Genetic Regulation of Daylength Adaptation and Bulb Formation in Onion (*Allium cepa* L.)

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DECLARATION

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work presented (including data generated and data analysis) was carried out by the author. A small amount of data based on the preliminary results presented in this thesis was used to produce a short article for *Acta Horticulturae* (Appendix XV), which will be published in a book titled 'VII International Symposium on Edible Alliaceae'.

The thesis is the final product of my participation in the Midlands Integrative Biosciences Training Partnership (MIBTP) PhD Programme for the session from 2012-2016 based at the School of Life Sciences, the University of Warwick, UK.

ABSTRACT

Genetic studies aimed at onion improvement have been limited because of outcrossing, high heterozygosity and a very large genome size with a high level of repetitive DNA. Onion bulb initiation is photoperiod-dependent, which places a significant barrier to adapting new varieties for growth at different latitudes. In comparison to photoperiodic regulation of flowering, relatively little is known about genetic regulation of the bulbing process. This project aims to test the hypothesis that the genetic regulation of bulb formation in response to daylength is analogous to the daylength regulation of flowering and to identify genes involved in daylength adaptation in onion. A comprehensive set of developmental, diurnal and spatial mRNA and quantitative expression experiments were carried out to investigate the bulbing response.

Bulbing ratios were used to measure the bulbing response of onion plants and the reversibility of the bulbing process under long day (LD) and short day (SD) conditions. RNA-Seq analysis provided a large number of differentially expressed transcripts in onion in response to daylength. Five *FT* and three *COL* genes were identified in onion including two novel *COL* sequences. *AcFT1* was expressed in LD, which might induce bulb formation, while *AcFT4* was expressed in SD, which might inhibit bulb formation. *AcFT5* and *AcFT6* were expressed in LD and might also be involved in bulb formation itself. *FKF1, GI* and *COL2* showed good diurnal expression patterns consistent with photoperiod sensing and regulation of *FT1*. All *FT* genes exhibited different diurnal expression patterns peaking at different times of the day. *FKF1*, *COL2*, *COL3*, *FT1*, *FT4*, *LFY* and *GA3ox1* genes showed distinctive patterns of tissue specific expression in onion. *FT* genes did not show any variation in expression that would account for the difference in critical daylength between the LD and SD varieties of onion.

LIST OF ABBREVIATIONS

α	alpha
β	beta
<	less than
>	greater than
=	equals
°C	degrees Celsius
%	per cent
µg	micrograms
µl	microlitres
µM	micromolar
AcCOL1	*Allium cepa CONSTANS LIKE 1*
AcCOL2	*Allium cepa CONSTANS LIKE 2*
AcCOL3	*Allium cepa CONSTANS LIKE 3*
ACT	*ACTIN*
ACT2	*ACTIN 2*
ACT12	*ACTIN 12*
AcTUB	*Allium cepa TUBULIN*
AcFKF1	*Allium cepa FLAVIN-BINDING, KELCH REPEAT, F-BOX 1*
AcGI	*Allium cepa GIGANTEA*
AcFT1	*Allium cepa FLOWERING LOCUS T 1*
AcFT2	*Allium cepa FLOWERING LOCUS T 2*
AcFT3	*Allium cepa FLOWERING LOCUS T 3*
AcFT4	*Allium cepa FLOWERING LOCUS T 4*
AcFT5	*Allium cepa FLOWERING LOCUS T 5*
AcFT6	*Allium cepa FLOWERING LOCUS T 6*
AcLFY	*Allium cepa LEAFY*
ALL	*ALLINASE*
ANOVA	analysis of variance
AP1	*APETALA 1*
AP2	*APETALA 2*
AP3	*APETALA 3*

ASCO	S-alk(en)yl-L-cysteine sulphoxides
AtGA3ox1	*Arabidopsis thaliana GIBBERELLIN 3-OXIDASE 1*
AtGA3ox2	*Arabidopsis thaliana GIBBERELLIN 3-OXIDASE 2*
AtGA3ox3	*Arabidopsis thaliana GIBBERELLIN 3-OXIDASE 3*
AtGA3ox4	*Arabidopsis thaliana GIBBERELLIN 3-OXIDASE 4*
ATPS	*ATP-SULPHURYLASE*
BBSRC	Biotechnology and Biological Sciences Research Council
B locus	bolting gene locus
bp	base pairs
CCT	*CO*, *CO*-like and *TOC1*
CDF1	*CYCLING DOF FACTOR 1*
CDL	Critical day length
CDS	Coding sequence
CE	Controlled environment
cDNA	copy DNA
cm	centimetre
CO	*CONSTANS*
COL	*CONSTANS LIKE*
conz1	*CONSTANS OF ZEA MAYS 1*
CRD	Completely Randomised Design
CRY1	*CRYPTOCHROME 1*
CRY2	*CRYPTOCHROME 2*
CTAB	cetyltrimethylammonium bromide
d	day
DAM	*DORMANCY ASSOCIATED MADS-BOX*
DFS	Days from sowing
DAT	Days after transfer
df	degrees of freedom
DNA	Deoxyribonucleic acid

dNTP	Deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EF1 α	*ELONGATION FACTOR 1 ALPHA*
EST	Expressed Sequence Tag
et al.	and others
FKF1	*FLAVIN-BINDING, KELCH REPEAT F-BOX*
FLC	*FLOWERING LOCUS C*
FST-1	*SUCROSE 1-FRUCTOSYLTRANSFERASE-LIKE*
FT	*FLOWERING LOCUS T*
g	grams
GA	Gibberellic acid
GA3ox	*GIBBERELLIN 3-OXIDASE*
GA3ox1	*GIBBERELLIN 3-OXIDASE 1*
GENT	Gentamycin
GFP	Green Fluorescent Protein
GGCS	*GAMMA-GLUTAMYLCYSTEINE SYNTHETASE*
GGTP	*GAMMA-GLUTAMYL TRANSPEPTIDASE*
GGTF	*GAMMA-GLUTAMYL TRANSFERASE*
GI	*GIGANTEA*
GSTF	*GLUTATHIONE S-TRANSFERASE*
G6PDH	*GLUCOSE-6-PHOSPHATE DEHYDROGENASE*
Hd1	*Heading date 1*
Hd3a	*Heading date 3a*
h	hour
kb	kilobase pairs
kg	kilogram
LB	Luria Broth
ID	Intermediate day
ICA	Independent Component Analysis
LD	Long day
L*er*	Landsberg *erecta*

LFS	*LACHRYMATORY FACTOR SYNTHASE*
LFY	*LEAFY*
LKP2	*LOV KELCH PROTEIN 2*
LOV	light, oxygen and voltage
Mbp	Megabase pairs
mg	milligrams
$MgSO_4$	magnesium sulphate
MIBTP	Midlands Integrative Biosciences Training Partership
min	minutes
ml	millilitre
MLSRF	Medical and Life Sciences Research Fund
mm	millimetre
mM	millimolar
NaCl	sodium chloride
NaOAc	sodium acetate
NASC	Nottingham Arabidopsis Stock Centre
NC	Natural Condition
NCBI	National Centre for Bioinformatic Information
ng	nanograms
OsGA3ox1	*Oryza sativa GIBBERELLIN 3-OXIDASE 1*
OsGA3ox2	*Oryza sativa GIBBERELLIN 3-OXIDASE 2*
PBF	Phytobiology Facility
PCR	polymerase chain reaction
PEBP	phosphatidylethanolamine-binding protein
PHYA	*PHYTOCHROME A*
PHYB	*PHYTOCHROME B*
PPFD	Photosynthetic Photon Flux Density
PP2A1	*PROTEIN PHOSPHATASE TYPE 2A*
PP2AA3	*PROTEIN PHOSPHATASE 2A REGULATORY SUBUNIT 3*
qRT-PCR	Quantitative real-time PCR
RIF	Rifampicin
RNA	Ribonucleic acid

rpm	revolutions per minute
RT-PCR	reverse transcription- polymerase chain reaction
SATF	*SERINE ACETYLTRANSFERASE*
SD	Short day
SDW	Sterile distilled water
SEM	standard error of the mean
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*
SPEC	Spectinomycin
SPS	*SUCROSE-PHOSPHATE SYNTHASE*
TFL1	*TERMINAL FLOWER 1*
tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
tris-HCl	2-Amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride
TIP-41	*TONOPLASTIC INTRINSIC PROTEIN-41*
TSF	*TWIN SISTER OF FT*
TUA-5	*ALPHA TUBULIN 5*
TUB-7	*BETA TUBULIN 7*
UBC9	*UBIQUITIN CONJUGATING ENZYME 9*
UBL	*UBIQUITIN-LIKE PROTEIN RUB2*
UBQ1	*UBIQUITIN EXTENSION PROTEIN*
UPL	ubiquitin-protein ligase
UK	United Kingdom
USA	United States of America
UTR	untranslated region
VeGIN	Vegetable Genetic Improvement Network
VRN1	*VERNALISATION 1*
VRN2	*VERNALISATION 2*
v/v	volume by volume
w/v	weight by volume
ZT	Zeitgeber time
ZTL	*ZEITLUPE*

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Onion (*Allium cepa* L.) is a monocotyledonous bulbous perennial (often biennial), belonging to the family *Alliaceae* and is one of the most important vegetable and spice crops cultivated worldwide under a wide range of climatic conditions (Brewster, 1994; McCallum, 2001).

1.1 Classification, origin and distribution of onion

Following the development of molecular techniques, the botanical classification of the genus *Allium* has been revised (Friesen et al., 2006) and it occupies the following taxonomic classification shown in Table 1.1. *Allium cepa* is the most edible species of the genus *Allium,* which consists of about 780 species, with 650 having two or more synonyms (Friesen et al., 2006; Fritsch and Friesen, 2002). It is included in the order Asparagales, which is the second most economically important monocotyledons, next to Poales (which include the cereal crops) (Brewster, 2008; Mathew, 1996). Other important members of the order include garlic (*A. sativum* L.), shallot (*A. cepa* L. var. *aggregatum* G. Don.), leek (*A. ampeloprasum* L.), chive (*A. schoenoprasum* L.), agave (*Agave Americana* L.), aloe (*Aloe* spp.), asparagus (*Asparagus officinalis* L.), iris (*Iris germanica* L.) and vanilla (*Vanilla planifolia* L.) (Griffiths et al., 2002; Kuhl et al., 2004). Allium species of the family Alliaceae have been grouped into various subgenera and sections based on their visible features (Hanelt, 1990) which have been largely confirmed by recent molecular techniques which showed relationships and phylogeny of the different species (Friesen et al., 2006; Fritsch and Friesen, 2002).

Table 1.1 Classification of *Allium cepa*

Kingdom	Plantae
Sub-kingdom	Tracheobionta
Class	Monocotyledons
Order	Asparagales
Family	Alliaceae
Genus	*Allium*
Species	*A. cepa L.*

The species of the genus *Allium* are among the oldest cultivated crops and had been widely cultivated and used in diverse representations in Egyptian artifacts dating to 2700 B.C (Fritsch and Friesen, 2002). However, the current species, *A. cepa*, is known only from cultivation, but appears to have been primarily domesticated from wild ancestors in the Central Asian mountains particularly in the region of Iran and Pakistan (Figure 1.1) (Anonymous, 2003; Brewster, 1994). The near east Asiatic and Mediterranean regions are considered to be the secondary centres of origin and sites of great diversity (Anonymous, 2003; Fritsch and Friesen, 2002).

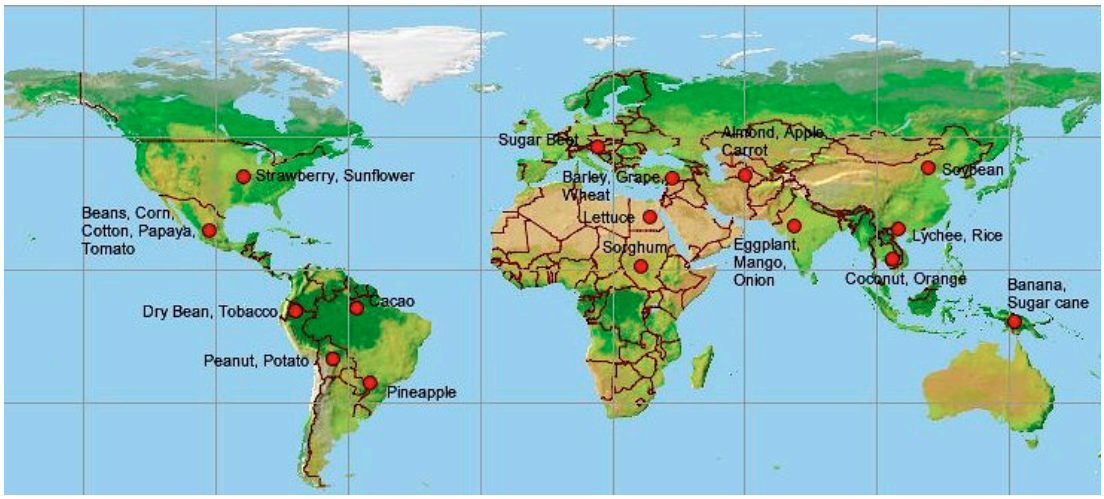


Figure 1.1 Map showing origin of onion and other important crop plants (Anonymous, 2003)

Onions are widely distributed through the temperate, tropical and sub-tropical regions in the world (Brewster, 2008). Numerous onion cultivars have been developed for size, form, colour, pungency, storability, resistance to pests and pathogens, and climatic adaptations (Griffiths et al., 2002). A few Southern European varieties of onion produce large sized bulbs of about 1 kg in weight (Fritsch and Friesen, 2002). Cultivars are divided into the Common Onion Group (*A. cepa* var. *cepa*), which contains most of the economically important varieties (including cultivars grown for green or salad onions) and the Aggregatum Group, which includes shallots and potato onions, and typically produce clusters of small bulbs (Brewster, 1994).

1.2 Importance

Onion is used both in the green and mature stage for salad and spice in a variety of flavoured dishes and soups. It is very important in cookery and widely used for

cooking in nearly all regions of the world, and has been used in diverse cultures and rituals throughout history; hence it is called the "Queen of kitchen" (Brewster, 1994).

1.2.1 Economic importance

Onions form part of the diet of millions of people across the globe, are in constant demand round the year and have a great impact on global food security. The Food and Agriculture Organization of the United Nations (FAO) reported that onion ranks second, next to tomato, among horticultural crops in terms of total annual world production (FAO, 2016) and comprise 15% of total gross production value of all vegetables (Figure 1.2) (FAOSTAT, 2016). Global production of onions in 2012 was 82.85 million tonnes from 4.2 million hectares of land, of which the UK produced 388,050 tonnes (Table 1.2) (FAOSTAT, 2016). China alone produced more than 22 million tonnes; other leading producers were India, the USA, Turkey, Pakistan and Bangladesh (Table 1.3) (FAOSTAT, 2016). However, data showed that about 582,000 tonnes of onions were consumed in the UK in 2011 (Table 1.4) (FAOSTAT, 2016). The majority of onions produced in the UK are consumed within the country, with only 9902 tonnes being exported in 2011 (Table 1.5) (FAOSTAT, 2016). So, the production of onions in the UK is far less than the consumption and the shortage of demands is filled by imports of 338,636 tonnes a year. The majority of fresh onions in the UK are exported from Netherlands.

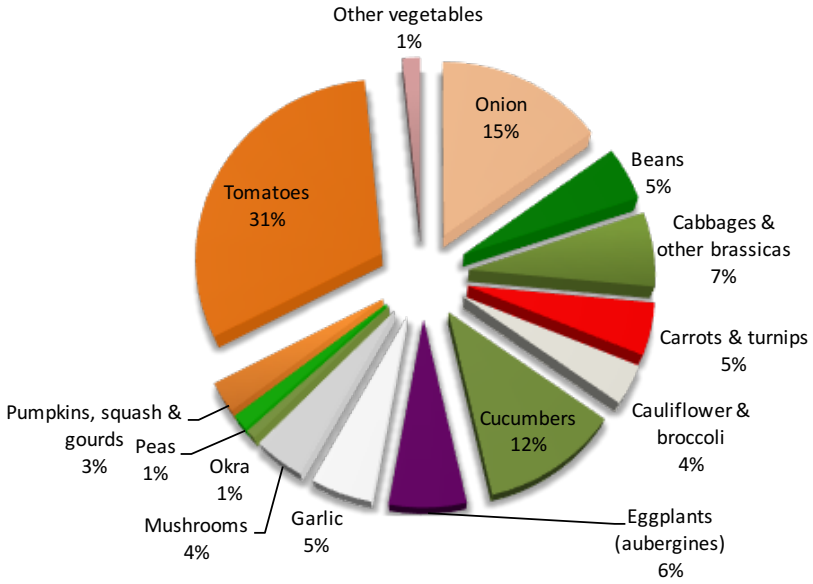


Figure 1.2 Gross production data (FAOSTAT, 2016)

Table 1.2 Total UK and worldwide production of onions (tonnes) from 2008-2012 (FAOSTAT, 2016)

Year	UK	World
2008	349200	74,484,149
2009	354,900	73,640,837
2010	364,500	78,865,614
2011	328,000	85,008,230
2012	388,050	82,851,732

Table 1.3 Leading onion producing countries in the world in 2012 (FAOSTAT, 2016)

Countries	Production (tonnes)
China	22,244,986
India	16,813,000
USA	3,277,460
Turkey	1,735,857
Pakistan	1,691,800
Bangladesh	1,159,259

Table 1.4 Consumption of onions (tonnes) in the UK and Worldwide, 2007-2011 (FAOSTAT, 2016)

Year	UK	World
2007	588,067	66,271,480.01
2008	643,863	67,317,649.76
2009	630,940	66,791,820.29
2010	643,977	71,291,553.05
2011	582,464	76,763,043.81

Table 1.5 Import/Export statistics for onions in the UK (tonnes), 2008-2012 (FAOSTAT, 2016)

Year	Import	Export
2008	373,728	6772
2009	353,487	6608
2010	361,878	9763
2011	356,748	9419
2012	338,636	9902

1.2.2 Nutritional, medicinal and health importance

Numerous potential health and nutritional benefits arising through consumption of onions have been investigated over many years. Onions provide flavour and contain valuable phytochemicals with health-related properties including various sulphur-containing compounds such as alkenyl cysteine sulphoxides, anti-oxidant molecules, which are probably used for defense against fungi and insects, that, together with their breakdown products, produce the distinctive odour, flavour, and lachrymatory (tear-stimulating) properties of onion (Brewster, 1994). Sulphur enters the plant as sulphate from the soil solution, and after being absorbed by a high sulphate-affinity transporter protein it moves in the vascular tissue to the leaves and there undergoes sequential enzymatic reduction and transfer reactions to be incorporated in the amino acid cysteine, which may then combine with glutamic acid and then glycine to form glutathione (Brewster, 2008). Evidence showed that there are two possible biosynthetic pathways to form the S-alk(en)yl-L-cysteine sulphoxides (ASCO) flavour of precursors in onion (Griffiths et al., 2002; Jones et al., 2004). Further investigation showed that in the short day (SD) treatment, ASCO's accumulated in the foliage leaves, while in the long day (LD) treatment, they initially accumulated in the foliage leaves and then moved to the bulb during bulb development (Mallor and Thomas, 2008). In addition, SD plants accumulated more ASCO's than those in LD, indicating that the length of the vegetative growth phase can drastically affect the level of flavour compounds in the plant, which is supported by the theory of a recycling of ASCO's within the plant (Lancaster et al., 1986). Onion also contains flavanols, which contribute to flower colour, and fibre, which may limit the risk for cancer, blood clots, asthma and infection (Griffiths et al., 2002). From history, it was reported that onions have been used in folk medicine for purposes ranging from treating wounds and stomach ailments to treating infertility (Brewster, 2008). The evidence found from the Scientific and pharmacological studies since World War II showed that onions, or their derived compounds, have antimicrobial and antifungal properties, and may also be of benefit in preventing or treating heart disease and atherosclerosis, diabetes, cancer, and possibly asthma (Brewster, 1994; Griffiths et al., 2002). Despite the benefits of onion to humans, they are toxic to cattle, cats, and dogs, and to a lesser extent, sheep and goats (Cope, 2005; Merck, 2011). Large amounts of onions consumed by these animals may lead to anemia and impaired oxygen transport.

1.3 Growth and life cycle of onion plants

Onion is a biennial, outcrossing and highly heterozygous crop plant, which is propagated by seeds, bulbs or sets (Eady, 1995). An onion plant is composed of leaves, which arise alternately from a base plate or small flattened stem (bulb), or base plate, which is a vegetative overwintering stage in the life cycle of the plant. The older leaves are on the outside and younger leaves on the inside of the stem (Lancaster et al., 1996). Onion is grown annually in terms of crop production. The onion bulb is a storage organ and leaves are composed of a photosynthetic leaf blade and a fleshy leaf sheath/storage leaf base or scale. When bulb initiation occurs, the leaf sheaths swell and initiation of bladeless bulb scales occurs to form the storage tissue of the bulb. Two or three foliage leaf initials are produced at the base at the maturity of the bulb, which will be elongated in the following season, producing bulb sprouts (Brewster, 1990). The basic structure of an onion bulb is shown in Figure 1.3.

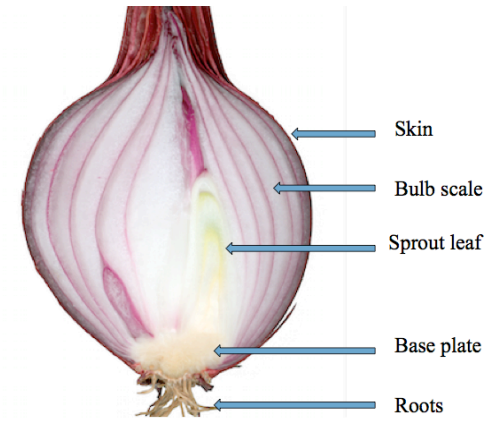


Figure 1.3 Cross-section of an onion bulb.

Onion has a fairly complex life cycle, compared to many other crops, that can be divided into three main stages, namely seedling, bulb and flowering stages (Bosch Serra and Casanova, 1979; Brewster, 1990). In the life cycle of temperate onion (Figure 1.4), during the first growth phase onion seed will start to germinate after sowing (Brewster, 1994). During the germination stage, the primary root starts to grow downwards while the cotyledon pushes upwards through the soil surface as a loop or a hook and this stage is referred to as the loop stage. After few days of germination, the seedlings/sets are planted in the spring and the plants undergo a juvenile phase of growth during which plants will not bulb regardless of being

exposed to inductive conditions. In addition to that, onion leaves must be exposed continuously to an bulb-inductive photoperiod in order to initiate and complete bulbing (Brewster, 2008). When the onion plant became mature and the daylength has reached a critical length, bulb formation, which is promoted by LD during summer, begins (Lee et al., 2013). The onion plant then overwinters as a bulb and during this time, if the environment is favourable, flowering is induced in response to prolonged cold temperature during winter, a process called vernalisation. The onion plant then flowers and sets seed during the spring/summer of its second year of growth and thus its life cycle is completed (Brewster, 1994; Lee et al., 2013).

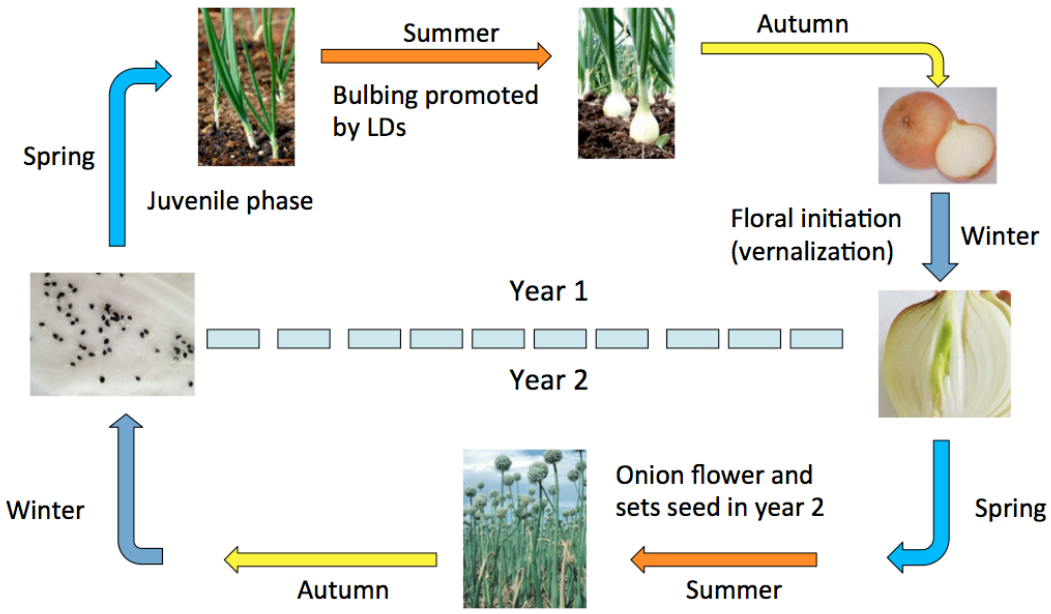


Figure 1.4 Life cycle of temperate onion (Brewster, 1994)

1.4 Factors involved in the bulbing process of onion

1.4.1 Daylength

Daylength is defined as the daily duration (hours) of light a plant is exposed to (Denisen, 1979). Bulb formation in temperate onion is daylength-dependent and the leaves of the plant are the photoperiodic stimulus receptor (Okporie and Ekpe, 2008). LD of at least 14 h of light is required to stimulate bulbing (Mettananda and Fordham, 1997).

1.4.2 Homeotic conversion

Bulbing involves homeotic conversion whereby photosynthetic leaves are transformed to swollen bulb scales at the responsive meristem (Thomas et al., 2006). With the reception of the photoperiodic stimulus, bladed green leaves formation near the apical meristem ceases and they are transformed into only bladeless leaves. Bulb sheath cells expand in response to a photoperiod signal from the leaf blade and act as a sink for the leaf carbohydrates such as glucose, fructose, sucrose and fructans. This could increase the osmotically active solutions in the outer sheath cells, thereby drawing in water and driving cell expansion at the base of the leaf, thus developing into a storage organ, the bulb (Mettananda and Fordham, 1999; Mondal et al., 1986; Thomas et al., 2006). With the maturity of the bulb, the outer (oldest) one to four leaf scales dry out and become protective skin (Brewster, 1994).

