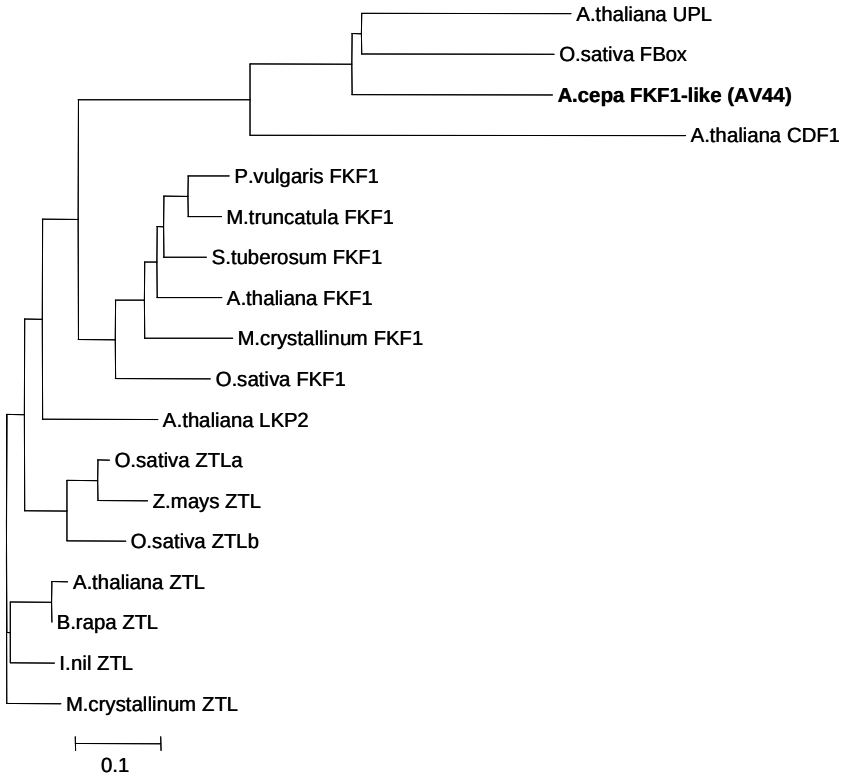


(b)

Fig. 3. Onion DQ45 (putative GI). (a) Differential expression in a 24-hour period in LD (16 hours of light) and SD (8 hours of light) conditions. (b) Phylogenetic analysis of GI genes from different plant species.



(a)



(b)

Fig. 4. Onion AV44 (putative FKF1). (a) Differential expression in a 24-hour period in LD (16 hours of light) and SD (8 hours of light) conditions. (b) Phylogenetic analysis of FKF1 genes from different plant species. Other closely related genes are included in this analysis. *ZTL=ZEITLUPE*; *UPL=UBIQUITIN-PROTEIN LIGASE*; *CDF1= CYCLING DOF FACTOR 1*; *LKP2= LOV KELCH PROTEIN 2*.