1.4.3 Reversibility

Bulb initiation can be defined as the point at which the 'bulbing ratio', the ratio of the maximum bulb diameter at the base to the minimum at the neck/sheath, (Figure 1.5) increases to greater than two (>2) (Brewster, 1997). Bulbing is a reversible process and plants grown under inductive conditions promote bulbing but if transferred to non-inductive condition, they revert back to vegetative growth (Brewster, 1997; Sobeih and Wright, 1986).

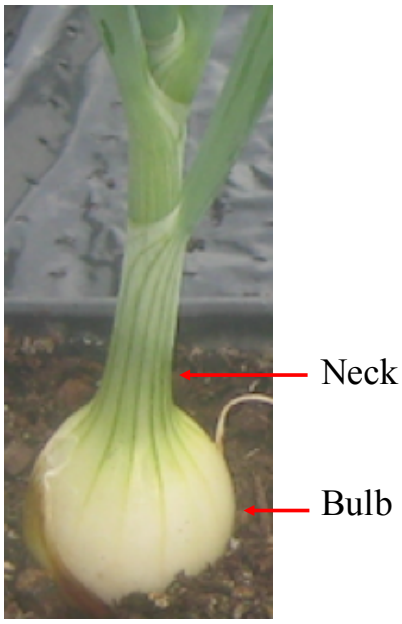


Figure 1.5 Measurements to be taken for the calculation of bulbing ratios.

1.4.4 Temperature

Temperature is a significant factor for bulb formation in SD onions. Elevated day or night temperatures accelerate bulb formation with varieties being highly adapted to their local conditions (Brewster, 1977; Heath, 1945; Kato, 1964; Magruder, 1937; Steer, 1980). The best temperatures for growing onions range from 13-25°C (55-75°F). The best temperatures for growing onions in the UK range from 20-25°C. It has been suggested that bulb initiation in onion requires a combination of a minimum thermal time and a minimum daylength (Lancaster et al., 1996). Every phase of development requires a minimum accumulation of temperature before that stage can be complete and the plant can move to the next stage. In effect, the plant senses the temperature every day and adds the average for that day to a running total up to the total required for the stage. This running total is called thermal time for the phase and the thermal units are degree days (°Cd). It can be calculated in the same way by totalling the mean temperatures (maximum + minimum divided by 2) for each day during the phase. So, if the day had a maximum of 35° C and a minimum of 15° C the mean would be 25° C (35 + 15 divided by 2) and the thermal time for the day would be 25° Cd. Bulb diameter at bulbing was related to thermal time accumulated prior to bulbing. Bulbing only occurred when dual thresholds of a minimum thermal time of 600 degree days and a photoperiod of 13.75 h were reached (Lancaster et al., 1996).

1.4.5 Light quality and quantity

Light quality is an important factor for bulb formation in onion (Austin, 1972). In bulbing, photoperiodic signals are detected by phytochrome(s) in the leaves (Lercari, 1984; Sobeih and Wright, 1987). Bulb initiation in a particular daylength varies with the quantity of light received by the plants and high levels of far-red light accelerate bulbing. Studies showed that the lower the ratios of red:far-red light the faster the rate of bulbing and crop maturity is accelerated (Mondal et al., 1986). Blue light has little influence on onion bulb formation (Terabun, 1965). In addition to quality, light quantity also affects bulb formation and high irradiance accelerates bulbing in LD onion (Sobeih and Wright, 1986). It has also been observed that elevated levels of carbon dioxide also accelerate the bulbing process (Daymond et al., 1997).

1.4.6 Age of the plant

The age of the plant also affects bulbing in onion and experimental results showed that older plants bulb more rapidly than the younger ones, which will not bulb unless they have at least 4 leaves (Sobeih and Wright, 1986). On the other hand, an increased amount of time is required for bulb initiation with the age of the plants when photoperiods decrease (Wiles, 1989).

1.4.7 Growth substances

Many hormones such as auxin, cytokinin, gibberellins and ethylene have been implicated in bulb formation, though many attempts to correlate hormone levels with bulbing have been unsuccessful (Brewster, 2008). Ethylene has been externally applied as a spray of ethephon, which induces a swelling of leaf sheaths and a small increase in bulbing ratios, but the normal elongation of leaf sheaths which cause bulb swelling does not occur and normal typical bulbs do not develop (Sobeih and Wright, 1987). In addition, ethylene antagonists do not repress bulb initiation in inductive photoperiods (Lercari, 1983). Thus, it was regarded that ethylene is responsible for pseudobulbing rather than true bulbing. Several studies also have been aimed at the implication of gibberellins in onion bulb formation (Brewster, 2008). Results showed that an inhibitor of gibberellin biosynthesis promotes bulbing in non-inductive photoperiods which suggests an inhibitory role in bulb initiation (Mita and Shibaoka, 1984). Gibberellin also has an inhibitory role on flowering in strawberry (Thompson and Guttridge, 1959). However, gibberellin was successful to promote flowering in Arabidopsis (*Arabidopsis thaliana*) through the activation of the promoter of the floral integrator gene *LEAFY* (*LFY*) (Blazquez et al., 1998) and is also involved in onion flowering (Rabinowitch, 1990). It was also reported that cytokinin has some effect in bulb initiation under inductive photoperiod (Lercari and Micheli, 1981).

1.5 Adaptation to daylength

Adaptations are special features that allow a plant to live in a particular place or habitat. These adaptations might make it very difficult for the plant to survive in a different place. This explains why certain plants are found in one area, but not in another. The daylength requirement for onion bulb formation largely varies with the type of cultivar, ranging from 10 to 16 hours (van den Berg et al., 1997). Adaptation to daylength largely depends on the adaptation of onion cultivars to a certain

production area and the daylength requirement of the specific cultivar (Wiles, 1989). Onions are commercially categorized into LD, intermediate-day (ID), and SD varieties and it is in reality a continuum within which an onion variety will initiate bulbing only when it gets a certain number of hours of daylight each day for a certain number of days. The critical daylength (CDL) is very important for flowering in any plant species. CDL can be defined as the period of daylight, specific in length for any given species that appears to initiate flowering in LD plants or inhibit flowering in SD plants (http://botanydictionary.org/critical-day-length.html). The CDL is thus the point at which the photoperiod switches from being noninductive to inductive, and the value of the CDL varies considerably among species and among plants within the same species (Jackson, 2009).

LD onions begin forming bulbs when the daylength reaches at least 14 or more hours and are well adapted to areas between 45^{o} to 60^{o} latitude, while, ID varieties require at least 12 or more hours for bulbing and can be planted in areas between 30^{o} to 45^{o} latitude. SD onions will start making bulbs at only 10 hours or more of daylight and can be planted in the tropics (30^{o}N and S from the equator) (Figure 1.6) (van den Berg et al., 1997; Wiles, 1989). This CDL for bulb formation indicates the adaptability of different onions in different latitudes in the world (Thomas et al., 2006).

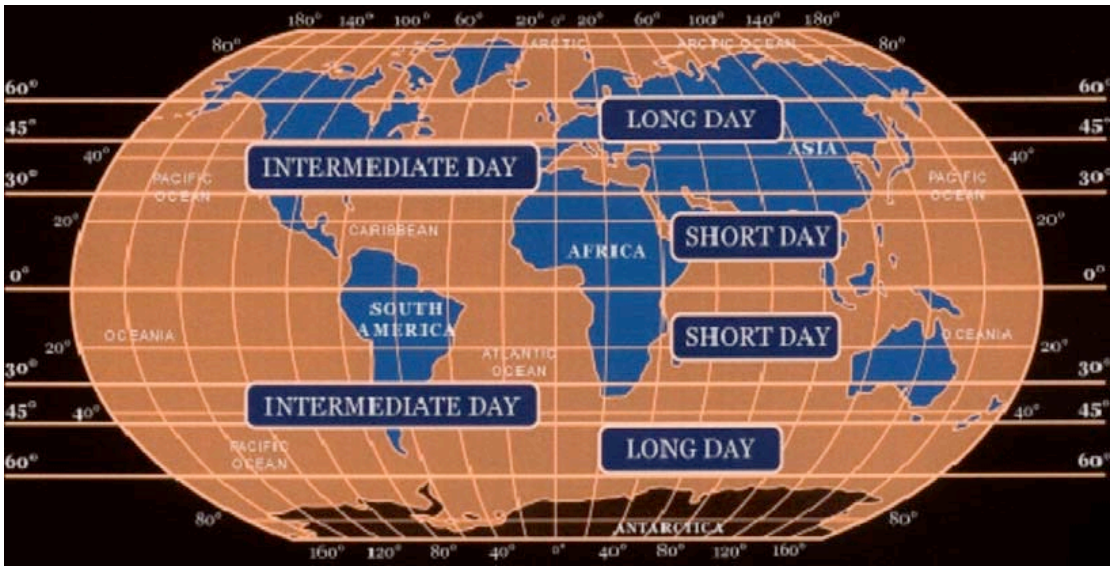


Figure 1.6 Adaption of onion cultivars to different latitudes (Hydrotech, 2010)

In the United Kingdom, LD cultivars are planted, although; ID and SD cultivars can be grown. The reason for this being that the longest average daylength in the UK is about 15 hours or more. However, if an onion cultivar is exposed to daylength shorter than the CDL, plants will not bulb, will continue to form leaves (Taylor et al., 2010; Wiles, 1989) and show a high percentage of bolting accompanied by thick bulb necks (Gonzalez, 1997). Studies showed that if bulbing plants are transferred or re-exposed to short, non-inductive photoperiods, onion plants could revert to green leaf production, even in plants at an advanced stage of bulb development (Brewster, 2008). On the other hand, when an onion cultivar is grown in areas where the daylength is longer than the required, premature bulb formation occurs and bulb development and maturity rates are increased, which ultimately results in smaller bulbs and low yields (Wickramasinghe et al., 2000). Therefore, the daylength at a specific production area or latitude at the time of bulb initiation will influence on the selection of onion cultivars.

1.6 Onion flowering

In addition to bulbing, flowering is an essential prerequisite for seed production in onion (Brewster, 2008). Flowering is not generally important when onions are grown for bulb or vegetative shoots for food with the exception to Chinese chives where both leaves and inflorescence are eaten. Apart from this, understanding of flowering and its interaction with vegetative growth and bulbing are very important for successful crop as well as seed production. Flowering in onion plants will normally occur in the second growing season, but can occur in the first growing season under favourable conditions (Rabinowitch, 1990). Onion flowering also involves different stages from the initiation of the inflorescence to seed ripening. For each stage of this cycle, different cultivars require different environmental optima and different daylengths to pass through the stage.

Temperature is an important phenomenon for onion flowering as a particular temperature required for vernalisation, which enhances flowering (Brewster, 1997). The optimum temperature for vernalisation is 8-12 ^{o}C, and is slower at 6 ^{o}C or less, however, the number of days required to induce vernalisation varies with cultivar, but is in the range of approximately 20-40 d. The rate of elongation of an inflorescence developed within the onion bulb depends on temperature. However,

after sprouting and growing of the bulb, it depends on daylength and LD photoperiods along with a cool temperature of 10-15 ^{o}C could enhance and shorten the vernalisation period, though it depends on the cultivars (Brewster, 1983). So, there is a competition between inflorescence development and bulb initiation that largely depends on daylength. The inflorescence of a SD cultivar will be suppressed by a shorter daylength than a long day cultivar. Also, plants raised from bulbs or sets produce bulbs more rapidly than those raised from seeds, unless they have been 'heat treated' at the end of storage, and therefore competition from bulbing probably suppresses inflorescences in bulb-raised plants at shorter photoperiods and lower temperatures than in seed-raised plants (Brewster, 2008). So, bulb initiation is a photoperiod response in onion and the photoperiod response genes characterised in Arabidopsis, are hypothesised to be involved in bulb initiation (Taylor et al., 2010). A similar situation appears to be the case in potato where the Arabidopsis *CONSTANS* (*CO*), when overexpressed in potato, impairs tuberisation (Martínez-García et al., 2002). Tuberisation is a photoperiod response in potato, and is initiated by SD conditions.

In onion, the abnormal inflorescence such as small bulbs, called 'bulbils' or 'top-sets' are frequently observed instead of normal flowers and seed capsules (Brewster, 2008). Studies showed that production of bulbils was promoted by onion bulb storage for 6 weeks at 28 ^{o}C, following an inflorescence-inducing cold treatment of 5-6 months. Therefore, it was observed that high temperature causes inflorescence initiation to revert to vegetative leaf formation and if the inflorescence is already developed to some extent, it diverted to bulbil development. This result proved that daylength is not responsible for bulbil development but accelerates bulb initiation in onion.

1.7 Bulbing vs Flowering

Arabidopsis flowering and onion bulb formation are both photoperiodically driven processes (Thomas et al., 2006). At the physiological level, bulb initiation in LD onion is regulated in a similar way to the photoperiodic regulation of flowering in Arabidopsis (Taylor et al., 2010). Both processes are induced by LD, signal perception is in the leaf and response is at the apex. Studies showed that the leaf blade in onion is the receptor for the photoperiodic control of bulb initiation and

when parts of leaf blades are removed, an increased number of leaves are formed before bulb initiation (Brewster, 1990; Kato, 1964).

Arabidopsis flowering and onion bulb formation can be compared in terms of the involvement of phytochrome, and both processes are promoted by far-red light, through PHYA (Brewster, 1977). In addition to far-red, blue light also acts in the photoperiodic control of flowering in Arabidopsis through the photoreceptor genes *CRYPTOCHROME 1* (*CRY1*) and *CRYPTOCHROME 2* (*CRY2*), regulating the expression of *CO* (Jackson, 2009). In contrast, blue light is ineffective in promoting bulbing in onion.

Flowering and bulbing can be compared in terms of the presence of a juvenile phase, and during both processes, initiation will not occur regardless of being exposed to favourable environmental conditions until this phase has been passed (Massiah, 2007). In certain tree species, it was reported that the juvenile phase has been shortened by the overexpression of the *FLOWERING LOCUS T* (*FT*) gene (Hsu et al., 2006).

The reversible nature of flowering was seen in some plants, e.g., soybean and wild Vigna species, where the process could be reversed even well after development of floral buds and in the case of garden balsam (*Impatiens balsamina*) reversibility of reproductive stage to vegetative growth stage, serves a function in the perenniality of the plant (Summerfield et al., 1991). However, these are exceptional cases and for the vast majority of plants, including Arabidopsis, flowering is an irreversible process once initiated. Bulbing in onion is a fully reversible process where plants can revert back to vegetative growth when transferred from inductive photoperiod to non-inductive conditions even at an advanced stage of bulbing (Brewster, 1997; Sobeih and Wright, 1986).

Both flowering and bulb formation involves homeotic conversion, meaning the conversion of photosynthetic leaves to other structures, such as sepals, petals, stamens and carpels in flowers and bulb scales in bulbs. In Arabidopsis, the leaf primordia and an inflorescence meristem, which is a floral structure, are produced from the shoot apical meristem until environmental and internal signals cause a

change of fate (Komeda, 2004). However, in onion, leaf sheaths swell and produced bladeless bulb scales, which is a storage structure (Lancaster et al., 1996; Summerfield, 1991).

1.8 Daylength perception in Arabidopsis

Flowering in Arabidopsis has been characterised at the molecular and genetic level and is regulated by 6 major separate pathways viz., photoperiodic, convergent autonomous, sucrose, gibberellin, temperature and light quality pathway (Jack, 2004; Thomas et al., 2006). However, this study focuses on the photoperiodic pathway, which is mediated by the circadian clock, an autonomous mechanism that generates endogenous rhythms in a 24-hour period (Jackson, 2009) and is controlled by various feedback loops (Hayama and Coupland, 2003). The mechanism is shown in Figure 1.7.

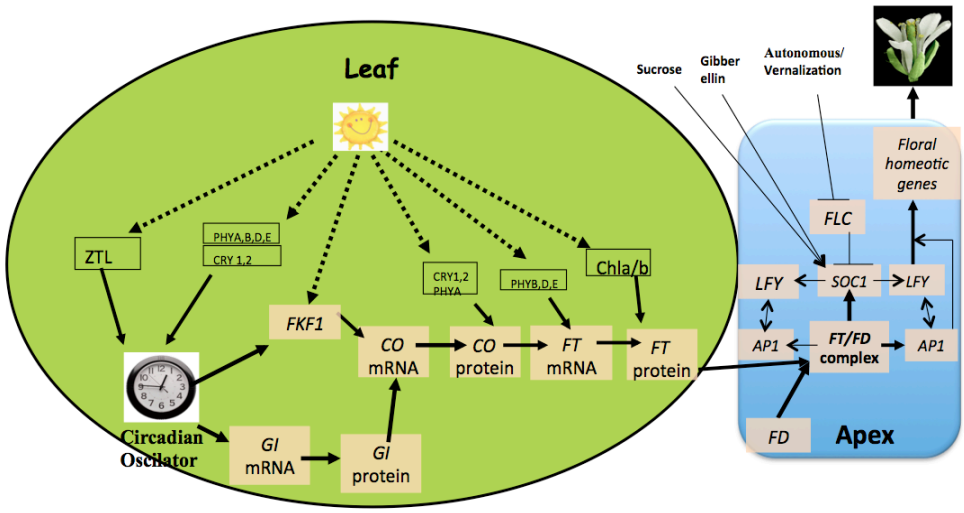


Figure 1.7 Daylength perception in *Arabidopsis* (Thomas et al., 2006)

Numerous responses are mediated by the clock including shade avoidance, stomatal opening/closing, photosynthesis and floral initiation (Hotta et al., 2007). Light plays an important role in the photoperiodic response in Arabidopsis and interacts with the circadian clock as part of the photoperiodic flowering pathway (Michael et al., 2003). In the leaf, light is perceived by different photoreceptors, both cryptochromes in blue light and phytochromes in red/ far-red light and inputs into the circadian clock. The clock derives the rhythmic expression of key genes *FLAVIN-BINDING, KELCH REPEAT, F-BOX* (*FKF1*), *GIGANTEA* (*GI*) and *CO* (Somers et al., 1998). *FKF1* and *GI* promote *CO* expression (Sawa et al., 2007) and this *CO* positively

regulates *FT* (Jung et al., 2007). The FT protein is then translocated to the apical meristem through the phloem and forms a FT/FD complex (Abe et al., 2005; Pnueli et al., 2001; Purwestri et al., 2009; Taoka et al., 2011; Wigge et al., 2005). This complex activates the *APETALA 1* (*AP1*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) genes, which trigger *LEAFY* (*LFY*) gene expression and cause flowering (Greg et al., 2015; Nakamichi, 2011; Yoo et al., 2005). The sucrose and gibberellin pathways, promote *SOC1* expression.

In addition to daylength perception, homologues of some major genes namely *CO*, *FT* and *GI* controlling photoperiodic flowering pathway in Arabidopsis are proposed to be conserved in many plant species, as summarised by a previous study (Table 1.6) (Taylor, 2009).

Table 1.6 Conservation of the Arabidopsis flowering time genes *CO*, *FT* and *GI*, which have been functionally characterised across plant species (Taylor, 2009).

Species	Genes	Key References
Rice	*CO, FT, GI*	(Hayama et al., 2003; Kojima et al., 2002)
Wheat	*CO, FT, GI*	(Nemoto et al., 2003; Xiang et al., 2005)
Barley	*FT, GI*	(Dunford et al., 2005; Faure et al., 2007)
Pea	*CO, FT, GI*	
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*	(Hayama et al., 2007; Liu et al., 2001)
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)

1.9 Daylength perception in other plants with vegetative storage organs

Many vegetatively propagating storage structures are important food crops and the formation of vegetative storage organs in those species depends on, or is accelerated by, particular daylength (Thomas and Vince-Prue, 1997). Vegetative storage organs of plants arise by lateral swelling of a number of different tissues including stems (tubers, corms), roots (tuberous roots or root-tubers) and leaves (bulb), however, physiologically, they all have a similar function as perrenating organs and are regions into which storage materials are mobilized (Thomas and Vince-Prue, 1997). In addition to the bulb formation in Allium species, which is favoured by LD, most photoperiodically induced storage organs are favoured by exposure to SD, with the exception of the runner formation in strawberries, which is favoured by LD and inhibited by SD (Guttridge, 1969).

Potato tubers, organs of vegetative propagation, arise by a swelling of a cluster of internodes, often at the tips of underground stems or stolons, which are important for energy and essential resource stocks for the progeny in potato (*Solanum tuberosum*) (Thomas and Vince-Prue, 1997) Tuberisation in potato involves the integration of certain environmental (especially daylength and gibberellin pathways) and internal cues (Martínez-García et al., 2001). Tuberisation in potato appears to be hastened by SD and the plants initiate tubers when the daylength is 12 h or less (Jackson, 1999), although cultivars differ considerably in the extent to which they are affected. It was reported that tuberisation in LD is inhibited by the photoreceptor *PHYTOCHROME B* (*PHYB*), though it is not responsible for induction of tuberisation in SD (Jackson, 1999). Studies suggested that differences found in the tuberisation in different cultivars of potato largely varies with the CDL (Kopetz and Steineck, 1954). It was observed that tuberisation occurred in potato when tobacco leaves from a flowering-induced plant were grafted onto potato plants kept under non-inductive conditions, which suggested that the genetic control of tuberisation is similar to the genetic control of photoperiodic flowering (Jackson, 1999). Recent studies showed that tuberisation is inhibited in SD conditions when Arabidopsis *CO* is overexpressed in potato (Martínez-García et al., 2002). In addition to that, two potato genes have been identified which showed a high level of homology to Arabidopsis *FT* (Abelenda et al., 2014; Rodríguez-Falcón et al., 2006).

In other tuber-forming plant species, SD either favour tuberisation or have no effect on it. However, some positive influence of SD was demonstrated on the tuberisation in *Dahlia* spp., *Begonia* spp. and some cultivars of *Helianthus tuberosus* (Nitsch, 1966). In *H. tuberosus,* tubers developed below ground when only the stem tips were covered to give SD, provided that leaves up to 2 inches long were included in the treated apical region (Hamner and Long, 1939). When different areas of *B. evansiana* leaves were exposed to SD or LD, tuberisation decreased as the ratio of the area in SD to that in LD decreased (Esashi, 1961a).

Daylength may not play a major role in the tropical root and tuber crops since differences in natural daylengths within the tropical belt are rather small and daylength in general is short compared to that in the growing conditions of temperate climate in the summer. Nevertheless, it is possible that artificial shortening of the day might accelerate tuberisation in some crops. Greater root production was observed during shorter daylengths in the yam bean *(Pachyrrhizus erosus* L. Urb.) in the Philippines and Hawaii was greater in the cooler part of the year (Bautista and Cadiz, 1967; Ezumah, 1970). It was also reported that SD of 12 h has influenced on the tuberous root formation in cassava (*Manihot esculenta Crantz*) (Bolhuis, 1966).

1.10 Onion Breeding

Being a biennial species, it takes more time to improve onion crops by conventional breeding methods such as hybridization, recombination and selection. Also the presence of severe inbreeding depression makes it difficult to produce and maintain a large number of near homozygous inbred lines ideal for genetic linkage and analysis (Brewster, 2008; Lawande, 2009). Daylength sensitivity places a significant barrier to onion breeding programmes as a trait in a particular daylength group cannot be transferred to another daylength group by cross breeding because the specific daylength response of the progeny will be unknown.

In addition, crossing onions with different daylength requirements is difficult, as the progeny will be compromised. In comparison to many other crop species, only a small number of quantitative genes with easily visible effects have been described in onion (Brewster, 2008). Therefore, identifying more genes responsible for daylength requirement of bulb formation will help understand the basis of the difference, which

is important for adapting new varieties for growth and development at different latitudes, and will provide molecular markers for breeding programmes. Moreover, through the techniques of transformation and somatic cell hybridization, it is currently possible to transfer novel genes into the vegetatively propagated species (Brewster, 2008; Schnell et al., 2015).

1.11 Focus on specific genes

An earlier study of Taylor et al. (2010) showed that some key genes such as *FKF1* and *GI* controlling photoperiodic flowering in Arabidopsis are conserved in onion. However, in this study more emphasis has been given to an onion *CO* homologue and *FT* as these genes play a central role in the circadian regulation and the photoperiodic regulation of flowering in both Arabidopsis and rice (Jackson, 2009).

1.11.1 FLAVIN-BINDING, KELCH REPEAT, F-BOX (FKF1)

In Arabidopsis, *FKF1* plays an important role in the flowering pathway through regulating daytime *CO* expression (Li et al., 2013). *FKF1* has three functional domains: the LOV (light, oxygen and voltage) domain, the F-box motif, and the Kelch repeats, all of which are highly conserved in two F-box proteins *ZEITLUPE* (*ZTL*) and *LOV KELCH PROTEIN 2* (*LKP2*) (Nelson et al., 2000; Schultz et al., 2001; Somers et al., 2000). *FKF1* interacts with *GI* through the LOV domain to form a complex in a blue-light dependent manner in the late afternoon under LD conditions (Sawa et al., 2007). In addition, the *FKF1* Kelch repeats can interact with *CYCLING DOF FACTOR 1* (*CDF1*) protein, which is a transcription repressor of *CONSTANS* (*CO*) (Imaizumi et al., 2005). In LD conditions, sufficient *FKF1-GI* complex is formed to activate *CO* transcription during the daytime, which is stabilized by light at the end of the day. This daytime *CO* expression triggers the expression of the floral integrators *FT* and *TWIN SISTER OF FT* (*TSF*), leading to floral initiation (Corbesier et al., 2007; D'Aloia et al., 2011; Jackson, 2009; Massiah et al., 2007). An earlier study showed that some key genes such as *GI* and *FKF1* controlling photoperiodic flowering in Arabidopsis are conserved in onion and suggest that homologues of these may play a role in the photoperiodic control of bulb initiation (Taylor et al., 2010).

1.11.2 GIGANTEA (GI)

In photoperiodism, the circadian clock is a timekeeping mechanism (Mizoguchi et al., 2005). *GI*, *CO*, and *FT* genes are involved in a circadian clock–controlled flowering pathway in Arabidopsis which promotes flowering specifically under LD (Mizoguchi et al., 2005). These genes were placed in Arabidopsis photoperiod pathways based on the genetic analysis (Bouché et al., 2016; Koornneef et al., 1998; Koornneef et al., 1991; Redei, 1962). However, loss-of-function mutations in each of these genes delay flowering under LD but have little or no effect under SD (Fowler et al., 1999). Mutant and wild-type backgrounds of these three genes placed them in the functional hierarchy *GI-CO-FT* (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach and Coupland, 2000; Suarez-Lopez et al., 2001) and within the pathway, *GI* regulates circadian rhythms and flowering and acts earlier in the hierarchy than *CO* and *FT* (Mizoguchi et al., 2005), suggesting that *GI* might regulate flowering indirectly by affecting the control of circadian rhythms (Fowler et al., 1999; Mizoguchi et al., 2005). *GI* encodes a protein containing six putative transmembrane domains, strongly suggesting that *GI* is a membrane protein (Park et al., 1999). *GI* appears to be expressed in every organ, however, it is highly conserved in seed plants, including monocotyledonous plants, such as rice, wheat and barley (Dunford et al., 2005; Hayama et al., 2003; Xiang et al., 2005) as well as dicotyledons such as radish and *Medicago truncatula* (Curtis et al., 2002; Paltiel et al., 2006) and gymnosperms, such as loblolly pine (*Pinus taeda*) (Hayama et al., 2002; Mizoguchi et al., 2005). By contrast, *GI* homologs appear to be absent from the genomes of the moss Physcomitrella, of Chlamydomonas, and of animals (Mittag et al., 2005). In onion epidermal cells, fusion proteins in which *GI* fused to the marker proteins green fluorescent protein (GFP) and β-glucuronidase were localized to the nucleus (Huq et al., 2000). Furthermore, in *gi* mutants, the abundance of *CO* mRNA is reduced (Suarez-Lopez et al., 2001), suggesting that *GI* plays a role, which might be direct or indirect, in promoting *CO* transcription (Mizoguchi et al., 2005; Song et al., 2014). However, the biochemical function of *GI* protein is unknown.

1.11.3 CONSTANS (CO) AND CONSTANS LIKE (COL)

CO, which probably acts as a transcriptional activator, was the first discovered member identified in Arabidopsis, leading to the activation of floral meristem identity gene *LFY* (Putterill et al., 1995). The Arabidopsis *CO* belongs to a family of

17 genes, including 16 *COL* genes, all of which are putative transcription factors (Griffiths et al., 2003). These genes are characterised by two conserved domains viz., a zinc-finger region near the amino terminus (B-box), which is necessary for protein-protein interactions, and a region near the carboxy terminus termed the CCT (CO, COL, TOC1) domain, which is necessary for nuclear localisation of the *CO* protein (Robson et al., 2001). The functions of only four Arabidopsis *COL* genes have been studied of which *COL1* and *COL2* are circadian regulated but do not affect flowering time (Ledger et al., 2001). However, overexpression of *COL1* affects circadian rhythms, which might have an effect in a light input pathway. *COL9* expression is circadian regulated and its overexpression leads to delayed flowering and might regulate *CO* expression (Cheng and Wang, 2005). *COL3* has been shown to be a positive regulator of red light signalling and root growth (Datta et al., 2006). *CO* and *COL* 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) and the moss *Physcomitrella patens* (Zobell et al., 2005). The presence of *COL* genes in SD 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 SD inductive conditions (Martínez-García et al., 2002). This suggests a broader role for *CO* than simply controlling flowering in LD and an involvement in bulb initiation. In addition, there are 16 *COL* genes present in rice where *Heading date 1* (*Hd1*) is the rice homolog of Arabidopsis *CO* (Griffiths et al., 2003). However in rice, *Hd1* plays the opposite function to Arabidopsis *CO*, suppressing the expression of *Hd3a* (the rice homolog of *FT*) and inhibiting flowering in LD (Hayama et al., 2003). It is particularly interesting that there is a *CO* homolog present in rice, which is more closely related to onion than Arabidopsis in terms of phylogenetic distances (Kuhl et al., 2004). This might provide resources for understanding the flowering and bulbing mechanisms and evolutionary pattern of *COL* genes.

1.11.4 FLOWERING LOCUS T (FT)

FT is expressed in leaves and is induced by LD treatment in Arabidopsis. *FT* integrates the photoperiod pathway, vernalisation pathway, sucrose pathway and

gibberillin pathway to promote flowering in plants; however, it is antagonistic with its homologous gene, *TERMINAL FLOWER 1* (*TFL1*) (Xu et al., 2012). *FT* is a target of *CO* and acts upstream of *SOC1*, which can induce flowering by long-distance transportation. In addition to Arabidopsis, the *FT* regulates flowering in other LD plants such as *Lolium temulentum* (King et al., 2006) and in SD plants such as rice, where, *FT* ortholog, known as *Heading date 3a* (*Hd3a*), plays an important role in the promotion of flowering (Kojima et al., 2002). *FT* has also been implicated in the regulation of flowering in other SD plants such as Cucurbits and Norway Spruce (Gyllenstrand et al., 2007; Lin et al., 2007). Interestingly, the tomato *FT* ortholog (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 article was retracted and the current theory is that FT protein is the mobile signal which is supported by data from both Arabidopsis and rice (Corbesier et al., 2007; Tamaki 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* in LD, SD and day-neutral plants, as well as evidence of signal transmission, suggested that 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.

Six *FT* genes (*AcFT1-6*) were identified in double haploid onion line CUDH2150 and the authors proposed that *AcFT1* and *AcFT4* genes promote and inhibit bulb formation, respectively, and the *AcFT2* promotes flowering in onion (Lee et al., 2013). However, other *FTs*, which might also affect onion bulb formation, did not complement Arabidopsis *FT1* and their functions were not studied. A recent study showed that *FT* transcript levels were greatly influenced by various environmental factors such as photoperiod, temperature and drought and suggested that *AcFT7* is a member of the *FT-LIKE PROTEIN* genes in onion that may be involved in regulation of onion bulbing, similar to other *FT* genes (Manoharan et al., 2016). In addition,

AcFT4 and *AcFT7* could be involved in establishing the difference in timing of bulb maturity between the two contrasting, early and late maturity onion lines.

1.11.5 GIBBERELLIN 3-OXIDASE (GA3ox)

Gibberellin (GA) 3-oxidase is a class of 2-oxoglutarate-dependent dioxygenases which catalyzes the final step in the synthesis of bioactive gibberellins (GAs), thereby playing a direct role in determining the levels of bioactive GAs in plants (Hu et al., 2008; Mitchum et al., 2006). Bioactive gibberellins (GAs) are important plant growth regulators which regulate plant growth and development by playing essential roles in seed germination, stem elongation, leaf expansion, flower, fruit and seed development (Davies, 2004; Singh et al., 2002). Cloning of the *GA3ox* genes has been done in several dicotyledonous species (Chiang et al., 1995; Itoh et al., 1999; Lester et al., 1997; Martin et al., 1997; Yamaguchi et al., 1998) and also in monocotyledons (Itoh et al., 2001). For most plant species, multigene families encode *GA3ox*, although it is unclear how many *GA3ox* genes are present in each genome. In rice (*O. sativa*) there are only two *GA3ox* genes, *OsGA3ox1* and *OsGA3ox2*, where, each gene has a distinct tissue-specific expression pattern (Itoh et al., 2001; Kaneko et al., 2003; Sakamoto et al., 2004) and in Arabidopsis, four *GA3ox* genes have been identified (*AtGA3ox1– AtGA3ox4*) which encode gibberellin 3-oxidase (Williams et al., 1998; Yamaguchi et al., 1998). However, it has yet to be investigated how each *AtGA3ox* gene contributes to optimizing bioactive GA levels during growth and development. Quantitative real-time PCR (qRT-PCR) analysis has shown that each *AtGA3ox* exhibits a unique organ-specific expression pattern; suggesting individual *AtGA3ox* members play distinct developmental roles. Results show that *AtGA3ox1* and *AtGA3ox2* are responsible for the synthesis of bioactive GAs during vegetative growth, but that they are dispensable for reproductive development. The stage-specific severe GA-deficient phenotypes of the ga3ox1/ga3ox2 mutant suggest that *AtGA3ox3* and *AtGA3ox4* are tightly regulated by developmental cues; *AtGA3ox3* and *AtGA3ox4* are not upregulated to compensate for GA deficiency during vegetative growth of the double mutant (Mitchum et al., 2006).

1.11.6 LEAFY (LFY)

LFY causes a group of undifferentiated cells named meristems to develop into flowers instead of leaves associated with shoots (Weigel et al., 1992). *LFY* encodes a plant-specific transcription factor, which is found in all plants and one of its exons have been used extensively in phylogenetic work on spermatophytes (Hollingsworth, 1999). Overexpression of the *LFY* causes the plant to be less sensitive to environmental signals and flowers earlier (Weigel and Nilsson, 1995). Experimental data suggested that the *LFY* is required for the development of metamers, changes in a basic unit cause variation in the shoot structure, which is necessary for the expression of downstream genes controlling floral organ identity and thus regulates inflorescence development in Arabidopsis (Schultz and Haughn, 1991). Metamer is a compound that contains the same number and type of atom as other compounds but with a different distribution of radicals. Many studies have been progressed on the regulation network and biological roles of *LFY* in Arabidopsis and its homologous genes in floral development (Wang et al., 2004). *LFY* is expressed widely in both vegetative and reproductive tissues in different higher plants, and plays an important role for promoting flower formation by interaction and coordination with other genes such as *TFL, AP1, AP2, FT, AP3, CO, GA1*, etc., where a critical level of *LFY* expression is essential. The *LFY* not only controls flowering-time and floral transition, but also plays an important role in inflorescence and floral organ development (Wang et al., 2004). *LFY* is one of the most important floral meristem identity genes, which is required to specify the lateral meristems as flowers. *LFY* encodes a plant-specific transcription factor that can act as either an activator or repressor depending on context, including what co-factors it is interacting with. It controls multiple aspects of oral morphogenesis, including phyllotaxis, organ number, organ identity and determinacy (Siriwardana and Lamb, 2012).

1.12 Onion genome

Genomic studies have investigated how genes function in controlling growth, development and adaptation and also considered the phylogenetic relationship between species (Brewster, 2008). Onion is a diploid plant (2n=2x=16) with a very large genome size (16,415 Mbp/1C, about 6, 36 and 107 times as large as maize, rice and Arabidopsis, respectively) and consists of a high percentage of repetitive DNA (Kuhl et al., 2004; McCallum, 2001). 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, 1998). However, it is reported to be less than the duplication in maize, soybean and some *Brassica* species (King, 1998). A set of 11,008 cDNA sequences generated from bulb, root, leaf and callus tissue of onion has been identified (Kuhl et al., 2004). These sequences are actively transcribed for RNA found in tissues, and all hence called 'expressed sequence tags' (EST). Little similarity and scant colinearity, was found in the comparison of ordering these onion ESTs with another monocotyledon, rice suggesting that genomic information from the vast grass family crops (order *Poales*) might not provide appropriate genomic models for crops in the *Asparagales* order such as onion or other alliums. This makes it necessary to develop more genomic information for these plants (Martin et al., 2005). Pilot sequencing of onion genomic DNA has been studied, however, the whole onion genome has not been sequenced because of the large genome size and high levels of duplicated DNA (Jakse et al., 2008).

In the past decades, multiple genes inducing flowering have been identified but there have been very limited genomic studies on daylength adaptation for bulbing. Onion bulb formation is daylength-dependent and is thus similar to the daylength response of flowering (Taylor et al., 2010). Complete genomes have been sequenced for several species, which include Arabidopsis (Arabidopsis Genome, 2000) and the crops rice (*O. sativa*), maize (*Zea mays*), wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), potato (*Solanum tuberosum*), legumes such as soybean (*Glycine max*), barrel medic (*Medicago truncatula*), fruits such as grape (*Vitis vinifera*), papaya (*Carica papaya*) and vegetables such as cucumber (*Cucumis sativus*) and tomato (*Solanum lycopersicum*) (Goff et al., 2002; Lunde et al., 2003; Shoemaker et al., 2002; Ware et al., 2002; Young et al., 2003; Yu et al., 2002; Michael and Jackson, 2013). However, due to difficulty in developing, maintaining and exchanging genetic stocks, high degrees of heterozygosity, and a dearth of sequence data, there are low numbers of genetic studies aimed at onion improvement (McCallum et al., 2012). In comparison to the knowledge gained regarding photoperiodic regulation of flowering, relatively little is known about genetic regulation of bulbing process (Lee et al., 2013; Osterlund and Paterson, 2002; Taylor et al., 2010). This project addresses this and will involve the identification of genes and regulatory mechanisms involved in the bulbing process.

1.13 Project Aims

Bulb initiation in LD onion at the physiological level is regulated in a similar way to the photoperiodic regulation of flowering in Arabidopsis as described in section 1.7 and the mechanism of daylength perception in Arabidopsis at the molecular and genetic level is shown in Figure 1.7.

1.13.1 Major aims: The overall aims of the project are;

i. to identify genes involved in daylength control of bulb formation in onion which is crucial for adapting new varieties for growth and development at different latitudes

ii. to test the hypothesis that genetic regulation of bulb formation in response to daylength is analogous to the daylength regulation of flowering

1.13.2 Specific aims

a. Establish experimental conditions to characterise daylength-dependent bulb initiation and development in onion by a comprehensive set of developmental expression experiments, and to generate materials for molecular analyses

b. Bioinformatic analyses to identify candidate genes in published database, e.g., *FT, CO* and to isolate the genes of interest in onion that have known functions in Arabidopsis flowering and regulate other important pathways

c. RNA-Seq analysis to generate onion transcriptome reference sequence to identify genes not in published database and for more widespread identification of genes differentially expressed in response to photoperiod

d. Carry out quantitative gene expression analysis in different response types associated with bulb formation, spatial expression and circadian regulation

e. Isolate, characterise and test the function of onion *CO* and *COL* genes by complementing Arabidopsis *CO*

CHAPTER 2: STANDARD MATERIALS AND METHODS

The investigation was carried out at the School of Life Sciences and Phytobiology Facility (PBF), the University of Warwick, UK, during the period from July 2013 to September 2016. The plant and biological materials that were used in conducting this study are presented in this chapter. The methods and experimental protocols presented in this chapter were used throughout the project.

2.1 Standard Materials

2.1.1 Plant materials

2.1.1.1 *Allium cepa L.*

The long day (LD) onion variety '*Renate F1*' (also called *Renate*) (Elsoms Seeds Ltd., Spalding, UK) was used throughout this project. Varieties with different daylength responses were originally sourced from the Warwick Genetic Resources Unit and the seeds provided by Dr. Andrew Taylor, Warwick Crop Centre. They were grown for different projects and based on the results (Chapter 3), '*Hojem*' was selected as the short day (SD) onion variety for this study. Seeds of *Hojem* were collected from the Vegetable Genetic Improvement Network (VeGIN, UK) project Diversity Set.

2.1.1.2 *Arabidopsis thaliana*

Seeds of *A. thaliana co-2* (Landsberg background) mutant as well as Landsberg *erecta* (L*er*) wild type were obtained from Dr. Stephen Jackson (School of Life Sciences, the University of Warwick). The original source of all Arabidopsis seed was the Nottingham Arabidopsis Stock Centre (NASC).

2.1.2 Other materials

Cloning products were generated using primers with att sites of a pDONR207 vector (Invitrogen Ltd.) specific to the Gateway® cloning system, and amplified using polymerase chain reaction (PCR) from pGEM-T easy. The pB2GW7 vector used for transformations was provided by Dr. Jemma Taylor (School of Life Sciences, the University of Warwick). Protocol for Plasmid DNA extracted from the colonies containing the correct sequence (in the pGEM-T vector, Promega Corporation) was provided by Dr. Sophie Piquerez (School of Life Sciences, the University of Warwick).

2.2 Standard Methods

2.2.1 RNA extraction, DNase treatment and cDNA synthesis

Total RNA was extracted from leaf and bulb material from onion grown under LD and SD using the Z6 buffer method, following the manufacturer's (Roche manufacturing Ltd., Republic of Ireland) guidelines. Samples were ground using pestle and mortar and then approximately 100 mg of frozen plant tissue was homogenised using a Dremel drill. In this step, Z6 buffer reagent and b-Mercaptoethanol were added which act to remove RNase. Two extra reagents, 3M Sodium acetate (NaOAC) and 7.5M Lithium chloride, which remove carbohydrates and polysaccharides, respectively, were included in this method to obtain high quality RNA. After isolation, the quality and quantity of total RNA was measured with the Thermo Scientific NanoDropTM 1000 Spectrophotometer (NanoDrop Technologies, Inc., USA).

The TURBO DNA-*free treatment* kit (Ambion, USA) was used to eliminate the genomic DNA contamination following the manufacturer's guidelines. A PCR, as described in section 2.2.4, was set up to check for genomic DNA contamination using primers for *ALLINASE* (*ALL*) gene and visualized on RNA gel electrophoresis.

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

2.2.2 Illumina Sequencing

Leaf and bulb material was harvested from *Renate F1* grown in LD or SD and used to prepare libraries for Illumina sequencing in the Life Sciences genome centre. Samples were multiplexed to obtain differentiation between long and short day samples and for biological replication.

2.2.3 Genomic DNA extraction from onion leaf

Genomic DNA was extracted from onion leaf using a modified CTAB method (Doyle and Doyle, 1987; Porebski et al., 1997). Leaf tissue was ground using a pestle

and mortar and then homogenised using a Dremel drill. The plant materials were further homogenised after adding 500 µl pre-warmed CTAB buffer. The reaction mixer was then put in a pre-heated block at 65 ºC for 10 minutes for incubation. Dichloromethane:isoamyl alcohol (24:1 v/v) was then added (500 µl) to the reaction followed by incubation and samples were then centrifuged at 16,500 x g for 2 min at room temperature (in a table top microcentrifuge). After centrifugation, the upper phase was transferred to a fresh tube and 300 µl of isopropanol was added. The reaction mix was then centrifuged at 16,500 x g for 2 min and the supernatant was removed. 500 µl wash buffer was added to the sample which was left at room temperature for 2 min. After incubation, the sample was centrifuged at 16,500 x g for 3 min and the supernatant removed again. A pulse spin was given using table-top centrifuge and the pellet was air dried for approximately 15 min. The pellet was then re-suspended in 200 µl of sterile distilled water. This protocol was provided by Dr. Andrew Taylor, Warwick Crop Centre.

2.2.4 PCR and Agarose gel electrophoresis

PCR reactions comprised 0.4 units KOD Hot Start DNA Polymerase (Merck Bioscience, Cat No. 71086-3), 1 x KOD Hot Start DNA Polymerase reaction buffer, 1 µl of template, 0.2 mM each dNTP, 2 mM $MgSO_4$, 0.3 µM of each forward and reverse primer in a total volume of total 20 µl made up with SDW (sterile distilled water). PCR amplification was carried out with an initial denaturation step of 95 °C for 2 min followed by 39 cycles (if not specified otherwise) of denaturation at 95 °C for 20 seconds, annealing at a temperature specific for the primer pair for 10 seconds and extension at 70 °C for 1 min per kb of product. For colony PCR, a pipette tip containing a single colony placed in 40 µl of SDW and then 1µl from that mixer used as a temple added to each reaction. A further extension of 10 min at 70°C was carried out at the end of the cycles.

Agarose gel electrophoresis was carried out to check the quality of RNA and DNA followed by PCR reactions. Agarose gels composed of 1-2% (w/v) ultra pure agarose (Invitrogen, USA) with 1x Tris-acetate-EDTA buffer (TAE buffer) and 0.05 µl/ml Gel Red™ Nucleic Acid Gel Stain, 10,000X in Water. Orange G ($C_{16}H_{10}N_2O_7S_2Na_2$, Sigma-Aldrich®, Cat. No. O3756, UK) loading dye was mixed with each sample prior to loading on a gel. 1Kb plus DNA ladder (Invitrogen Ltd., Cat. No. 10787,

USA) was also loaded on gel to estimate the product size. Gels were run in a tank filled with 1x TAE buffer at 100-120 mA for 45 to 90 min, depending on the sizes of the nucleic acids and the concentration of agarose in the gel. After electrophoresis, the samples on the gel were visualised and recorded by a G:BOX gel documentation system (Syngene, UK).

2.2.5 qRT-PCR

Extraction of total RNA and synthesis of cDNA was followed by the protocol as described in section 2.2.2. The expression of reference genes and genes of interest was analysed by qRT-PCR using the CFX384 Touch™ Real-time PCR machine from BioRad (Bio-Rad Laboratories Ltd., UK). For the CFX384 Touch™ Real-time PCR machine, each reaction contained 5 µl GoTaq® qPCR Master Mix, 2X (Promega, USA), 0.5 µl of cDNA, either 0.2 or 0.3 µM of each primer (Appendix VIII), 0.2 µL fluorescein dye and water to make up 10 µl reaction volume. Three replications were carried out for each sample. The protocol is provided in Table 2.1 and all primer details are presented in Appendix VII and VIII. qRT-PCR data were analysed after completion of each PCR run using three replications and data indicated as means and normalized against expression levels of the house keeping genes for each sample (Appendix XV). The acceptable PCR efficiency (standard curve, slopes on log amplification curves) is between 90-110%, R^2 higher than 0.985 were appropriate and the melt curve should show a unique peak to indicate that only a single type of expected amplicon is present (Eurogentec qPCR guide, http://www.eurogentec.com/uploads/qPCR-guide.pdf). PCR products were purified as described in section 2.2.6 and sequenced as described in section 2.2.7 to confirm the identity of the genes.

Table 2.1 The thermal cycling condition for qRT-PCR

qRT-PCR protocol
1: 95.0°C for 2:00 min 2: 95.0°C for 15 sec 3: X°C for 1:00 min (*Annealing temperature will be variable depending on the gene of interest*) Plate Read 4: GOTO 2, 39 more times 5: 95.0°C for 10 sec 6: Melt Curve 60.0°C to 95.0°C: Increment 0.5°C 5 sec

2.2.6 Purification of PCR products

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

2.2.7 Sequencing of PCR products

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

2.2.8 Cloning

Cloning was carried out using the cloning kit supplied by the manufacturer (Invitrogen Ltd., USA) as specified in the appropriate chapter. Following initial PCRs, an adenine (A) residue was added to the 3′ PCR products by incubating for 30 min at 72°C the PCR fragments with dNTPs and non proofreading TAQ DNA polymerase. PCR products of the expected lengths were purified as described in section 2.2.7. The reaction mix in a total volume of 10 µl was then ligated for overnight into the pGEM-T Easy vector (Promega Ltd., Cat. No. A1360, Australia) (Figure 2.1), following the manufacturer's guidelines.

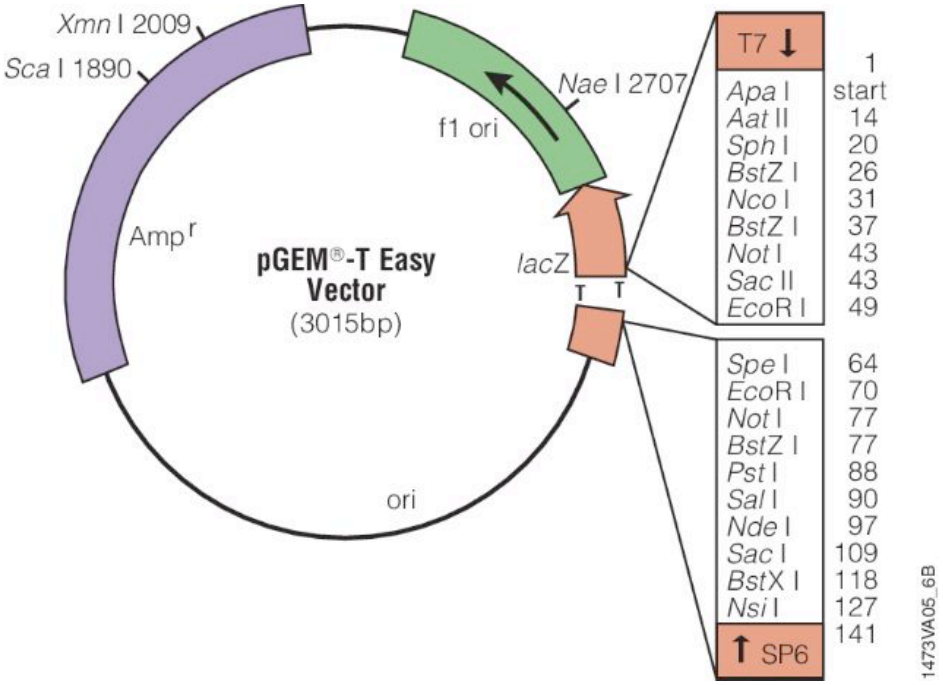


Figure 2.1 Map of pGEM®-t Easy transformation vector

Electroporation was used as the standard method of transformation. Overnight ligations were precipitated and re-suspended in 10 µl of sterile distilled water. One microlitre of vector products was then added to 20 µl of Electrocompetant EC100 *E. coli* cells (Cambio Ltd., Cat. No. EC10005) or electrocompetent *Agrobacterium* cells (AgC58pGV3101) in a 1 mm electroporation cuvette (Geneflow Ltd., Cat. No. E6-0050) and electoporated using the EC1 setting of a Bio-Rad laboratories Micropulser, using the appropriate setting at 1.8 kV for 5 ms following the manufacturer's guidelines. After electroporation, 1 ml of SOC medium (Appendix X) was added to the cuvette and the contents were mixed and transferred to a microcentrifuge tube and shaken at 250 rpm for 1 hour at 37 ºC or 28°C, for *E.coli* or *Agrobacterium* respectively. After this, aliquots of 10, 50 and 100 µl were plated on LB media (VWR International) containing 100 µg/ml ampicillin, 0.1 M isopropyl/-D-thiogalactoside (IPTG), and 20 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) for *E.coli* selection and 50 µg/µl Spectinomycin (SPEC), 20 µg/µl Gentamycin (GENT) and 50µg/µl Rifampicin (RIF) for *Agrobacterium* selection and left overnight at 37°C or 28°C, respectively. Colonies were screened by PCR for expected insert using gene specific primers presented in Appendix XIV. This protocol was provided by Dr. Jemma Taylor from the School of Life Sciences, the University of Warwick

2.2.9 Plasmid DNA extraction

Bacterial cultures were prepared by adding a single bacterial colony to approximately 5 ml liquid LB medium (VWR International, Cat. No. 1.10285) using seeding centrifuges tubes containing the appropriate antibiotic. Cultures were grown overnight at 37 ºC with shaking (250 rpm). Bacteria were harvested by centrifuging at 6000 x g for approximately 15 min. Plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (QIAGEN, Cat. No. 27106) following the manufacturer's guidelines and was eluted in 50 µl of SDW. This protocol was provided by Dr. Sophie Piquerez from the School of Life Sciences, the University of Warwick.

CHAPTER 3: BULB DEVELOPMENT IN ONION IN RESPONSE TO DAYLENGTH

3.1 Introduction

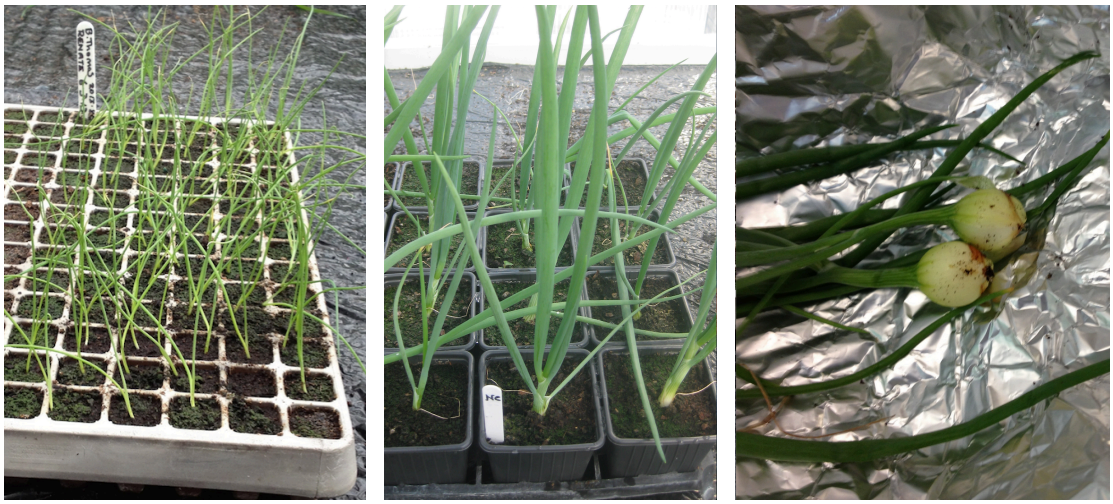
The bulbing process of the onion plant in response to daylength is described in Chapter 1. Bulb formation in temperate onions is daylength-dependent and LD of at least 14 h of light is required to stimulate bulb initiation (Mettananda and Fordham, 1997). Long day onions are grown in temperate regions and require at least 14 or more hours of light to stimulate bulb initiation, while, SD onions are grown in more tropical regions and require a daylength of only 10 h or more for bulbing (Brewster, 2008). The matter is further complicated as some onion varieties are intermediate where they need 12 h or more of daylight before they will start producing the onion bulb. This daylength-dependent bulb initiation is similar to the photoperiodic regulation of flowering in other plants (Mettananda and Fordham, 1997; Taylor et al., 2010). Therefore, it is hypothesised that the genes involved in the daylength regulation of flowering are also responsible for the daylength regulation of bulb formation in onion. Both processes are induced by LD, signal perception is in the leaf and response at the apex and both are promoted by far-red light, through PHYA (Lercari, 1984; Sobeih and Wright, 1987). Bulbing is a reversible process and plants grown under inductive conditions promote bulb formation but if transferred to non-inductive condition, they revert back to vegetative growth (Brewster, 1997; Taylor, 2009).

This chapter describes experiments designed to characterise bulb initiation and development in LD onion cv. *Renate F1* grown under natural conditions (NC), constant LD in controlled environment (CE, 16 h) and constant SD (CE, 8 h) conditions as well as in the SD onion cv. *Hojem* continuously grown at 12 h of daylight. In natural conditions, no artificial light was provided for plant growth and which are similar to outside environment/field conditions. Bulb initiation can be measured by bulbing ratio. The major objective of these experiments was to establish the experimental conditions for daylength-dependent bulb initiation and development in onion by a comprehensive set of developmental expression experiments and generate materials for molecular analyses.

3.2 Materials and Methods

3.2.1 Time-course experiment for Renate F1 during development

Experiment 1: This experiment was conducted to characterise the response of bulb initiation in relation to daylength as a prelude to more detailed later experiments. Onion plants were grown in natural conditions within a glasshouse at the Crop Centre in Wellesbourne during the period from 6th March to 7th May 2013 when daylight was 11 h 15 min to 15 h 37 min, respectively. Initially, *Renate F1* seeds were sown in modular trays and after 4 weeks plants were potted up into 9 cm pots containing Levington M2 compost (Figure 3.1). At 48 d when plants had initiated bulbing, half of them were transferred to constant LD (16 h photoperiod including 8 h fluorescent followed by 8 h incandescent light) and the other half to constant SD (8 h fluorescent light) in SANYO 2279 controlled environment cabinets at the Phytobiology Facility for another 2 weeks. Both SANYO cabinets were set at 22°C day and 18°C night temperatures with 60% relative humidity and ambient CO_2 concentration, and provided with a Photosynthetic Photon Flux Density (PPFD) of 100 Wm^{-2}. Sampling was carried at 62 d at Zeitgeber time 10 (ZT10) and involved removing the middle part of the first newly expanded leaf and the middle to the basal portion of bulb, chopped into small pieces, and freezing in liquid nitrogen before storing at -80°C. The harvested materials were used for molecular analyses.



Plants at 28 d | Plants at 48 d | Plants at 62 d

Figure 3.1 Growth of *Renate F1* plants under national conditions in Phytobiology Facility to generate materials for molecular analyses. LD-grown bulbs are shown. A similar method was employed for plants grown in SD and other developmental experiments.

Experiment 2: Based on the timing of the bulb response in experiment 1, this experiment was conducted for measuring bulbing ratio and generating materials for molecular analyses (Figure 3.2). Onion plants were grown in natural conditions in a glasshouse at Phytobiology Facility during the period from 19th June to 6th August 2013 when daylight was 16 h 38 min to 15 h 7 min, respectively. Initially, *Renate F1* seeds were sown in modular trays and after 4 weeks; plants were potted up into 9 cm pots containing Levington M2 compost. At 48 days from sowing (DFS), the rest of the plants were divided into 3 groups, one group was transferred to constant LD, one to constant SD, using similar controlled condition as described in Experiment 1, and one group kept in NC. From 30 d, measurements of bulb and neck diameter, using slide callipers, were taken at weekly interval for 2 weeks and then twice in a week for the rest of the growth period. Onion leaf and bulb material was harvested and sampling (3 plants were pooled together for replication using a Completely Randomised Design, CRD) was carried out at ZT10 and involved removing the middle part of the first newly expanded leaf and the middle to the basal portion of bulb, chopped into small pieces, and freezing in liquid nitrogen before storing at -80°C. Plants were selected for harvesting using a random number generator (Haahr, 2006). The harvested materials were used for molecular analyses. 'Bulbing ratio' was calculated by dividing the maximum bulb diameter by the minimum neck diameter and bulb initiation was considered to have been initiated when the bulbing ratio reached a value greater than 2 (Clark and Heath, 1962). Means, standard deviations and standard errors were calculated for all data points using Microsoft Excel and the significance of the differences in bulbing ratio between treatments were assessed by using factorial analysis of variance (ANOVA) with repeated measures. ANOVA was carried out using statistical software package SPSS.

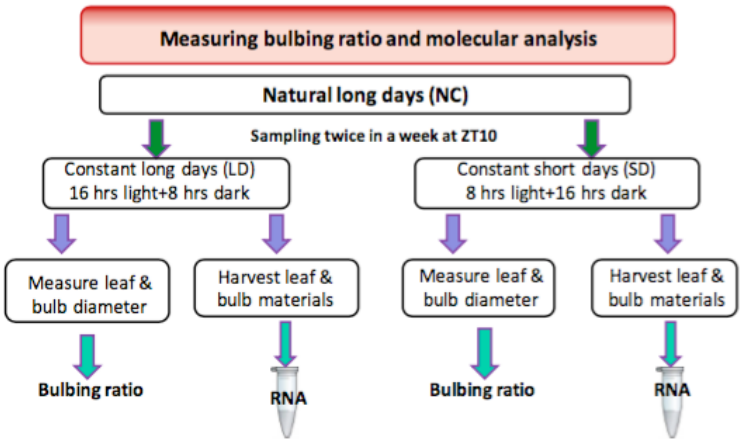


Figure 3.2 Method of transfer experiment during development of onion cv. *Renate F1*.

3.2.2 Daylength adaptation and selection of SD onion variety

This experiment was conducted for measuring bulbing ratio in response to daylength for the selection of a SD onion cultivar. The plants were grown in a photoperiod controlled glasshouse compartment of Phytobiology Facility to give 12 h daylight and provide other environmental conditions as described in 3.2.1 during the period from 14th October 2013 to 6th March 2014. Initially, seeds of 17 varieties of onion were sown in modular trays and after 4 weeks plants were potted up into 9 cm pots containing Levington M2 compost. Measurements of bulb and neck diameter were taken from three uniform plants under each variety at 104, 118, 132 and 148 DFS using slide callipers. 'Bulbing ratio' was calculated as described in section 3.2.1 for Experiment 2.

3.2.3 Time-course experiment for Hojem during development

This experiment was conducted for measuring bulbing ratio and generating materials for molecular analyses using cultivar *Hojem* and also to compare the bulbing ratio with cultivar *Renate F1* under ID conditions. The plants were grown in a photoperiod controlled glasshouse compartment of Phytobiology Facility to give 12 h daylight and provide other environmental conditions as described in 3.2.1 during the period from 17th March to 8th August 2014. Initially, seeds of both varieties were sown in modular trays and after 4 weeks; plants were potted up into 9 cm pots containing Levington M2 compost. From 35 d, measurements of bulb and neck diameter were taken from both varieties at weekly interval throughout the growth period using slide callipers. Harvesting and sampling was carried out in the similar way as described in section 3.2.1 for Experiment 2 before storing at -80°C. Plants were selected for harvesting using a random number generator (Haahr, 2006). The harvested materials were used for molecular analyses. 'Bulbing ratio' was calculated and statistical analysis was conducted in the similar way as described in section 3.2.1.

3.3 Results and Discussion

3.3.1 Bulb development in LD onion cv. Renate F1

This experiment was conducted to see the response of onion bulbing ratio to different daylengths. Plants were transferred to LD (CE) and SD at 48 d as well as kept under LD (NC). A clear difference was observed in bulb development between plants grown at different daylengths (Figure 3.3).

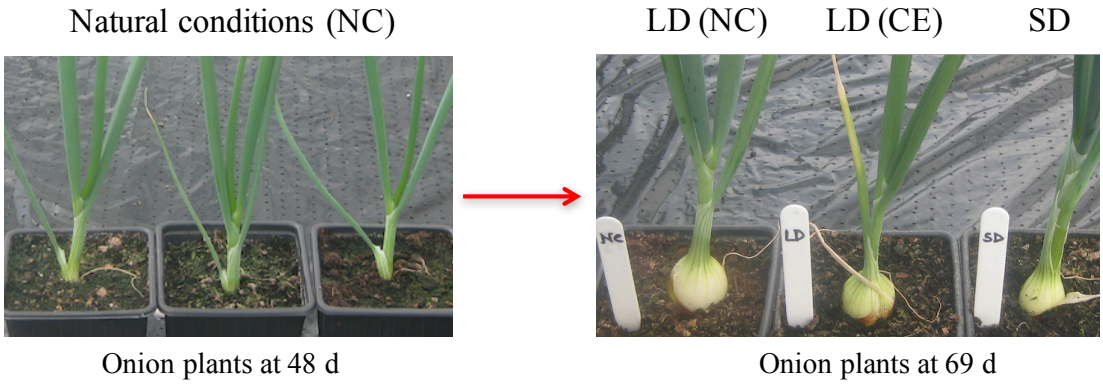


Figure 3.3 Comparison of *Renate F1* plants grown under different daylengths at different stages of development. The left panel shows plants at the time of transfer at 48 DFS. The right panel shows plants from different treatments at 21 days after transfer (DAT).

At 21 DAT, the LD (NC) and LD (CE) plants showed increased bulb diameter, which consequently increased bulbing ratio (Figure 3.4), whereas, the plants in SD showed a small increase in bulb diameter, which was less than in LD, and continued producing new leaves, which resulted in an increase in neck diameter.

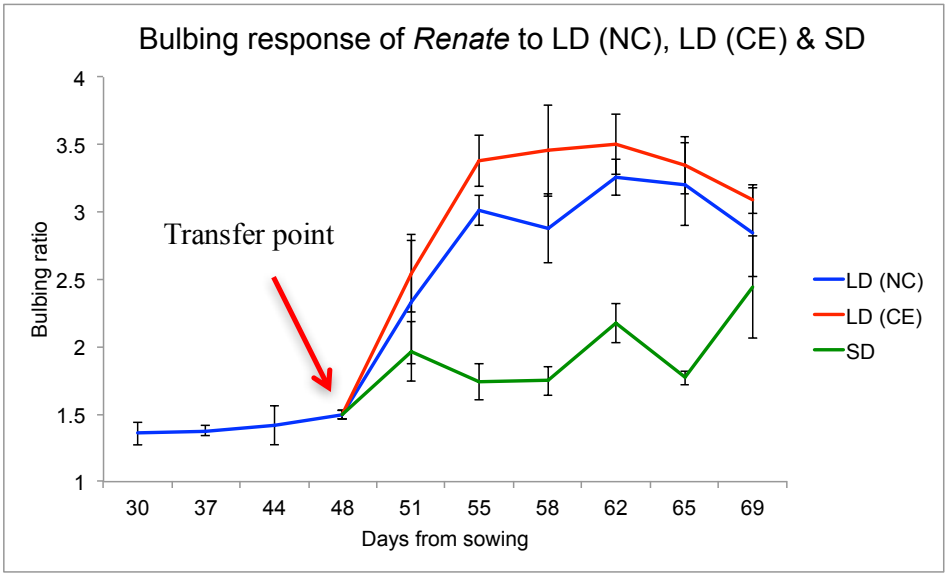


Figure 3.4 Bulbing response of *Renate F1* plants grown under different daylengths. Error bars represent the SEM. Three plants were used in each data point.

In addition, plants grown under LD (NC) produced a larger sized bulb than those grown in LD (CE) which is probably due to the higher light integrals and longer days in the glasshouse in mid summer, both conditions having been shown to accelerate rapid bulb formation (Wright and Sobeih, 1986). So, light quantity also affects bulb initiation. Light quantities used under each daylength conditions in this experiment are shown in Figure 3.5. Light quality also 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). It seems that light quality has a more pronounced effect on bulb initiation in onion. However, daylength is the major factor controlling bulb initiation in LD onions and this will usually be the limiting factor (Taylor, 2009). 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). Samples from this experiment were used for gene expression studies.

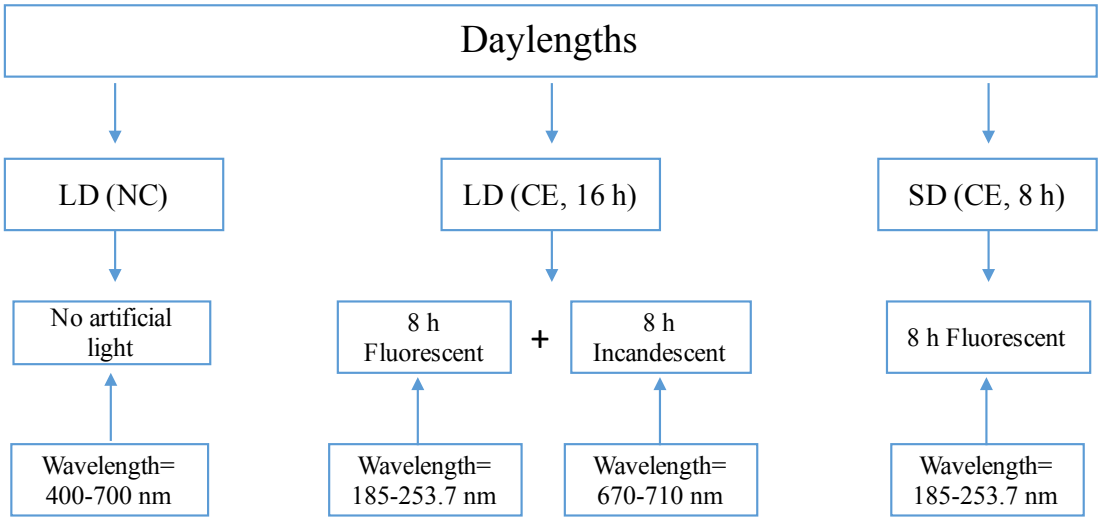


Figure 3.5 Light quantities used for growth of *Renate F1* under different daylength conditions. (NC: natural condition, LD: long day, SD: short day, CE: controlled environment, nm: nanometre).

From ANOVA results, it was also confirmed that LD and SD treatments were significantly different from each other and the number of days from sowing had a significant affect on bulbing ratio (Table 3.1). Moreover, the interaction between DFS and daylength was shown to be significant suggesting that the pattern of bulbing ratio during the period of development of onion is affected by daylength. Therefore, it was confirmed that bulb initiation in *Renate F1* was controlled by daylength with LD conditions stimulating the bulbing process (Taylor, 2009). This result was consistent with previous studies conducted for other onion varieties (Lancaster et al., 1996). The multiple comparisons between DFS are presented in Appendix I.

Table 3.1 Assessment of the significance of differences in bulbing ratio in *Renate F1* between daylength treatments and their interactions with DFS.

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Daylength	9.143	2	4.572	32.174	<0.05
Days from sowing	40.670	9	4.519	38.397	<0.05
Daylength x Days from sowing	8.537	18	0.474	4.030	<0.05
Error	6.355	54	0.118		

3.3.2 Bulb development in response to daylength and selection of SD onion variety

The plants of 17 onion varieties were grown in a photoperiod controlled glasshouse compartment of Phytobiology Facility at 12 h daylight. A clear difference was observed in bulbing ratio between all varieties (Table 3.2). Taking a bulbing ratio of 2 as representing bulb formation, bulbing was first observed in *Hojem* at 104 DFS than to other varieties of onion. Bulbing ratio of *Hojem* was also higher during rest of the growth and development than to other onion cultivars. This reflects CDL requirement, where SD onions start making bulbs at 10 to 12 h of daylight, while ID and LD onion requires at least 12 to 14 h and 14 to 16 h of daylight to stimulate bulbing process (Mettananda and Fordham, 1997). Therefore, *Hojem* was selected as a SD variety for later time-course experiment and molecular analysis. These data were also used for the VeGIN Diversity Project, UK.

Table 3.2 Response of bulbing ratio of different onion varieties at different stages of development grown under 12 h daylength (VeGIN, UK). Three plants were used in each data point.

Onion variety	Bulbing ratio at days from sowing (DFS)			
	104	118	132	148
1. Agrifound Rose	1.22	1.48	1.67	1.94
2. Babosa Jeram	1.95	2.34	2.63	3.41
3. Ben Shemer	2.1	2.1	1.73	2.35
4. California Red	1.58	1.92	2.19	3.22
5. Early Red	1.49	1.86	2.47	3.55
6. Excel	1.97	2.35	3.34	4.28
7. Giza 20	1.88	2.16	1.89	2.42
8. Granoble	1.98	2.43	3.16	4.29
9. Hojem	2.11	2.48	3.64	4.35
10. Mitzri Haemele	1.43	1.43	2.1	2.02
11. New Mexico Yellow Grano	1.55	1.87	2.38	2.36
12. Numex BRI	1.51	1.84	2.48	3.45
13. Pompei	1.42	1.77	1.94	2.25
14. Red Synthetic	1.38	1.57	2.85	3.73
15. While Creole	1.58	1.83	2.1	2.83
16. Yellow Bermuda	1.96	2.24	3.33	4.14
17. Renate F1	1.61	1.91	1.98	2.26

3.3.3 Bulb development in SD onion cv. Hojem

The plants were grown in a photoperiod controlled glasshouse compartment of Phytobiology Facility at 12 h daylight. A clear difference was observed in bulb development between *Hojem* and *Renate F1* plants (Figure 3.6).

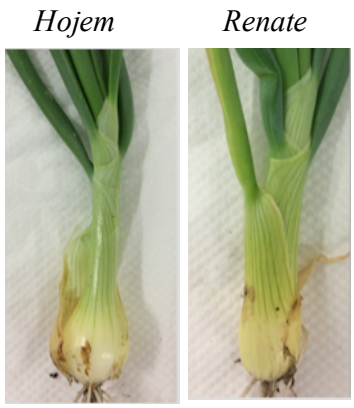


Figure 3.6 Comparison of *Hojem* and *Renate F1* at 104 d under 12 h.

Taking a bulbing ratio of 2 as representing bulb formation, bulbing was observed in *Hojem* at around 104 DFS, but was not seen in *Renate F1* even at 132 DFS (Figure 3.7). This reflects CDL requirement, where SD onions start making bulbs at 10 to 12 h of daylight, while temperate (LD) onion requires at least 14 h of daylight to stimulate bulbing process (Mettananda and Fordham, 1997). Even though 12 h daylength would be long enough for bulbing in SD varieties of onion, there was a long period of time (104 DFS) to bulb initiation in *Hojem*, compared to the time to bulbing in *Renate F1* plants grown initially in NC. This could be due to the reduced insufficient light level during the early stage of plant growth in the 12 h daylength chamber compared to NC (Thomas and Vince-Prue, 1997).

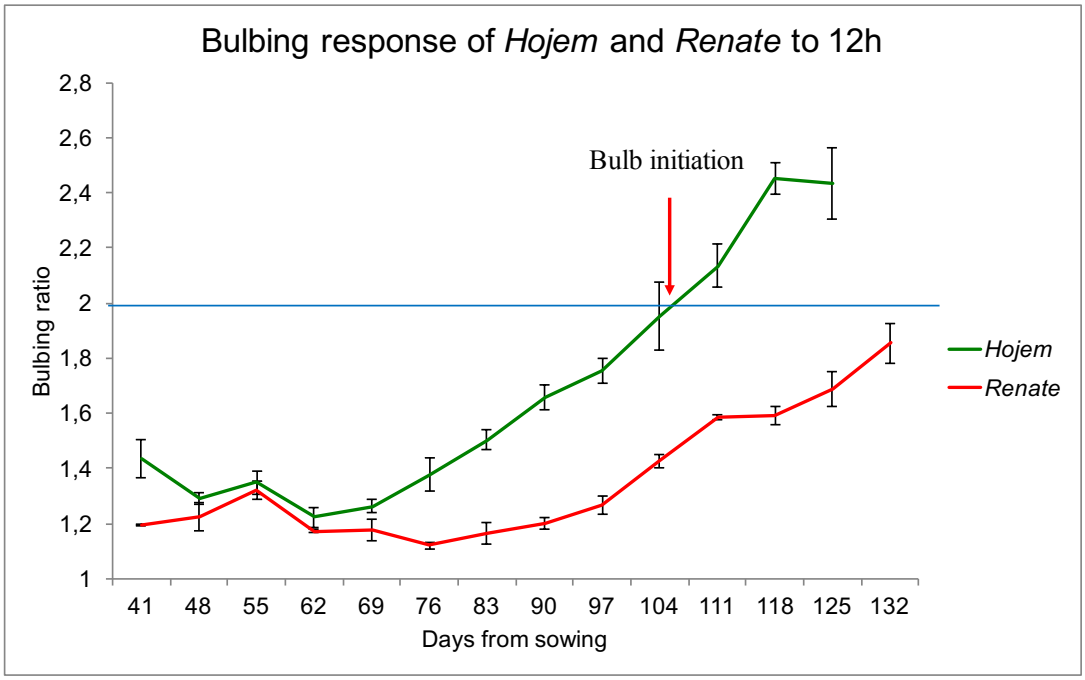


Figure 3.7 Bulbing response of *Hojem* and *Renate F1* plants grown at 12 h. Error bars represents the SEM. Three plants were used in each data point.

The reason behind the delay in the bulb formation could be due to bulb scales being formed quite quickly but it taking longer to detect bulb swelling because the scales expand slowly (Heath and Hollies, 1965). In addition, it has been suggested that plants grown in constant SD could accumulate an inhibitor of bulb formation, so that plants would then take a longer time to initiate bulbs, as the inhibitor would have to be degraded. It is not surprising that sometimes, onions sown in springtime fail to complete bulb formation and they revert to leaf blade production, resulting in thick-necked plants. It was also clearly observed that immediately after bulb formation in

Hojem, bulbs grew more rapidly in older plants than to younger ones (Figure 3.8) which might be due to the age of plants, which has been shown to affect the rate of bulb formation (Sobeih and Wright, 1986).

48 d 62 d 76 d 90 d 104 d 118 d 132 d

Figure 3.8 *Hojem* plants showing bulb development at different days from sowing under 12 h daylength.

Statistical analysis also showed that bulbing ratios were significantly different between *Hojem* and *Renate F1* varieties in response to the daylength (Table 3.3). Samples from this experiment were used for gene expression. The multiple comparisons in DFS between varieties are presented in Appendix II.

Table 3.3 Assessment of the significance of differences in bulbing ratio at 12 h between variety (*Renate F1* and *Hojem*) treatments and their interactions with DFS.

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Variety	4.23	1	4.523	720.70	<0.05
Days from sowing	14.22	13	1.09	100.08	<0.05
Variety x Days from sowing	3.45	13	0.27	24.29	<0.05
Error	0.57	52	0.011		

3.4 Conclusions

Daylength-dependent bulb initiation and development were characterised in LD and SD varieties of onion namely *Renate F1* and *Hojem*, respectively. Bulb initiation and development in *Renate F1* was controlled by daylength and 16 h of light promoted bulbing. In *Renate F1*, the bulbing ratio increased in plants kept in LD (NC) or transferred to LD (CE) at the time that bulbing was just beginning, but not in those transferred to SD at that time. The results presented in this chapter are consistent with the data from previous studies and showed that LD onions require longer days throughout the period of bulb development to reach maturation (Kedar et al., 1975). The age of the plants had a significant effect on bulbing ratio and in addition plants grown under LD (NC) produced a larger sized bulb than those grown in LD (CE), presumably because of the higher light integrals.

In *Hojem*, 12 h was sufficient for bulbing, although bulbs were not seen in *Renate F1* at 12 h even after 132 DFS. However, it was observed that there was a delay in bulbing response in *Hojem* when plants were grown in constant 12 h daylength. This is consistent with the theory that an inhibitor of bulb initiation accumulates when plants are in SD conditions, delaying initiation, although, measurements have not been shown for inhibitory activity. However, it was also clearly observed that immediately after bulb formation in *Hojem*, bulbs grow more rapidly in older plants than in younger ones.

CHAPTER 4: GENE IDENTIFICATION AND ISOLATION

4.1 Introduction

This chapter describes the identification, isolation and differential expression of the key genes that have known functions in Arabidopsis flowering and regulate other important pathways such as the sucrose and gibberellin pathways. This chapter also discusses the characterisation of the key genes that are particularly linked to the circadian or diurnal regulation and involved in the bulb initiation and development in onion. Furthermore, to achieve another important project aim, RNA-Seq analysis was performed to generate onion transcriptome reference sequence and more widespread identification of genes differentially expressed in response to photoperiod, which is also discussed in this chapter. The introduction to the specific genes on which this experiment has been focused was described in section 1.11.

An earlier study of Taylor et al. (2010) showed that some key genes such as *FKF1* and *GI* controlling photoperiodic flowering in Arabidopsis are conserved in onion. However, in this chapter more emphasis has been given to an onion *CO* homologue and *FT* as these genes play a central role in the circadian regulation and the photoperiodic regulation of flowering in both Arabidopsis and rice (Jackson, 2009). It has also been shown that these genes play an important role in the tuberisation in potato, a different response to photoperiod (Martínez-García et al., 2002). *LFY* is a floral homeotic gene which is involved in the photoperiodic regulation of flowering in Arabidopsis (Wang et al., 2004) and has been cloned and functionally identified in onion (Yang et al., 2016). Some other genes, such as *GA3ox1*, play a direct role in determining the levels of bioactive GAs in plants (Mitchum et al., 2006) were also present in the growth and reproductive organs in onion (Shiraiwa et al., 2011). *SUCROSE 1-FRUCTOSYLTRANSFERASE* (*FST-1*), which plays an important storage activity in carbohydrate pathway in approximately 15% of flowering plant species, was also cloned form onion where structurally defined fructans molecules are synthesised from sucrose (Vijn et al., 1998). These will help to provide the information in relation to the involvement of those genes in onion bulb initiation and development.

4.2 Materials and Methods

4.2.1 Identification and isolation of onion genes

First of all key genes were selected that have known functions in Arabidopsis flowering and regulate other important pathways such as the sucrose and gibberellins pathways (Figure 4.1). The sequences of each gene in Arabidopsis were obtained from National Center for Biotechnology Information (NCBI) database (NCBI., 2016). An onion EST sequence was obtained by blasting the sequence of each gene in Arabidopsis homologs against the onion EST database (www.ncbi.nlm.nih.gov/nucest/?term=onion). After obtaining the EST sequences, they were aligned with Arabidopsis sequences using MegAlign™. From alignment information, the positions of sequence identity were obtained.

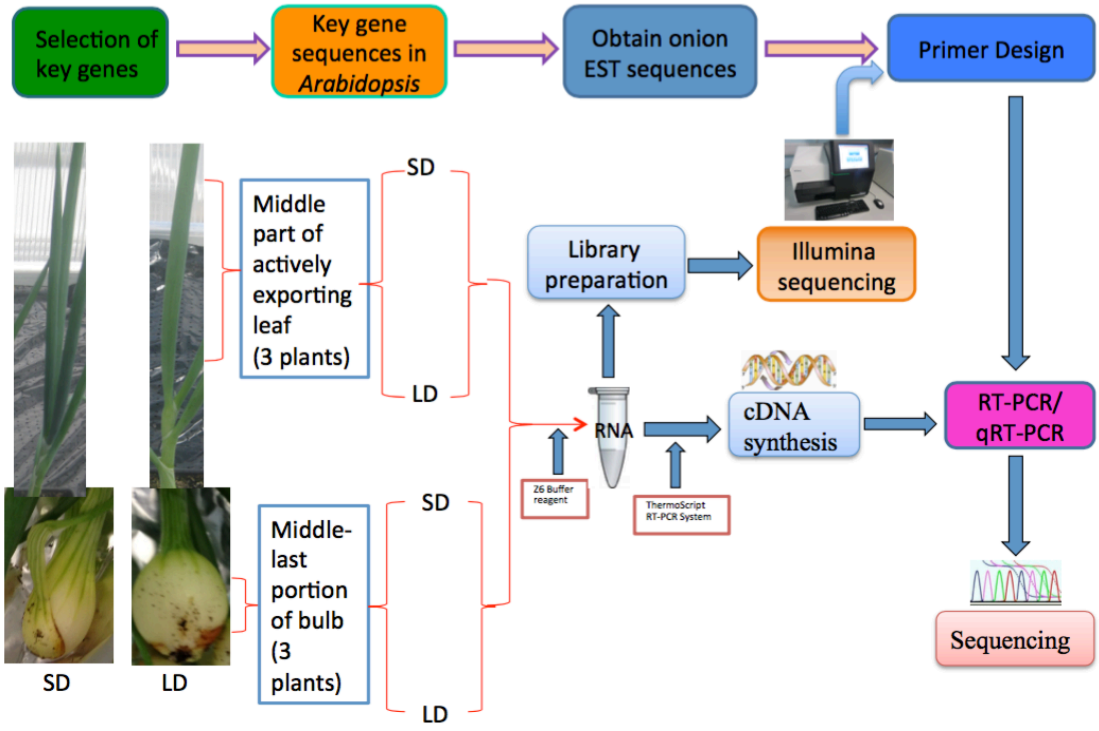


Figure 4.1 Gene identification and isolation from onion cv. *Renate F1*

At the same time, onion plants were grown at the Phytobiology Facility glasshouse providing the conditions as described in section 3.2.1 for Experiment 1. Leaf and bulb materials were harvested from LD and SD conditions, and were used for RNA extraction to prepare libraries for Illumina sequencing as described in section 2.2.1 and 2.2.2, respectively. Onion ESTs and transcriptome sequences obtained from RNA seq

analysis were used to design primers (Forward and Reverse) for each gene amplification using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and synthesised by Invitrogen Ltd and Sigma-Aldrich® (UK).

Primers used for obtaining the full-length of key genes in onion are presented in Appendix III and Appendix IV. cDNA was also synthesised from DNase treated RNA and used for RT-PCR using the designed primers as described in section 2.2.1 and 2.2.4, respectively. Small amounts of cDNA from 4 individual samples (LD leaf, LD bulb, SD leaf & SD bulb) were pooled together for preliminary isolation of the genes. Full-length cDNA was also very useful in designing primers for qRT-PCR. Primers used for reference genes were designed from the full-length sequences obtained from onion transcriptome sequences (Appendix VII). qRT-PCR primers for other key genes (Appendix VIII) were designed from the full-length cDNA obtained from gene isolation together with the EST sequences. The mfold web server (http://mfold.rna.albany.edu/?q=mfold) was used to check primer characteristics in order to avoid unwanted secondary structures in the PCR product. PCR products were purified as described in section 2.2.6 and sequenced as mentioned in section 2.2.7 to confirm the identity of the genes.

4.2.2 Characterisation of onion genes

4.2.2.1 Sequencing and comparison

Onion gene sequences were obtained as mentioned in section 4.2.1. A contig was constructed for each gene using the SeqMan package of DNAStar (DNAStar Inc.). Sequences of the genes from other plant species were also obtained from publicly available NCBI database (NCBI., 2016). Various boxes and domain regions of the specific genes were separated into different data files using the EditSeq package of DNAStar (DNAStar Inc.). Sequences were also stored in FASTA format in Notepad, alignments carried out and phylogenetic trees constructed using predicted amino acid sequences. Alignments for nucleotide and amino acid were carried out by Clustal W method using the MegAlign program of DNAStar, and percentage identities of the genes with homologue genes in other plant species were also calculated.

4.2.2.2 Expression of the onion genes using RT-PCR

The expression of the key genes in pooled as well as individual tissues of LD leaf, LD bulb, SD leaf and SD bulb was examined using RT-PCR as described in section 2.2.4. The primers used for RT-PCR were abbreviated as RT-FOR (Forward) and RT-REV (Reverse), respectively. The primers used for qRT-PCR were abbreviated as qRT-FOR and qRT-REV, respectively. An appropriate annealing temperature and a cycle number of 35 (if not specified otherwise) were used for each specific gene (see Appendices III, IV, VII and VIII for sequences and annealing temperatures). Both cDNA and genomic DNA templates were originated from the *Renate F1* and *Hojem* varieties of onion. All the PCR products were purified and sequenced using both forward and reverse primers to confirm the identity of the genes, as described in section 2.2.6 and 2.2.7.

4.2.2.3 Cloning of *FKF1*, *EF1α*, *LEAFY*, *GA3ox1*, *FT-LIKE PROTEIN 1* and *FT-LIKE PROTEIN 2*

The template used for the cloning reaction was 1 µl of a previously purified PCR product obtained as described in section 4.2.2.2. Cloning was carried out using the cloning kit supplied by the manufacturer as described in section 2.2.8. Colony PCR amplification was carried out using M13 forward and reverse primers on six colonies under each gene with an initial denaturation step of 94 °C for 2 min followed by 34 cycles (if not specified otherwise) of denaturation at 94 °C for 45 seconds, annealing at a temperature of 57 °C for 30 seconds and extension at 72 °C for 40 seconds per kb of product. A further extension of 10 min at 72°C was carried out at the end of the cycles. A pipette tip containing a single colony placed in 40 µl of SDW and then 1µl from that mixture used as a template added to each reaction. Colonies that produced a positive PCR product were cultured and plasmid DNA isolated (as described in section 2.2.9). Sequencing was carried out using M13 forward (GTTGTAAAACGACGGCCAGT) and reverse (CACAGGAAACAGCTATGACC) primers as described in section 2.2.7. Gene-specific primers were used to confirm the double-stranded sequence for each gene (see Appendix III for sequences). Contigs were constructed using the SeqMan package and alignments carried out using the MegAlign of DNAStar programme (DNAStar Inc.) as described in section 4.2.2.1.

4.3 Results and Discussion

4.3.1 Identification and isolation of onion genes

In preliminary experiment, twenty-two of the twenty-five genes of interest including two putative *FT* genes from published databases were identified and isolated in pooled samples of *Renate F1* using RT-PCR except *ACT*, *TUB7* and *RAN2* (Appendix III). PCR products were run on a gel (Figure 4.2-6). The data indicated that the cDNA samples used for the analyses were of good quality and suitable sources for sequence amplification. All were shown to represent the expected gene through sequencing of PCR products. However, in the first PCR, *ALLINASE* was not found in the first PCR reaction (Figure 4.2), but a band was found in the re-amplified PCR (Figure 4.3).

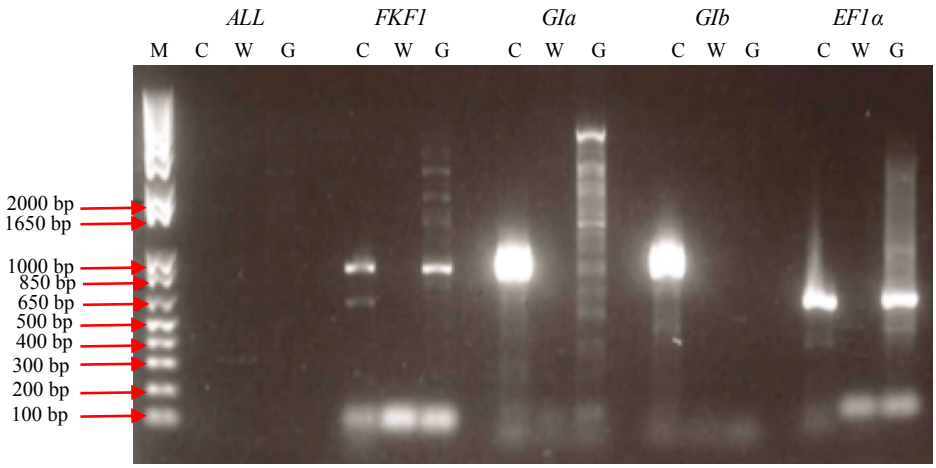


Figure 4.2 Amplification of the 3' end of *ALL*, *FKF1*, *GIa*, *GIb* and *EF1α* in *Renate F1*. M= marker, C= cDNA, W= water control, G= genomic DNA.

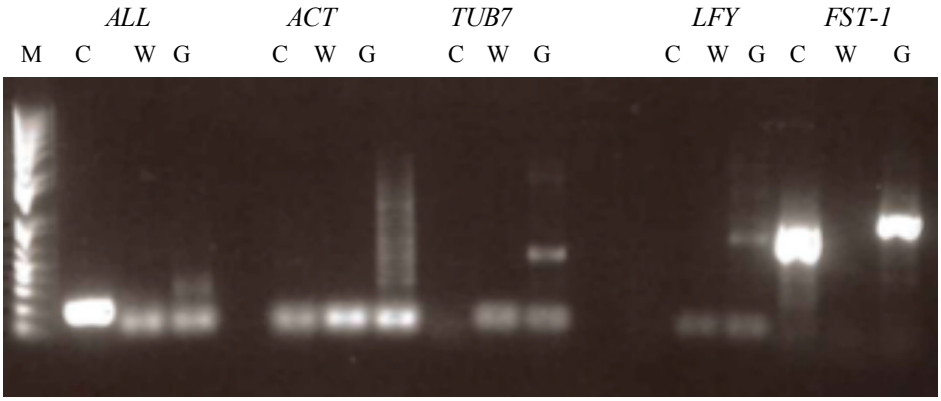


Figure 4.3 Amplification of the 3' end of *ALL*, *ACT*, *TUB7*, *LFY* and *FST-1* in *Renate F1*. M= marker, C= cDNA, W= water control, G= genomic DNA. The same marker was used as shown in Figure 4.2.

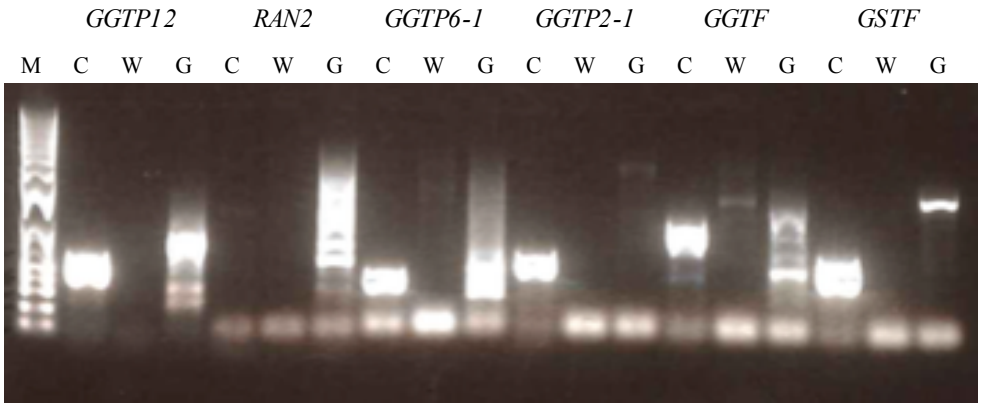


Figure 4.4 Amplification of the 3' end of *GGT12*, *RAN2*, *GGTP6-1 GGTP2-1* and *GSTF* in *Renate F1*. M= marker, C= cDNA, W= water control, G= genomic DNA. The same marker was used as shown in Figure 4.2.

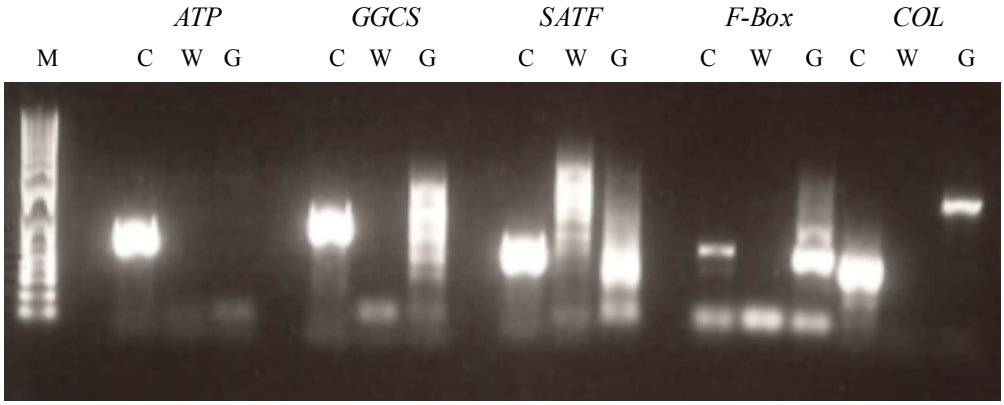


Figure 4.5 Amplification of the 3' end of *ATP*, *GGCS*, *SATF, F-Box* and *COL* in *Renate F1*. M= marker, C=cDNA, W= water control, G= genomic DNA. The same marker was used as shown in Figure 4.2.

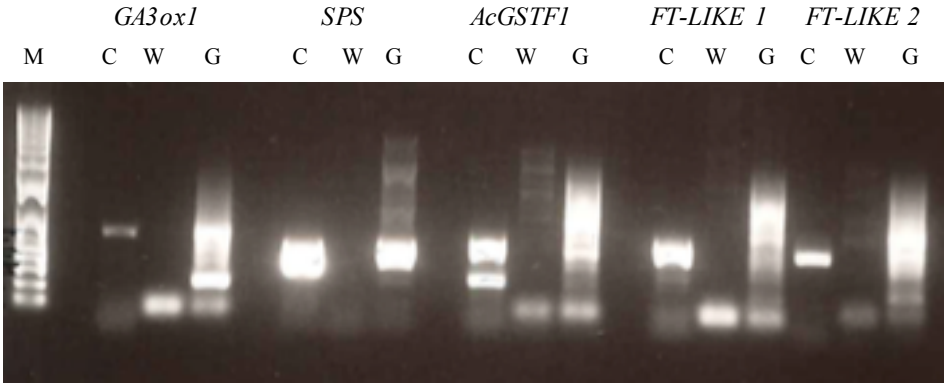


Figure 4.6 Amplification of the 3' end of *GA3ox1*, *SPS*, *AcGSTF1, FT-LIKE 1* and *FT-LIKE 2* in *Renate F1*. M= marker, C= cDNA, W= water control, G= genomic DNA. The same marker was used as shown in Figure 4.2.

4.3.2 Characterisation of onion genes of interest

4.3.2.1 Expression of *AcFKF1*

PCR was carried out to obtain full-length gene sequence of *AcFKF1* (section 4.2.2.2). Result showed that two amplification products of 1 kilobase pairs (kb) and 650 base pairs (bp) were found for *AcFKF1* (Figure 4.2). The gene was then cloned to get the full-length cDNA and sequenced. Contigs were constructed in order to obtain information on the entire gene sequences of the gene. Sequencing analysis showed that the *AcFKF1* clone obtained covered the coding region of this gene, which spanned 826 bp (275 amino acids, Figure 4.7). This is about 50% similar to Arabidopsis *FKF1,* which contains a coding region that spans 1860 bp (620 amino acids). The sequence of *AcFKF1* in onion is presented in Appendix V.

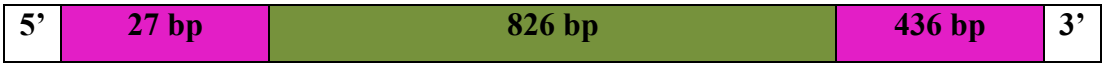


Figure 4.7 Structure of *AcFKF1* mRNA in *Renate F1*. The green box represents the coding region; pink boxes represent the untranslated regions.

Homology is the existence of shared common ancestry between a pair of structures, or genes, in different taxa (Pearson, 2013). A common rule of thumb is that two sequences are homologous if they are more than 30% identical over their entire lengths. Studies have shown that sequences that share more than 40% identity are very likely to be considered as high homology or functional similarity as judged by Enzyme Commission (E.C.) numbers (Pearson, 2013). Nucleotide and amino acid alignments were also carried out to produce percentage identities with *AcFKF1* homologues from Arabidopsis (Accession number NM_105475.3). *AcFKF1* showed 66.1% nucleotide and 66.7% acid amino acid sequences similarity with Arabidopsis which could be considered as high. Therefore, it could be confirmed that *Renate F1 FKF1* is homologous to Arabidopsis *FKF1* (Nelson et al., 2000; Somers et al., 2000; Taylor et al., 2010).

4.3.2.2 Expression of *AcGI*

AcGI mRNA band was found in *Renate F1* pooled cDNA (Figure 4.2). The expression level was high in this pooled sample, which was originated from RNA extracted from leaf and bulb materials under LD and SD conditions. The expression

of *AcGI* in cDNA suggested that this is a functional gene and not a pseudogene (Taylor et al., 2010). Sequencing of the PCR product confirmed that it was amplified from *AcGI*. However, there was no PCR product obtained from genomic DNA using this primer pair, which might be due to the huge size of the genome in onion (Kuhl et al., 2004). Another possible reason could be the used primer pair amplifies a region containing an intron. The insert size from cDNA was 956 bp. The presence of an intron in the genomic DNA may have made the resulting product too large to be amplified. However, the expression of *AcGI* in cDNA allowed for qRT-PCR to be undertaken.

Nucleotide and amino acid alignments were also carried out which produced percentage identities with *AcGI* homologues from Arabidopsis (Accession number NM_102124.3). *AcGI* showed 67% nucleotide and 60.9% amino acid sequences similarity with Arabidopsis homologue, which could be considered as high. Therefore, data revealed that *Renate F1 GI* might be homologous to Arabidopsis *GI.* Sequence of *AcGI* in onion is presented in Appendix V.

4.3.2.3 Expression of *AcLFY*

In the preliminary RT-PCR, no *LFY* mRNA band was found in *Renate F1* pooled cDNA (Figure 4.3). Cloning was carried out using re-amplified PCR products to obtain full-length gene sequence of *AcLFY* (Figure 4.8). Contigs were constructed in order to obtain information on the entire gene sequences of *AcLFY*. Sequencing analysis showed that the *AcLFY* clone obtained covered the coding region of this gene, which spanned 663 bp (221 amino acids, Figure 4.9). This is about 55% similar to Arabidopsis *LFY* (Accession number KF051022.1), which contains a coding region that spans 1263 bp (420 amino acids). The sequence of *AcLFY* in onion is presented in Appendix V.

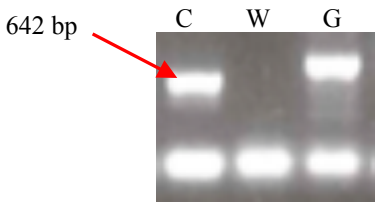


Figure 4.8 Gel red stained agarose gel showing expression of *AcLFY* in *Renate F1* using re-amplified PCR. C=cDNA, W=water control, G=genomic DNA.

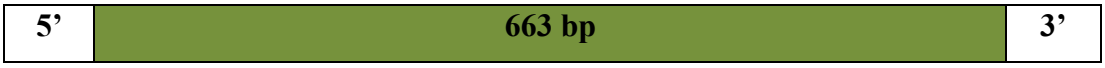


Figure 4.9 Structure of *AcLFY* mRNA in *Renate F1*. The green box represents the coding region.

Nucleotide and amino acid alignments were also carried out which produced percentage identities with *LFY* homologues from Arabidopsis. *AcLFY* showed 61% nucleotide and 62.4% amino acid sequences similarity with Arabidopsis homologue, which could be considered as high. Therefore, data revealed that *Renate F1 LFY* might be homologous to Arabidopsis *LFY*.

4.3.2.4 Expression of *AcCOL*

At the beginning of this study, only one CO-like gene (*AcCOL*) had been identified in onion (Taylor et al., 2010). This *AcCOL* (Accession number GQ232751) sequence was expressed in the pooled cDNA sample (Figure 4.5). The expression level was high in this sample, which was originated from RNA extracted from leaf and bulb materials under LD and SD. Sequencing of the PCR product confirmed that it was amplified from *AcCOL*. Further sequence analysis revealed that the gene contains both a B-Box and CCT domain, which are found in all *CO* and *CO*-like genes (Robson et al., 2001; Taylor et al., 2010). However, the expression of *AcCOL* in cDNA allowed for qRT-PCR to be undertaken. The sequence of *AcCOL* in onion is presented in Appendix V.

4.3.2.5 Expression of *GA3ox1*

The preliminary RT-PCR result showed a slightly visible *GA3ox1* band in *Renate F1* (Figure 4.6). Cloning was carried out using re-amplified PCR products to obtain full-length gene sequence of *GA3ox1* (Figure 4.10). Contigs were constructed in order to obtain information on the entire gene sequences of *GA3ox1*. Sequencing analysis revealed that *GA3ox1* clone obtained covered the coding region of this gene, which spanned 1029 bp (343 amino acids). This is very similar to Arabidopsis *GA3ox1* (Accession number NM_101424), which contains a coding region that spans 1077 bp (358 amino acids).

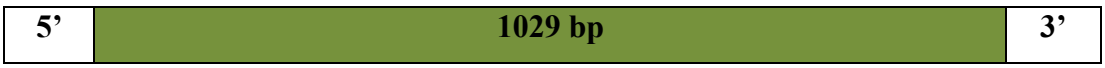


Figure 4.10 Structure of *GA3ox1* mRNA in *Renate F1*. The green box represents the coding region.

Nucleotide alignments were also carried out which showed percentage identities with *GA3ox1* homologues from Arabidopsis. *GA3ox1* showed 77.7% nucleotide and 72.8% sequences similarity with Arabidopsis which could be considered as very high. Data suggest that *GA3ox1,* which has a distinct role in the growth and reproductive organs development in bunching onion (Shiraiwa et al., 2011), might be homologous to Arabidopsis *GA3ox1*. The Sequence of *GA3ox1* in onion is presented in Appendix V.

4.3.2.6 Expression of *FT-LIKE PROTEIN*

At the beginning of this study, only two FT-like genes (*FT-LIKE PROTEIN 1* and *FT-LIKE PROTEIN 2*) had been identified in onion (Taylor, 2009). PCR was carried out to obtain full-length gene sequence of *FT-LIKE PROTEIN 1* and *FT-LIKE PROTEIN 2* (section 4.2.2.2). Results showed that two amplification products were found for *FT-LIKE PROTEIN 1* and no particular genomic DNA band was found for both *FT-LIKE PROTEIN 1* and *FT-LIKE PROTEIN 2* genes (Figure 4.6). The genes were then cloned to get the full-length cDNA and sequenced. Contigs were constructed in order to obtain information on the entire gene sequences of the gene. Sequencing analysis for *FT-LIKE PROTEIN 1* showed that the clone obtained covered the coding region of this gene, which spanned 528 bp (176 amino acids, Figure 4.11). Sequencing analysis for *FT-LIKE PROTEIN 2* showed that the clone obtained covered the coding region of this gene, which spanned 522 bp (174 amino acids, Figure 4.12). Data suggest that these genes are very similar to Arabidopsis *FT* (Accession number AB027504.1), which contains a coding region that spans at 528 bp (175 amino acids). Sequences of *FT-LIKE PROTEIN 1* and *FT-LIKE PROTEIN 2* in onion are presented in Appendix V.

Figure 4.11 Structure of *FT-LIKE PROTEIN 1* mRNA in *Renate F1*. The green box represents the coding region; pink box represents the untranslated region.

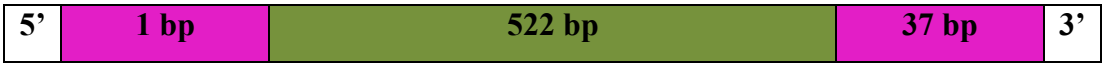


Figure 4.12 Structure of *FT-LIKE PROTEIN 2* mRNA in *Renate F1*. The green box represents the coding region; pink boxes represent the untranslated regions.

Nucleotide and amino acid alignments were also carried out which produced percentage identities with *FT* homologues from Arabidopsis. *FT-LIKE PROTEIN 1* showed 60.2% nucleotide and 84.9% amino acid sequences similarity with Arabidopsis, while *FT-LIKE PROTEIN 2* showed 61.1% nucleotide and 65.7% amino acid sequences similarity with Arabidopsis. It was clearly observed that both *FT-LIKE PROTEIN 1* and *FT-LIKE PROTEIN 2* showed almost up to 85% and 70% amino acid identities, respectively, with the Arabidopsis sequence which could be considered as very high. Therefore, both genes might be homologues of Arabidopsis *FT*.

4.3.3 Differential expressions of onion genes of interest

RT-PCR was also carried out to see the expression of twenty-two genes of interest in the leaf and bulb tissues under SD and LD conditions as mentioned in section 4.3.1 of which several genes showed evidence of differential expression (Table 4.1 and Appendix VI). This experiment gives an indication of gene expression patterns, which would need to be confirmed by qRT-PCR.

FKF1 was expressed in bulb material but not in the leaf (Figure A1). This was unexpected, as Taylor et al. (2010) found the *FKF1* was expressed in onion leaf samples at ZT10, which was the time of sampling the leaf in this experiment. This pattern therefore needs to be confirmed by qRT-PCR. *FST-1* mRNA bands were found in all four samples, although the expression intensity was higher in bulb than in leaf at the right product size of 813 bp (Figure A2). No *LFY* mRNA bands were found in leaf samples under either SD or LD conditions but were found in bulb under both conditions at the right product size as expected (642 bp) (Figure A3). *GGTF* mRNA bands at 936 bp were found in all four samples, though the expression intensity was highest in SD leaf compared to other samples (Figure A4). *COL* mRNA bands were found in all four samples; however, the expression intensity was higher in leaf than to bulb at the right product size as expected (522 bp) (Figure A6). *GA3ox1* was only found for SD leaf at the right product size as expected (644 bp) but not found for SD bulb, LD leaf and LD bulb (Figure A6). *FT-LIKE PROTEIN 1* and *2* mRNA bands were found in all 4 samples under both SD and LD conditions.

Table 4.1 Differential expressions of 22 genes of interest in the individual tissues of onion cv. *Renate F1*. Genes highlighted in red are those identified as being differentially expressed. Asterisks indicate relative levels of expression as measured by relative intensity of PCR products on gels.

GENE	GeneBank Accession	mRNA Expression				Genomic DNA control
		SD Leaf	SD Bulb	LD Leaf	LD Bulb	
1. ALL: ALLINASE	L48614.1	**	**	**	**	**
2. FKF1: FLAVIN-BINDING KELCH REPEAT PROTEIN	GQ232754	-	*	-	*	*
3. GIa: GIGANTEA	GQ232756	***	***	***	***	*
4. GIb: GIGANTEA	GQ232757	***	***	***	***	*
5. EF1α: ELONGATION FACTOR 1 ALPHA	CF437531	*	*	*	*	**
6. FST-1: SUCROSE 1-FRUCTOSYLTRANSFERASE-LIKE	GQ214178	**	***	*	***	**
7. LFY: LEAFY	JX275963	-	*	-	*	*
8. GGTP12: GAMMA-GLUTAMYL TRANSPEPTIDASE	AY517548	**	**	**	**	**
9. GGTP: 6-1 GAMMA-GLUTAMYL TRANSPEPTIDASE	AY517547	**	**	**	**	*
10. GGTP: 2-1 GAMMA-GLUTAMYLTRANSPEPTIDASE	AY517549	*	*	*	*	-
11. GGTF: GAMMA-GLUTAMYLTRANSFERASE	AF401622	*	**	**	**	-
12. GSTF: GLUTATHIONE S-TRANSFERASE	AF401623	***	***	***	***	*
13. ATPS: ATP-SULPHURYLASE	AF403295	-	-	-	-	-
14. GGCS: GAMMA-GLUTAMYLCYSTEINE SYNTHETASE	AF401621	-	-	-	-	-
15. SATF: SERINE ACETYLTRANSFERASE	AF212156	**	**	*	**	-
16. F-Box: ACAAV44 F-box	GQ232752	*	*	*	*	*
17. COL: ACABR20 CONSTANS-LIKE PROTEIN	GQ232751	***	**	***	**	*
18. GA3ox1: GIBBERELLIN 3-OXIDASE 1	AB303422	*	-	-	-	*
19. SPS: SUCROSE-PHOSPHATE SYNTHASE	EU164758	***	***	***	***	**
20. ACGSTF1: GLUTATHIONE S-TRANSFERASE 1	AB300334	*	**	*	**	**
21. FT-LIKE PROTEIN 1: FLOWERING LOCUS T-LIKE PROTEIN 1	JX145040	*	*	**	*	**
22. FT-LIKE PROTEIN 2: FLOWERING LOCUS T-LIKE PROTEIN 2	JX145039	**	*	**	*	*

The *Renate F1* partial cDNA sequences of differentially expressed genes obtained from gene isolation were blasted against an onion EST database (www.ncbi.nlm.nih.gov/nucest/?term=onion) to determine their similarity (Table 4.2).

Table 4.2 Similarity between onion EST and onion partial sequence obtained in *Renate F1*

Gene	Onion partial cDNA sequence obtained from gene isolation (bp)	Onion partial EST sequence (bp)	Similarity between onion EST and onion partial sequence obtained	
			% Nucleotide	% Amino acid
FKF1	814	2054	99	98
FST-1	813	1830	97	99
LFY	642	1081	99	98
GGTF	936	1905	99	97
COL	522	1109	99	100
GA3ox1	645	1208	97	98
FT-LIKE 1	515	807	99	99
FT-LIKE 2	579	834	100	100

4.3.4 Isolation of FT genes

During the project, Lee et al. (2013) published a paper in which they identified 6 FT-like genes (*FT1-6*). Using the sequence information from that paper and the primers in Appendix IV, we were able to detect five out of 6 *FT* genes in *Renate F1* and *Hojem* (Figure 4.13). The identity of the genes was confirmed through sequencing of PCR products. *FT2* was not detected in *Renate F1* which might be because the material used was vegetative and its expression was linked to flowering in the previous study (Lee et al., 2013). However, *FT2* was detected in *Hojem*. Even though *Hojem* is a SD variety and vegetative material was also used in this experiment, some evidence showed that SD varietes are likely to flower under SD in the glasshouse conditions. *FT5 is* identical to *FT-LIKE PROTEIN 2* and *FT6* is identical to *FT-LIKE PROTEIN 1*. For the remainder of the thesis, we use the numbering proposed by Lee et al. (2013) and call the genes *AcFT1-6*. Overall, *AcFT* genes showed about 53% identity to amino nucleotide level with the exception of *FT3,* which shared 83% identity with *FT5*.

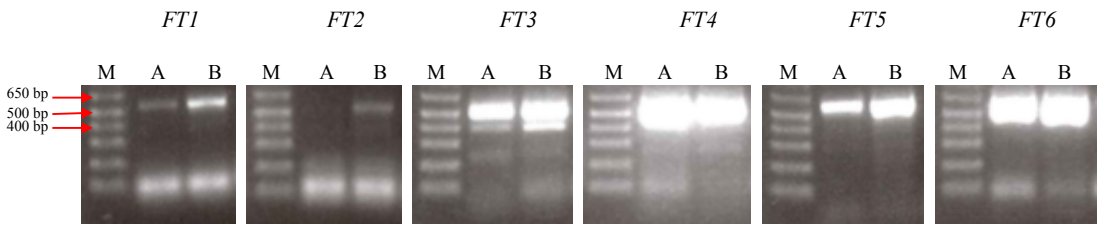


Figure 4.13 Gel red stained agarose gel showing amplification of *FT* genes in onion. M= marker, A= *Renate F1* cDNA, B= *Hojem* cDNA.

4.3.5 Transcriptome analysis in Renate F1

RNA-Seq analysis was performed to generate an onion transcriptome reference sequence and for more widespread identification of genes differentially expressed in response to photoperiod. Leaf and bulb samples were multiplexed to obtain differentiation between long and short day samples and for biological replication (Table 4.3). Two multiplex combinations were run: Multiplex 5 = Leaf (SD groups 3 & 4 and LD groups 5 & 6) and Multiplex 6 = Bulb (SD groups 3 & 4 and LD groups 5 & 6).

Table 4.3 Samples multiplexed for Illumina sequencing

Group	Plant no.	Bulb diameter (mm)	Neck diameter (mm)	Bulbing ratio
Group 3 (SD)	9	10	5	2.0
	10	10	4	2.5
	11	7	7	1.0
Group 4 (SD)	12	14	7	2.0
	13	10	6	1.7
	14	14	7	2.0
Group 5 (LD)	15	17	6	2.8
	16	13	8	1.6
	17	15	5	3.0
Group 6 (LD)	18	15	8	1.9
	19	17	6	2.8
	20	20	8	2.5

All sequences obtained from RNA seq analysis were used for onion gene assembly with the assistance of the Life Sciences Bioinformatics support officer Mr. Siva Samavedam using Galaxy Biotinformatics Platform (http://galaxyproject.org/). 12604 differential expressed transcripts were detected in LD leaf vs bulb, 13665 in SD leaf vs bulb, 484 in SD leaf vs LD leaf and 964 in SD bulb vs LD bulb of onion. Some of the genes were upregulated and some were downregulated, and differentially expressed in leaf vs bulb and LD vs SD or vice versa (Figure 4.14).

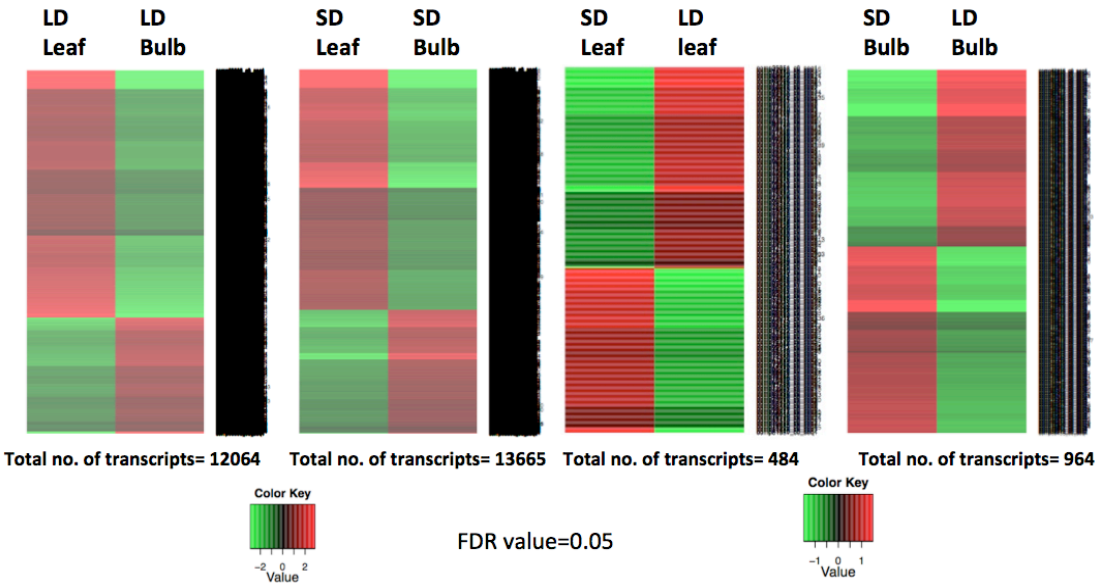


Figure 4.14 Heat Map showing differential expressed transcripts in *Renate F1* grown under different daylengths.

Comparison of transcriptome sequences with sequence databases allowed us to identify sequences for genes that have known function in the daylength regulation of flowering, e.g. *FT* and *CO*. Five out of six *FT* genes were detected in *Renate F1* transcriptome sequence and used to design primers for qRT-PCR. *FT2* was not detected, and *FT3,* which shared 83% identity with *FT5* could not be distinguished in the qRT-PCR analysis. Three *COL* genes including two novel sequences (*AcCOL2* & *AcCOL3*) were identified through RNA-Seq analysis. *AcCOL2* showed 52.5% nucleotide and 23.1% amino acid sequences similarity with Arabidopsis *CO* (Accession number X94937.1). *AcCOL2* showed 46.5% nucleotide and 30.9% amino acid sequences similarity with Arabidopsis *CO* which could still be considered as high. Therefore, both genes might be homologues of Arabidopsis *CO*.

In addition, the global genes that were significantly different between leaf and bulb in *Renate F1* are listed in the tables below (Table 4.4). These genes were selected based on their function to provide more bulb-specific genes, such as those for quality traits such as carbohydrates and organosulphur content, although bioinformatics analysis is yet not complete. The difference in expression level between leaf and bulb samples can provide a clearer understanding of the functions of these genes in respect of quality traits.

Table 4.4 Differential gene expressions between leaf and bulb in *Renate F1* onion. All differences are highly significant (P value < 0.01).

Gene name	P value	FDR	Expression level		Ratio
			Leaf	Bulb	
PREDICTED: gibberellin-regulated protein 14-like [*Cicer arietinum*]	7,98E-38	1,07E-35	0,892	43,68	0,02
PREDICTED: glutathione transferase GST 23-like [*Setaria italica*]	1.13E-37	1.50E-35	0.282	55.707	0.01
PREDICTED: LOW QUALITY PROTEIN: sucrose:sucrose 1-fructosyltransferase-like [*Setaria italica*]	2.26E-24	1.67E-22	17.308	143.972	0.12
PREDICTED: LOW QUALITY PROTEIN: sucrose:sucrose 1-fructosyltransferase-like [*Setaria italica*]	3.04E-24	2.22E-22	16.909	140.543	0.12
histone H4 [*Arabidopsis thaliana*]	1.70E-12	5.67E-11	2.918	24,535	0.12
PREDICTED: AP2-like ethylene-responsive transcription factor BBM2-like [*Cucumis sativus*]	0.00174731	0.013442924	0	1.092	0.00
PREDICTED: UDP-glycosyltransferase 73B5-like [*Brachypodium distachyon*]	0.004003958	0.026646789	0.057	1.052	0.05
PREDICTED: gibberellin 20 oxidase 1-like [*Vitis vinifera*]	0.005180718	0.03279684	0.022	0.461	0.05
PREDICTED: dihydroflavonol-4-reductase-like [*Vitis vinifera*]	0.00578575	0.035848727	0	0.448	0.00
PREDICTED: MADS-box transcription factor 2-like [*Setaria italica*]	0.006261258	0.038150999	0.097	1.424	0.07
fructokinase, putative [*Ricinus communis*]	0.006490755	0.039253685	0	0.941	0.00
PREDICTED: UDP-glycosyltransferase 85A1 [*Vitis vinifera*]	0.007170561	0.042455517	1.657	7.947	0.21
PREDICTED: cytokinin dehydrogenase 3-like [*Setaria italica*]	0.008374	0.048017163	0	0.35	0.00
glycoside hydrolase family 28 protein [*Arabidopsis lyrata* subsp. *lyrata*]	0.008440329	0.0483314	0.366	2.732	0.13
trehalose-6-phosphate synthase, putative [*Ricinus communis*]	0.008518127	0.048690615	0	0.416	0.00
R2R3-MYB transcription factor [*Medicago truncatula*]	0.008711944	0.04953714	0	0.203	0.00
PREDICTED: cell division cycle protein 27 homolog B-like, partial [*Cucumis sativus*]	0.008725432	0.049583262	0.051	0.645	0.08
PREDICTED: MADS-box transcription factor PHERES 2-like [*Setaria italica*]	0.008792126	0.049901936	0.131	1.139	0.12

Table 4.4 Continued

Gene name	P value	FDR	Expression level		Ratio
			Leaf	Bulb	
PREDICTED: LOW QUALITY PROTEIN: beta-fructofuranosidase, insoluble isoenzyme CWINV1-like, partial [*Cucumis sativus*]	1.13E-06	1.89E-05	1.405	7.951	0.18
PREDICTED: beta-fructofuranosidase 1-like [*Brachypodium distachyon*]	1.54E-06	2.54E-05	0.194	9.194	0.02
PREDICTED: beta-fructofuranosidase, insoluble isoenzyme 7-like isoform X2 [*Setaria italica*]	4.66E-06	7.02E-05	2.355	9.66	0.24
PREDICTED: LOW QUALITY PROTEIN: sucrose:sucrose 1-fructosyltransferase-like [*Setaria italica*]	0.000162359	0.001738632	0.408	5.375	0.08
PREDICTED: agamous-like MADS-box protein AGL62-like [*Glycine max*]	0.007709598	0.044919757	0.064	1.082	0.06
PREDICTED: LOW QUALITY PROTEIN: sucrose:sucrose 1-fructosyltransferase-like [S*etaria italica*]	1.78E-49	3.74E-47	19.943	0.121	164.82
PREDICTED: probable xyloglucan endotransglucosylase/hydrolase protein 30-like [*Cucumis sativus*]	1.97E-40	2.94E-38	38.269	0.792	48.32
PREDICTED: LOW QUALITY PROTEIN: sucrose:sucrose 1-fructosyltransferase-like [*Setaria italica*]	1.62E-14	6.36E-13	231.447	19.137	12.09
glutathione S-transferase TAU 18 [*Arabidopsis thaliana*]	4.12E-05	0.000512897	17.409	3.08	5.65
PREDICTED: trehalose-phosphate phosphatase [*Vitis vinifera*]	0.005299667	0.033429798	0.278	0	0
PREDICTED: LOW QUALITY PROTEIN: beta-fructofuranosidase, insoluble isoenzyme CWINV1-like, partial [*Cucumis sativus*]	1.13E-06	1.89E-05	1.405	7.951	0.18
PREDICTED: beta-fructofuranosidase 1-like [*Brachypodium distachyon*]	1.54E-06	2.54E-05	0.194	9.194	0.02

4.4 Conclusions

Twenty-two key genes including two putative *FT* genes were identified in *Renate F1* onion and some of them were shown to be differentially expressed in leaf and bulb tissues under LD and SD conditions. *AcFKF1* was isolated from bulb materials, which needs to be confirmed by qRT-PCR analysis. *AcFKF1* showed high percentage of nucleotide similarity with Arabidopsis homologues. *AcGI* was almost equally expressed in both leaf and bulb tissue under LD and SD conditions. No *LFY* mRNA bands were found in leaf samples under either SD or LD conditions but were found in bulb under both conditions. This result is similar to the expression of Arabidopsis *LFY* gene at the shoot apical meristem which together with *AP1* triggers the expression of the floral homeotic genes at the floral apical meristem and cause flowering (Yoo et al., 2005). In onion, the meristem is in the basal plate, which would be in the bulb samples. Sequencing of PCR products showed that *AcCOL* was expressed in *Renate F1*, which contains B-Box and CCT domain regions in the conserved domains that are present in all *CO* and *CO*-like genes (Robson et al., 2001; Taylor et al., 2010). *GA3ox1* was only found in leaf tissue under SD, which needs to be confirmed by qRT-PCR. However, *GA3ox1* showed greater amino acid identities with Arabidopsis *GA3ox1* homologues. *FT-LIKE PROTEIN 1* and *2* genes were found in both leaf and bulb tissues under both LD and SD conditions. Using the sequence information from Lee et al. (2013), five out of six *FT* genes were identified and isolated in *Renate F1* and *Hojem*. *FT5* is identical to *FT-LIKE PROTEIN 2* and *FT6* is identical to *FT-LIKE PROTEIN 1*.

RNA seq analysis identified a large number of differentially expressed transcripts on a daylength and tissue basis. Five *FT* and three *COL* genes were identified in *Renate F1* transcriptome sequences including two novel *COL* sequences (*AcCOL2* and *AcCOL3*). In addition, comparison of transcript abundance between the LD and the SD treatment would enable the identification of genes involved in the bulbing process itself. In addition, the global genes that were significantly different between leaf and bulb in *Renate F1* could provide a clearer understanding the functions of the genes for quality traits such as carbohydrates and organosulphur content.

CHAPTER 5: EFFECTS OF DAYLENGTH ON GENE EXPRESSION IN ONION DURING DEVELOPMENT

5.1 Introduction

Bulb initiation and development in response to daylength was characterised in onion as described in chapter 3. Onion is a biennial plant, where bulb formation, being an overwintering stage (Brewster, 2008) occurs in the first year of the life cycle and flowering occurs following a period of vernalisation (Brewster, 1997) in the second year of the life cycle. *FT*, first identified in *A. thaliana* (Kardailsky et al., 1999; Kobayashi et al., 1999) has been shown to be the major component of the floral signal molecule, florigen (Andres and Coupland, 2012). This *FT* plays a major role in the photoperiodic pathway for the initiation of flowering in the apical meristem with the help of other floral homeotic genes like *LFY* (Thomas et al., 2006). Furthermore, *FT* is a target of *CO* (Suarez-Lopez et al., 2001) and acts upstream of *SOC1* and this *FT* can induce flowering by long-distance transportation, thus acting as a mobile flowering signal (Corbesier et al., 2007; Purwestri et al., 2009). As mentioned earlier, for bulbing, as with flowering, daylength perception occurs in the leaves, while the response is in the meristem (Brewster, 2008). This suggests that a mobile signal with properties similar to *FT* might be involved (Taylor et al., 2010).

In addition to the regulation of flowering, *FT* genes have been found to be involved in a range of physiological processes, suggesting its more general function as a plant hormone (Lee et al., 2013). For example, an *FT* promotes vegetative growth and inhibition of bud set in poplar in response to warm temperatures and LD photoperiods (Bohlenius et al., 2006; Hsu et al., 2011; Hsu et al., 2006), while, in tomato and maize, *FT* genes have been found to function as general growth regulators (Danilevskaya et al., 2011; Lifschitz et al., 2006). Other than vegetative growth and flowering, *FT* is also involved in the SD induction of tuberisation in potato (Navarro et al., 2011).

Onions form bulbs in response to LD photoperiods, which was first reported by Garner and Allard (1920). However, in tropical regions, short day onions develop bulbs in response to SD photoperiods. During the period of this project, six *FT* genes

(*AcFT1-6*) were identified in a double haploid onion line CUDH2150 and the authors proposed that *AcFT1* and *AcFT4* genes promote and inhibit bulb formation, respectively, and the *AcFT2* promotes flowering in onion (Lee et al., 2013). In addition, *FKF1* and *GI*, which are involved in the photoperiodic regulation of flowering in Arabidopsis are also conserved in onion (Taylor et al., 2010).

This chapter details the preliminary RT-PCR expression of target genes in *Renate F1* and *Hojem* under study using qRT-PCR primers. This chapter also describes quantitative gene expression analysis in different response types of onion under a range of bulbing and non-bulbing conditions. The objective of these experiments was to characterise the developmental and spatial expression of the genes in order to further understand their potential roles in the daylength regulation of bulbing.

5.2 Materials and Methods

5.2.1 Developmental time-course experiment to study gene expression in Renate F1 under different daylengths

This experiment was conducted to see the effects of daylength on gene expression during bulb initiation and development in *Renate F1* by qRT-PCR. The methods for plant growing, experimental conditions, harvesting and sampling of materials for molecular analysis were carried out as described in section 3.2.1 for Experiment 2.

5.2.2 Developmental time-course experiment to study gene expression in Hojem and Renate F1 at 12 h

This experiment was conducted to study the gene expression during bulb initiation and development in relation to daylength adaptation in *Hojem* and also to compare it with cultivar *Renate F1* under ID condition by qRT-PCR. The methods for plant grow, experimental conditions, harvesting and sampling of materials for molecular analysis were carried out as described in section 3.2.3.

5.2.3 Spatial patterns of gene expression in leaves of Renate F1

An experiment was performed to determine the spatial expression of the genes of interest in *Renate F1*. Onion plants were grown under NC in the glasshouse at Phytobiology Facility during the period from 26th July to 27th September 2013 when daylight was 15 h 42 min to 11 h 52 min, respectively. The same growth and environmental conditions were provided as described in section 3.2.1 for Experiment 1. On the last day, plants were harvested at ZT10 and leaves were separated. The 5th number leaf was taken and cut into 12 pieces of 1 cm starting from the base and 6 plants were pooled together for both LD and SD conditions (Figure 5.1). The samples were immediately frozen in liquid nitrogen before storing at -80°C. The harvested materials were used for molecular analyses.

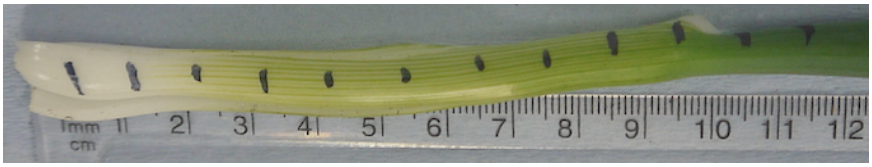


Figure 5.1 Leaf sections for spatial expression experiment in *Renate F1*

5.2.4 RT-PCR, qRT-PCR analyses and Statistical analysis

The identification and isolation of the genes of interest in onion was followed by the techniques as described in section 4.2.1. The methodology for designing qRT-PCR primers (forward and reverse) was also carried out as described in section 4.2.1. Extraction of total RNA, DNase treatment and synthesis of cDNA using 2 µg total RNA was followed by the protocol as described in section 2.2.1. The expression of the key genes was initially examined in pooled cDNA samples of *Renate F1* leaf and bulb samples under LD and SD conditions and *Hojem* leaf and bulb samples at 12 h, respectively, by RT-PCR. RT-PCR was carried out as described in section 2.2.4 using the designed qRT-PCR primers. The PCR products were purified followed by the protocol as described in section 2.2.6. The purified PCR products were then sequenced followed by the protocol as described in section 2.2.7 to confirm the identity of the genes. The relative expression of the key genes was further examined by qRT-PCR which was carried out using the CFX384 Touch^{TM} Real-time PCR machine from BioRad (Bio-Rad Laboratories Ltd., UK), as described in section 2.2.5, and PCR conditions were followed as mentioned in Table 2.1. Each primer pair (forward and reverse) used for the respective gene was initially tested to obtain the optimum primer and cDNA concentrations. Three replications (triplicate) were carried out for each sample and the average CT value calculated, which represents the number of PCR cycles when a product is first detected. qRT-PCR data were analysed after completion of each PCR run and data indicated as means and normalized against expression levels of the house keeping genes for each sample (Appendix XV). Normalisation was achieved by using Biogazelle qBase+ software (www.biogazelle.com). qbase+ software was developed by Biogazelle for analysis of qPCR data. It is based on the geNorm (Vandesompele et al., 2002) and qBase technology (Hellemans et al., 2007) from Ghent University. The averages of time-courses and spatial expression were calculated and standard errors included. Standard curves (using 10-fold serial dilutions) were plotted using cDNA synthesised from approximately 2 µg of toal RNA extracted from leaf material at various DFS and leaf segments at 62 DFS, respectively, as used for cDNA synthesis described in section 2.2.1. The significance of the differences in gene expression between treatments were assessed by using two-way analysis of variance (ANOVA). ANOVA was carried out using statistical software package Prism 7.

5.3 Results

5.3.1 Preliminary RT-PCR expression analysis

In preliminary RT-PCR experiment, the expression of the genes under study was determined and isolated in pooled samples of *Renate F1* using qRT-PCR primers (Appendix VIII). PCR products were run on a gel (Figure 5.2-12). The data indicated that the cDNA samples used for the analyses were of good quality and suitable sources for sequence amplification as these showed clear band at the right product size. All were shown to represent the expected gene through sequencing of PCR products except *FT2*. *FT3* shared 83% identity with *FT5*, which could not be distinguished in the qRT-PCR analysis. In the first PCR, *AcFKF1* was not identified through sequencing (Figure 5.2), which might be due to the poor primers. Therefore, new qRT-PCR primers were designed to identify and isolate *AcFKF1* in onion and later on identification and isolation was confirmed through gel electrophoresis and sequencing of PCR products (Figure 5.3). Furthermore, in the initial PCR, *AcFT1* and *AcFT4* mRNA bands of *Renate F1* pooled sample were not clearly visible on the gel (Figure 5.7). However, re-amplified PCR showed clearly visible bands on the gel (Figure 5.8). In addition, clear cDNA bands for *Hojem* and *Renate F1* grown under 12 h daylength were also found on gel (Figure 5.9 and 5.10).

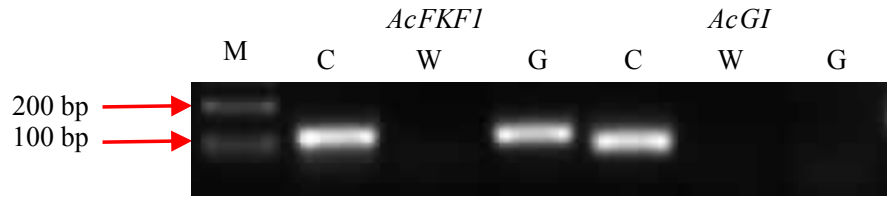


Figure 5.2. Gel red stained agarose gel showing amplification of *AcFKF1* and *AcGI* in *Renate F1*. M=marker, C= cDNA, W=water control, G=genomic DNA.

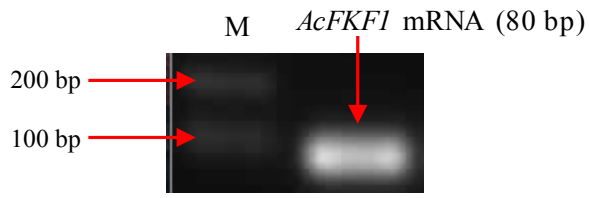


Figure 5.3. Gel red stained agarose gel showing amplification of *AcFKF1* in *Renate F1*. M=marker.

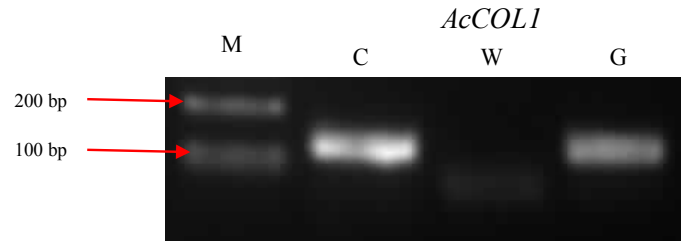


Figure 5.4 Gel red stained agarose gel showing amplification of *AcCOL1* in *Renate F1*. M=marker, C= cDNA, W=water control, G=genomic DNA.

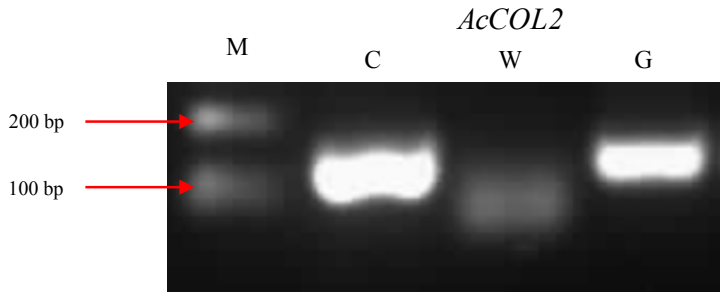


Figure 5.5 Gel red stained agarose gel showing amplification of *AcCOL2* in *Renate F1*. M=marker, C= cDNA, W=water control, G=genomic DNA.

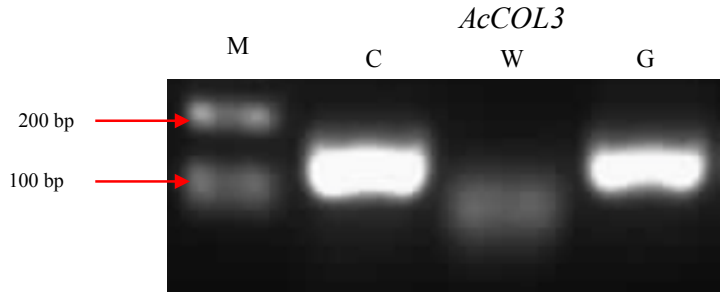


Figure 5.6 Gel red stained agarose gel showing amplification of *AcCOL3* in *Renate F1*. M=marker, C= cDNA, W=water control, G=genomic DNA.

Figure 5.7 Gel red stained agarose gel showing amplification of *FT* mRNA in *Renate F1*. 1) *AcFT1*, 2) *AcFT2*, 3) *AcFT3*, 4) *AcFT4*, 5) *AcFT5*, 6) *AcFT6*. M=marker.

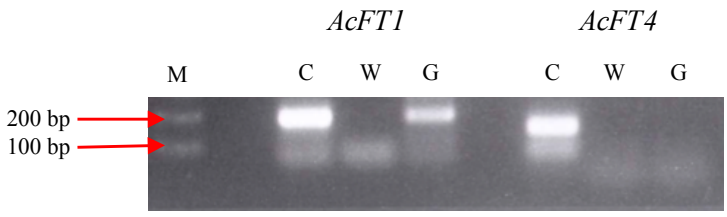


Figure 5.8 Gel red stained agarose gel showing amplification of *AcFT1* and *AcFT4* in *Renate F1*. M=marker, C= cDNA, W=water control, G=genomic DNA.

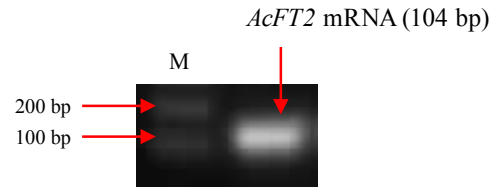


Figure 5.9 Gel red stained agarose gel showing amplification of *AcFT2* in *Hojem* at 12 h. M=marker.

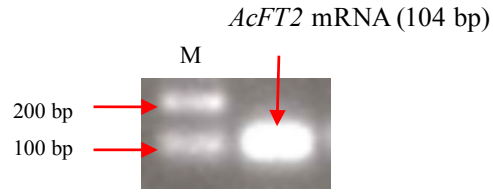


Figure 5.10 Gel red stained agarose gel showing amplification of *AcFT2* in *Renate F1* at 12 h. M=marker.

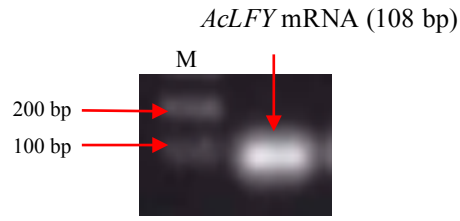


Figure 5.11 Gel red stained agarose gel showing amplification of *AcLFY* in *Renate F1*. M=marker.

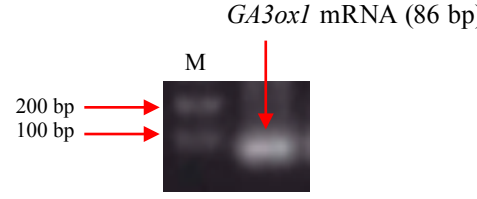


Figure 5.12 Gel red stained agarose gel showing amplification of *GA3ox1* in *Renate F1*. M=marker.

5.3.2 Developmental time-course expression of the genes in Renate F1 under different daylengths

This experiment was conducted to see the effects of daylength e.g. LD (NC), LD (CE) and SD on gene expression during bulb initiation and development in *Renate F1* by qRT-PCR. Particularly, this study focused on those genes that have known function in the photoperiodic regulation of flowering in Arabidopsis and regulate other important pathways such as the sucrose and gibberellin pathways.

5.3.2.1 Expression of *COL* genes

Three *COL* genes including two novel sequences (*COL2* & *COL3*) were identified through RNA-Seq analysis in *Renate F1* as described in section 4.3.5. *AcCOL1* was identified and isolated in the previous study of Taylor et al. (2010). Three *COL* genes were expressed throughout the growth and development period in LD (NC), LD (CE) and SD (Figure 5.13, 5.14 and 5.15). Two-way ANOVA confirmed that the difference in expression of all three *COL* genes between LD (NC), LD (CE) and SD conditions were significant (Table A1, A2 and A3). It was also confirmed that the number of days from sowing had a significant affect on gene expression (Table A1, A2 and A3). Finally, the interaction between days from sowing and daylength was also shown to be significant (Table A1, A2 and A3), showing that the relative expression of the genes over time is affected by daylength.

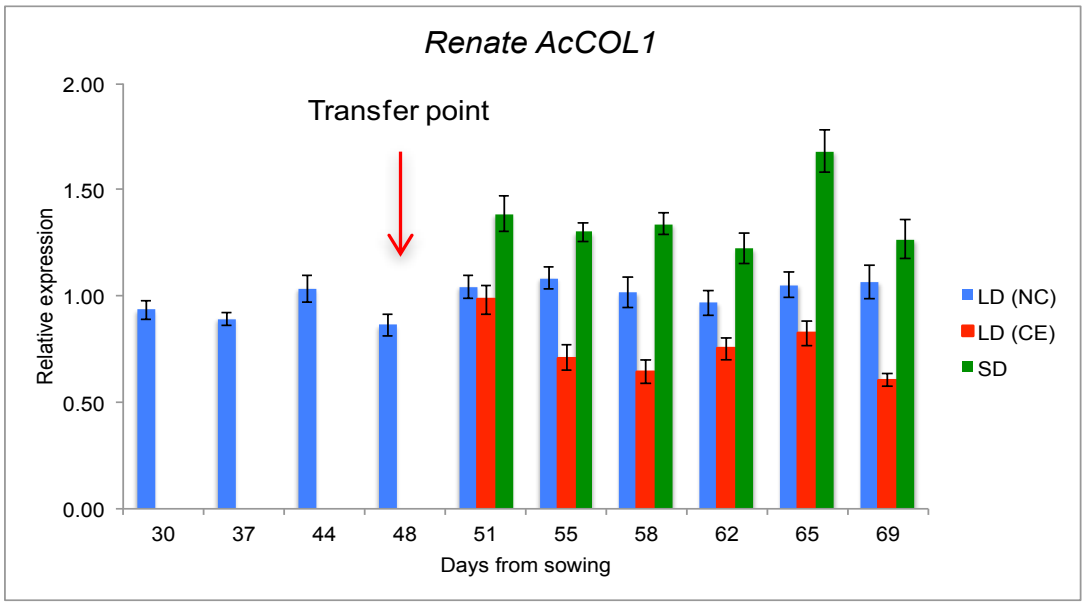


Figure 5.13 Expression of *AcCOL1* in *Renate F1* leaf tissue at different daylengths, relative to *PP2A1, UBL* and *TIP41*. All plants were grown under NC upto the transfer in different conditions. *AcCOL1* was expressed in all three conditions. Error bars represent the SEM.

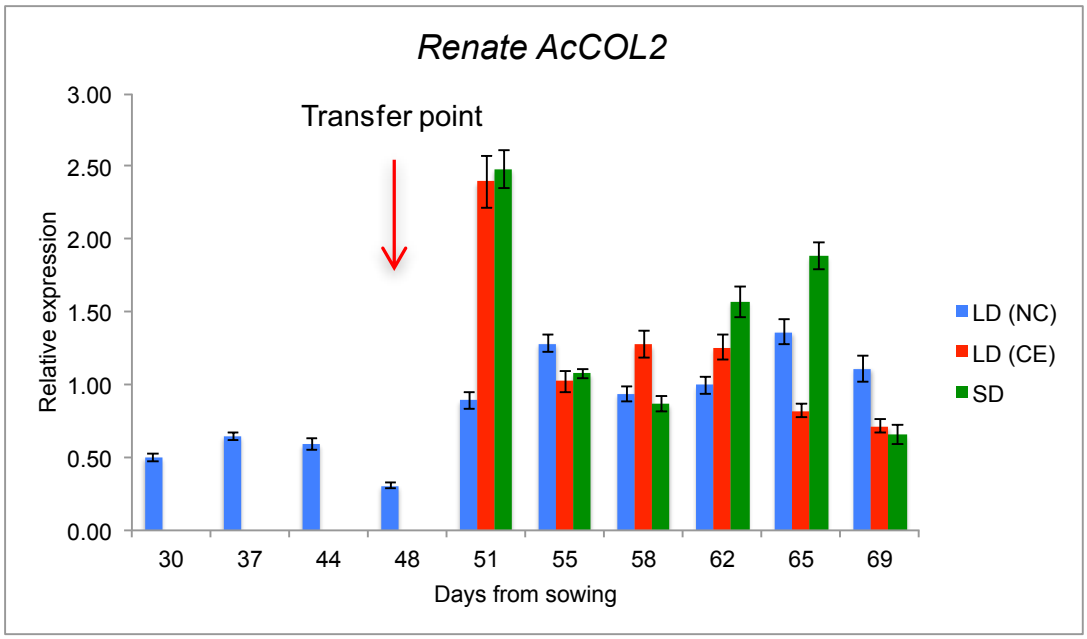


Figure 5.14 Expression of *AcCOL2* in *Renate F1* leaf tissue at different daylengths, relative to *PP2A1, UBL* and *TIP41*. All plants were grown under NC upto the transfer in different conditions. *AcCOL2* was expressed in all three conditions. Error bars represent the SEM.

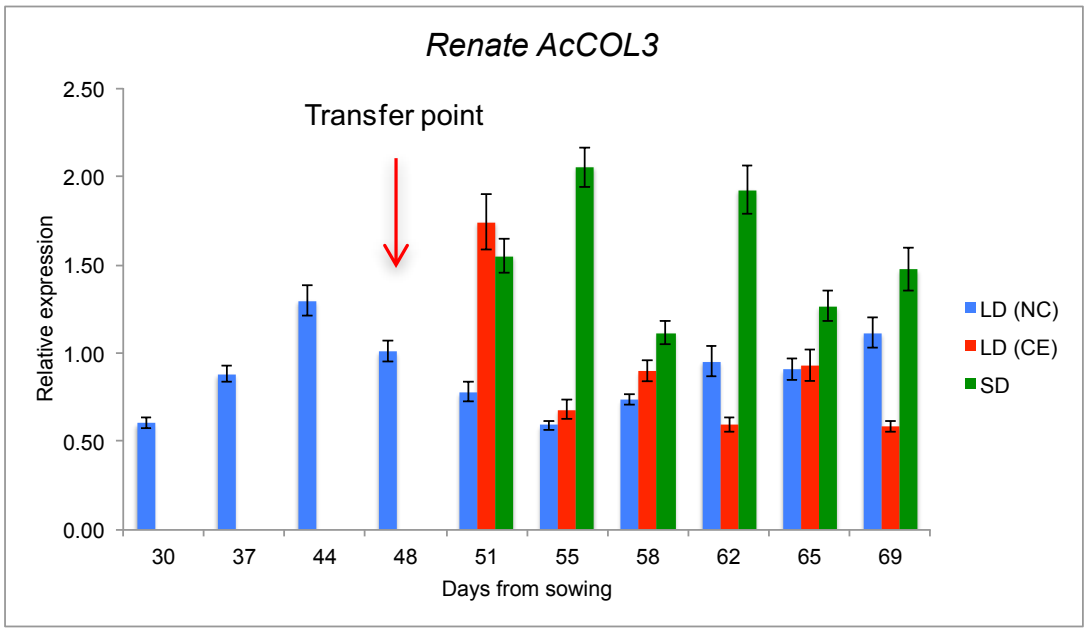


Figure 5.15 Expression of *AcCOL3* in *Renate F1* leaf tissue at different daylengths, relative to *PP2A1, UBL* and *TIP41*. All plants were grown under NC upto the transfer in different conditions. *AcCOL3* was expressed in all three conditions. Error bars represent the SEM.

5.3.2.2 Expression of *FT* genes

Five out of the 6 *FT* genes, with the exception of *AcFT2*, identified by Lee et al. (2013) were detected by PCR in *Renate F1* as described in section 4.3.4. The expression of these genes during onion development in bulbing and non-bulbing samples was followed using qRT-PCR.

5.3.2.2.1 Expression of *AcFT1*

In *Renate F1*, *AcFT1* was expressed in LD (NC) and LD (CE), i.e. the conditions that promoted bulbing, but not expressed in SD, where foliage leaves continued to be produced (Figure 5.16). It was observed that the level of expression of *AcFT1* was significantly higher in LD (NC) than to LD (CE). Two-way ANOVA confirmed that the difference in *AcFT1* expression between LD (NC), LD (CE) and SD conditions was significant (Table A4). However, it was confirmed that the number of days from sowing had no significant affect on *AcFT1* expression (Table A4). Finally, the interaction between days from sowing and daylength was shown to be significant (Table A4), showing that the expression of the *AcFT1* over time is affected by daylength.

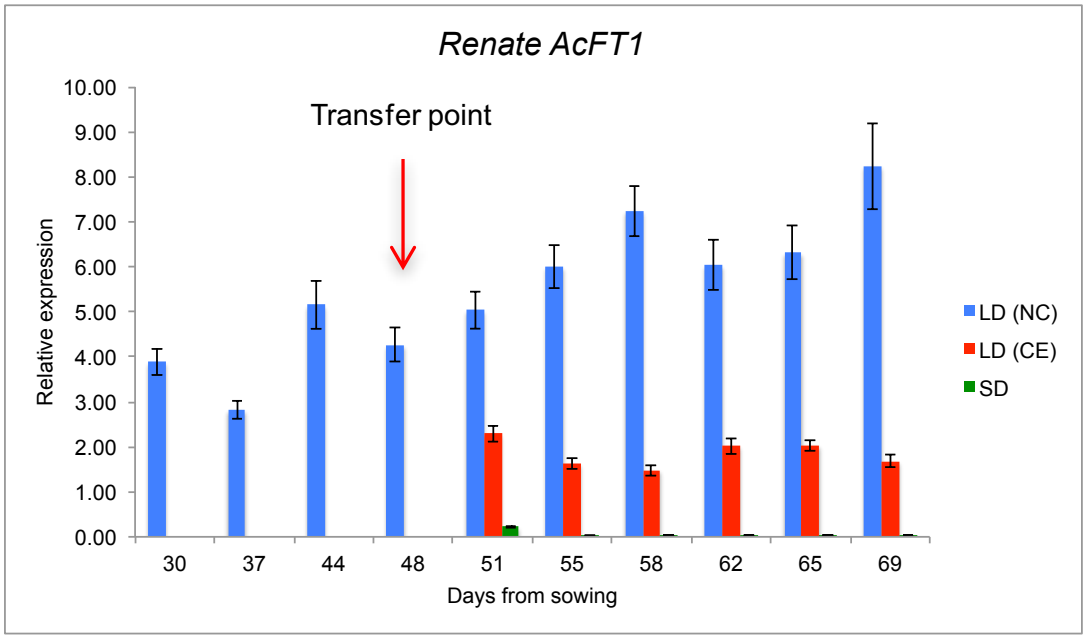


Figure 5.16 Expression of *AcFT1* in *Renate F1* leaf tissue at different daylengths, relative to *PP2A1, UBL* and *TIP41*. All plants were grown under NC upto the transfer in different conditions. *AcFT1* was expressed in LD (NC) and LD (CE) but not expressed in SD. Error bars represent the SEM.

5.3.2.2.2 Expression of *AcFT4*

AcFT4, in contrast, was expressed in SD but was not expressed in LD (NC) or LD (CE) conditions (Figure 5.17). This result is also consistent with the previous study conducted by Lee et al. (2013), where they proposed that *AcFT4* prevents *AcFT1* upregulation and inhibits bulbing in transgenic onions. It was also seen that *AcFT4* expression was not detected during the early stages of seedling growth, even though the natural daylength at about 12 h was significantly less than would promote bulbing.

Two-way ANOVA confirmed that the difference in *AcFT4* expression between LD (NC), LD (CE) and SD conditions was significant (Table A5). It was also confirmed that the number of days from sowing had a significant affect on *AcFT4* expression (Table A5). Finally, the interaction between days from sowing and daylength was also shown to be significant (Table A5), showing that the expression of the *AcFT4* over time is affected by daylength.

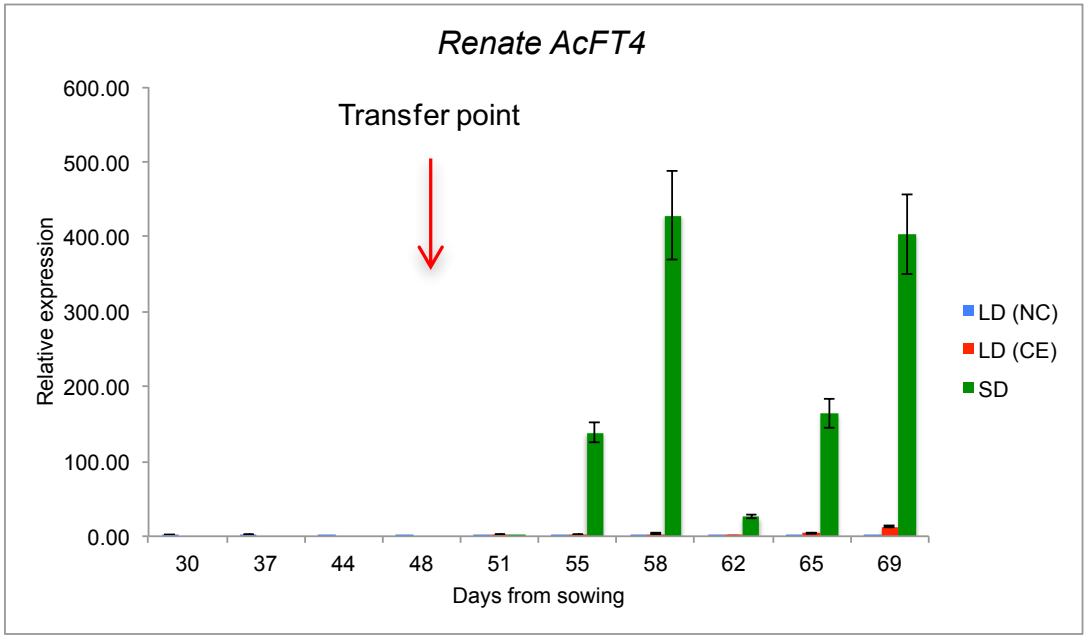


Figure 5.17 Expression of *AcFT4* in *Renate F1* leaf tissue at different daylengths, relative to *PP2A1, UBL* and *TIP41*. All plants were grown under NC upto the transfer in different conditions. *AcFT4* was only expressed in SD but was not expressed in LD (NC) and LD (CE). Error bars represent the SEM.

5.3.2.2.3 Expression of *AcFT5*

AcFT5 was strongly expressed throughout the development in LD (NC), but showed very limited expression in LD (CE) and it was not expressed in SD (Figure 5.18). The level of expression was higher in LD (NC) than to LD (CE). Considering plants under different daylengths, *AcFT5* showed an interesting pattern of expression, which is different to that of *AcFT1*. It was observed that *AcFT5* showed higher expression during early stage of growth and the expression was sharply decreased at the time of bulb formation and then sharply increaed during the rest of the bulb development period. However, this result is inconsistent with the previous study conducted by Lee et al. (2013), where the authors proposed that *AcFT5* expression did not appeared to be strongly affected by daylengths.

Two-way ANOVA confirmed that the difference in *AcFT5* expression between LD (NC), LD (CE) and SD conditions was significant (Table A6). It was also confirmed that the number of days from sowing had a significant affect on *AcFT5* expression (Table A6). Finally, the interaction between days from sowing and daylength was also shown to be significant (Table A6), showing that the expression of the *AcFT5* over time is affected by daylength.

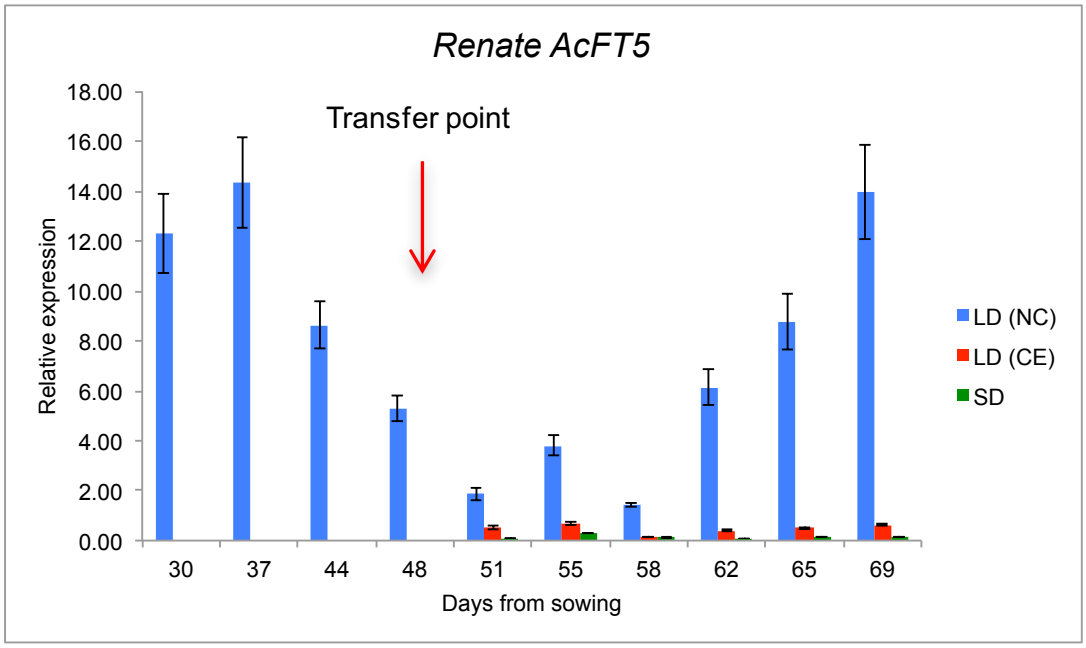


Figure 5.18 Expression of *AcFT5* in *Renate F1* leaf tissue at different daylengths, relative to *PP2A1, UBL* and *TIP41*. All plants were grown under NC upto the transfer in different conditions. *AcFT5* showed higher expression in LD (NC) and showed a limited expression in LD (CE) but showed no expression in SD. Error bars represent the SEM.

5.3.2.2.4 Expression of *AcFT6*

AcFT6 was only expressed in LD (NC) but not in LD (CE) or SD conditions (Figure 5.19). It may be due to the regulation of this gene by light quantity. In addition, the expression level of *AcFT6* in LD (NC) was very low during the early stage of plant growth and was only higher immediately after bulb initiation. This could be due to a positve correlation with *AcFT1* and *AcFT5* or negative correlation with *AcFT4* in *Renate F1* under different daylength conditions. Therefore, it could be confirmed that *AcFT6* might be involved in the bulb formation process under LD (NC), although the function of this gene is still unknown. Furthermore, a limited expression of *AcFT6* was found in both LD (CE) and SD conditions at the later stage of bulb development in *Renate F1*.

Two-way ANOVA confirmed that the difference in *AcFT6* expression between LD (NC), LD (CE) and SD conditions was significant (Table A7). It was also confirmed that the number of days from sowing had a significant affect on *AcFT6* expression (Table A7). Finally, the interaction between days from sowing and daylength was also shown to be significant (Table A7), showing that the expression of the *AcFT6* over time is affected by daylength.

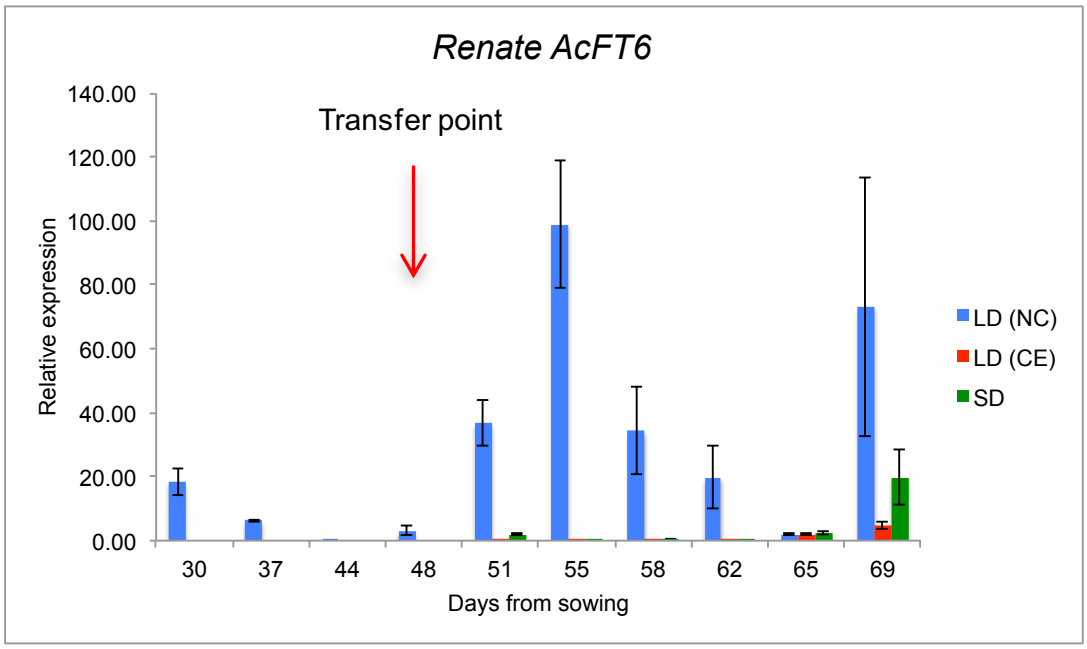


Figure 5.19 Expression of *AcFT6* in *Renate F1* leaf tissue at different daylengths, relative to *PP2A1, UBL* and *TIP41*. All plants were grown under NC upto the transfer in different conditions. *AcFT6* was expressed in LD (NC) and LD (CE) but not in SD. Error bars represent the SEM.

5.3.2.3 Expression of *AcLFY*

In this experiment bulb tissue was used instead of leaf to see the expression of *AcLFY* in *Renate F1*, because *LFY* was only found in bulb tissue as described in section 4.3.3 of chapter 4. It was observed that *AcLFY* was strongly expressed in bulb tissue under LD (NC) at the early stage of plant growth but was not expressed in either LD (CE) or SD conditions (Figure 5.20). Early stage expression suggest that *AcLFY* might be maintaining vegetative growth in the early stage under LD (NC) but it is inhibited when bulbing starts. Immediately after bulb formation, it was observed that the expression of *AcLFY* was undetectable in all three conditions.

Two-way ANOVA confirmed that the difference in *AcLFY* expression between LD (NC), LD (CE) and SD conditions was significant (Table A8). It was also confirmed that the number of days from sowing had a significant affect on *AcLFY* expression (Table A8). Finally, the interaction between days from sowing and daylength was also shown to be significant (Table A8), showing that the expression of the *AcLFY* over time is affected by daylength.

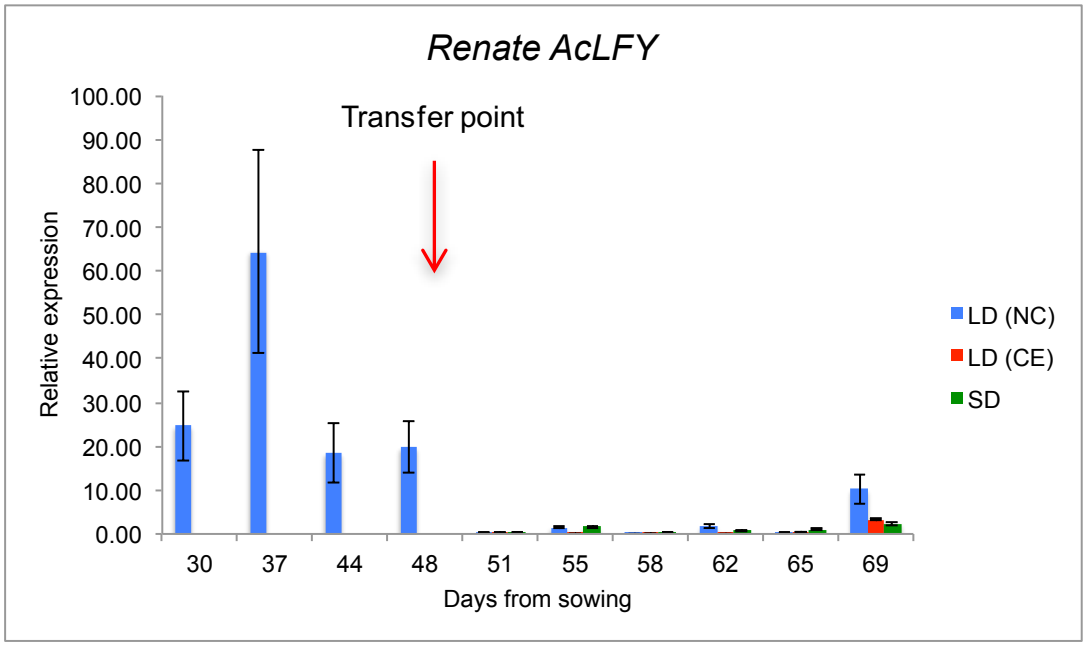


Figure 5.20 Expression of *AcLFY* in *Renate F1* bulb tissue at different daylengths, relative to *PP2A1, UBL* and *TIP41*. All plants were grown under NC upto the transfer in different conditions. *AcLFY* was expressed in LD (NC) but not in LD (CE) and SD. Error bars represent the SEM.

5.3.2.4 Expression of *GA3ox1*

In *Renate F1*, *GA3ox1* was expressed in all three conditions of LD (NC), LD (CE) and SD throughout the bulb initiation and development (Figure 5.21). Data suggest that *GA3ox1* was not directly involved in onion bulb initiation and development.

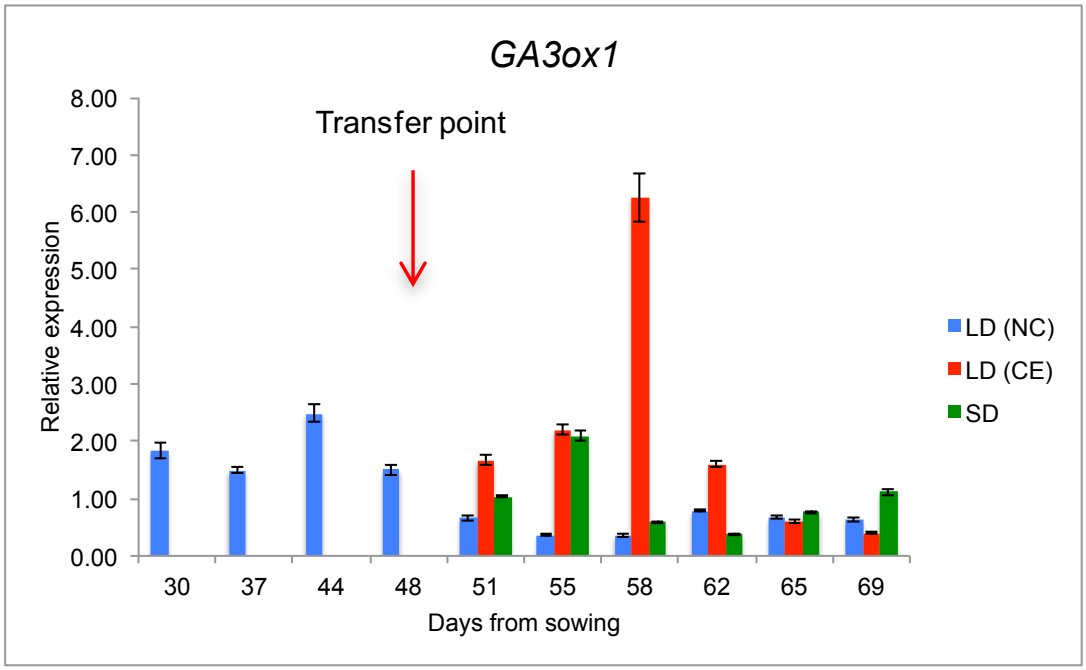


Figure 5.21 Expression of *GA3ox1* in *Renate F1* leaf tissue at different daylengths, relative to *PP2A1, UBL* and *TIP41*. All plants were grown under NC upto the transfer in different conditions. *GA3ox1* was expressed in all three conditions of LD (NC), LD (CE) and SD. Error bars represent the SEM.

5.3.3 Developmental time-course expression of the genes in Hojem and Renate F1 in 12 h daylength

This experiment was conducted to compare the development of the LD variety *Renate F1* with the SD variety *Hojem* when grown in 12 h, an intermediate daylength and to determine the pattern of gene expression in these conditions. This experiment again particularly focused on those genes, which have known function in Arabidopsis flowering and regulate other important pathways such as the sucrose and gibberellin pathways.

5.3.3.1 Expression of *COL* genes

All three *COL* genes were expressed throughout the growth, development and maturity in both *Hojem* and *Renate F1* under a 12 h daylength (Figure 5.22, 5.23 and 5.24). In addition, these genes did not show any difference in the expression level between two varieties of onion. However, *AcCOL2* showed similar level of expression throughout the early growth, bulb initiation and development in both *Hojem* and *Renate F1* in the ID.

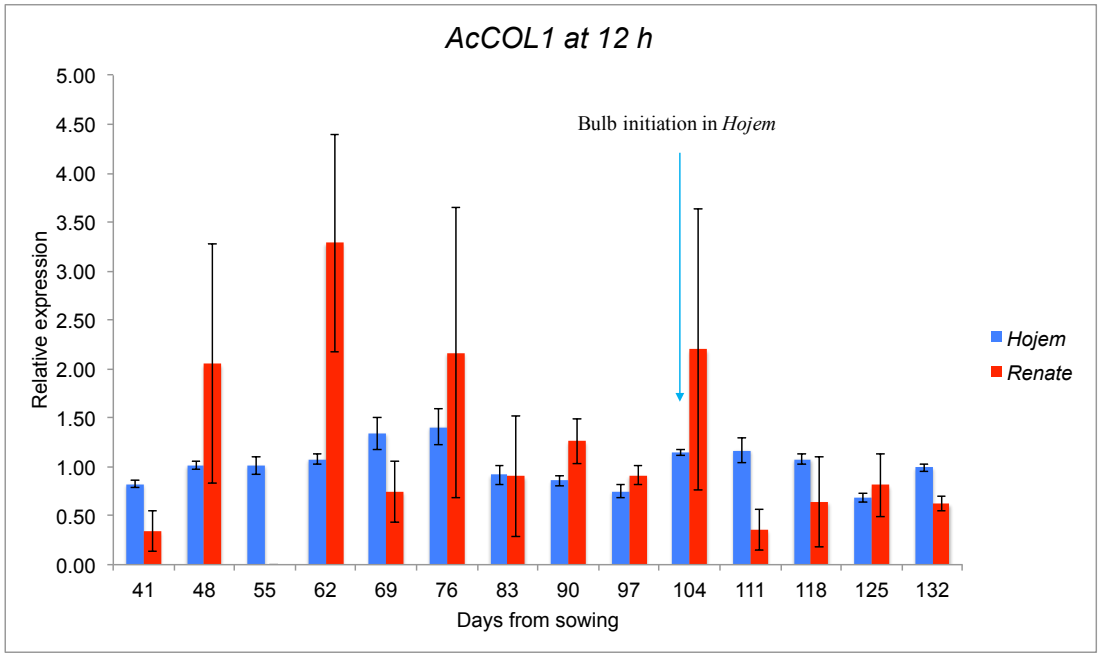


Figure 5.22 Expression of *AcCOL1* in *Hojem* and *Renate F1* at 12 h, relative to *PP2A1, UBL* and *PP2AA3*. *AcCOL1* was expressed in both *Hojem* and *Renate F1* throughout the development. Error bars represent the SEM.

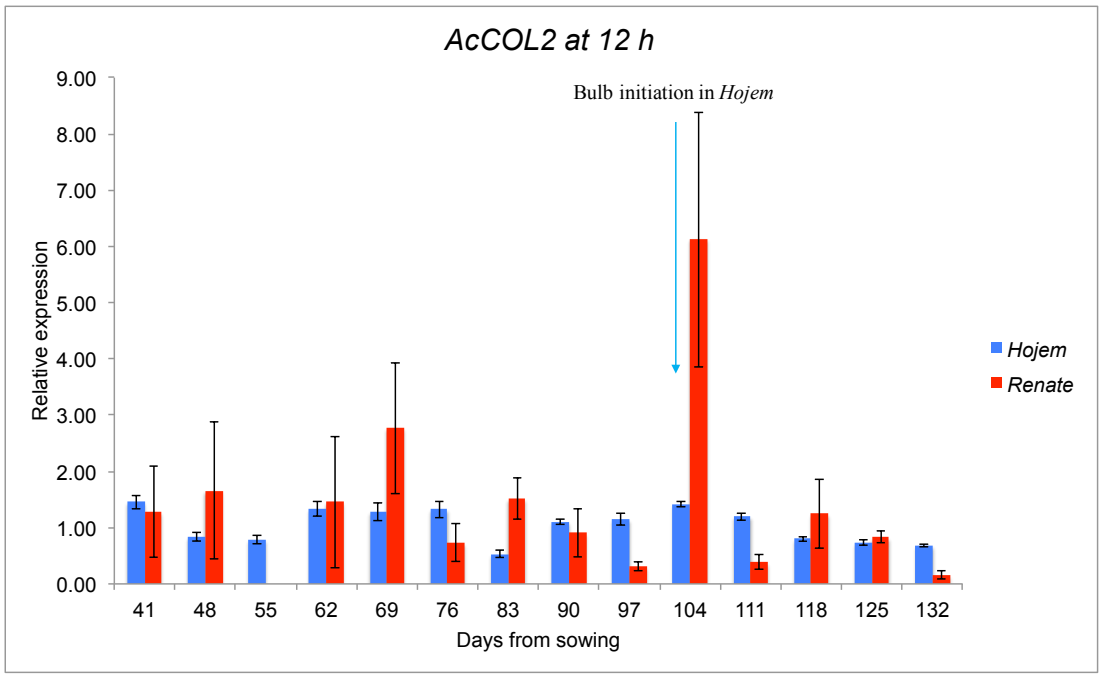


Figure 5.23 Expression of *AcCOL2* in *Hojem* and *Renate F1* at 12 h, relative to *PP2A1, UBL* and *PP2AA3*. *AcCOL2* was expressed in both in *Hojem* and *Renate F1* throughout the development. Error bars represent the SEM.

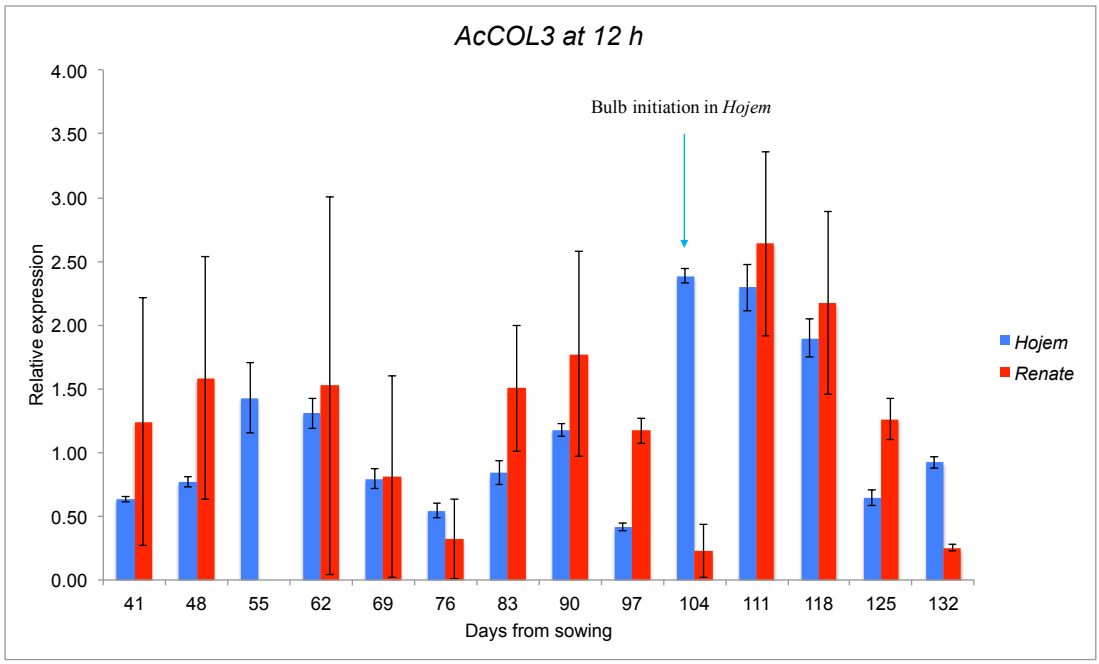


Figure 5.24 Expression of *AcCOL3* in *Hojem* and *Renate F1* at 12 h, relative to *PP2A1, UBL* and *PP2AA3*. *AcCOL3* was expressed in both in *Hojem* and *Renate F1* throughout the development. Error bars represent the SEM.

5.3.3.2 Expression of *FT* genes

5.3.3.2.1 Expression of *AcFT1*

AcFT1 showed higher levels of expression in *Hojem* at 12 h during the later stage of bulb development and maturity (Figure 5.25). In *Hojem*, bulbing takes place at about 104 DFS and come in detectable moment at 118 DFS. The data clearly suggest that *AcFT1* might induce bulb formation and development in *Hojem*. On the other hand, *AcFT1* showed very limited expression in *Renate F1* at 12 h throughout the development period. This result is consistent with the previous results in the experiment as described in section 5.3.2.2.1, where it was found that *AcFT1* was not expressed in *Renate F1* at 8 h daylength. Therefore, it could be confirmed that *AcFT1* is only expressed when the daylength is greater than 12 h in *Renate F1*, which is consistent with it having a role in the daylength dependence of bulbing. Two-way ANOVA confirmed that the difference in *AcFT1* expression between *Hojem* and *Renate F1* under 12 h daylength was significant (Table A9). It was also confirmed that the number of days from sowing had a significant affect on *AcFT1* expression (Table A9). Finally, the interaction between days from sowing and variety was also shown to be significant (Table A9), showing that the expression of the *AcFT1* over time is affected by daylength.

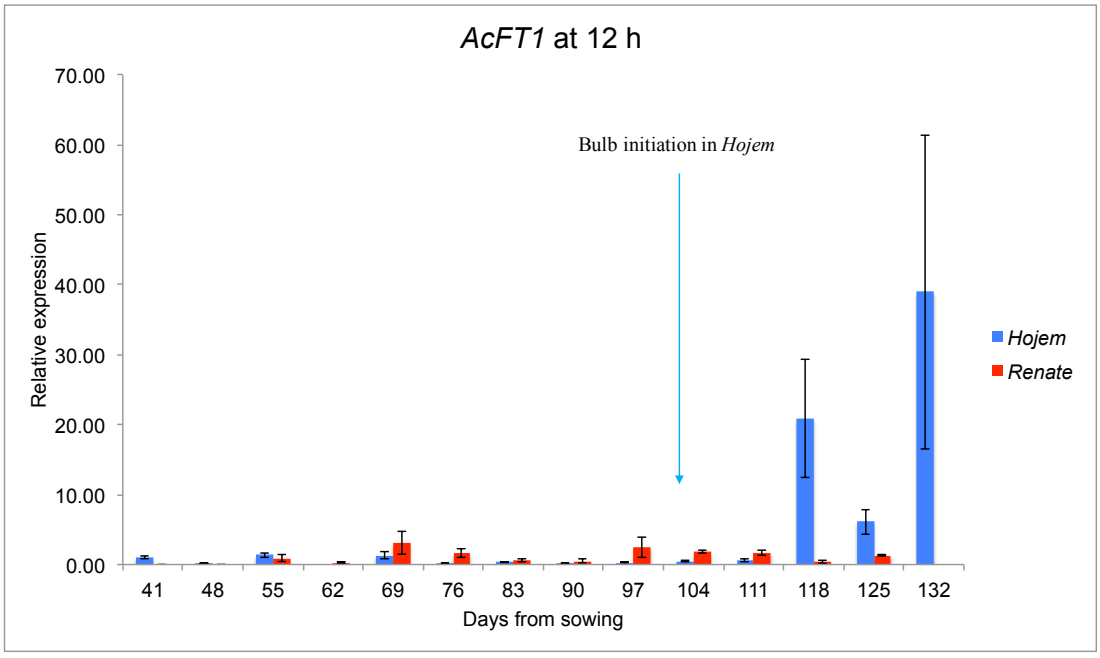


Figure 5.25 Expression of *AcFT1* in *Hojem* and *Renate F1* at 12 h, relative to *PP2A1, UBL* and *PP2AA3*. *AcFT1* was expressed in *Hojem* during bulb formation and development, while, showed very limited expression in *Renate F1*. Error bars represent the SEM.

5.3.3.2.2 Expression of *AcFT2*

AcFT2 was only expressed in *Hojem* at 12 h during the later stage of bulb development and maturity but not expressed in *Renate F1* (Figure 5.26). The expression of *AcFT2* is quite similar to the expression of *AcFT1* in *Hojem* at 12 h as described in section 5.3.3.2.1. Data may suggest that *AcFT2* has a role in bulb formation, development and maturity in the SD onion cultivar *Hojem*. Although, there was no evidence that *AcFT2* has a role in bulb formation or development in *Renate F1* at 12 h, which is consistent with the previous experiment as described in sections 4.3.4 and 4.3.5. Two-way ANOVA confirmed that the variety and days from sowing had no significant effects on *AcFT2* expression (Table A10). Finally, the interaction between days from sowing and variety was also shown to be insignificant (Table A10), showing that the expression of the *AcFT2* over time is not affected by daylength.

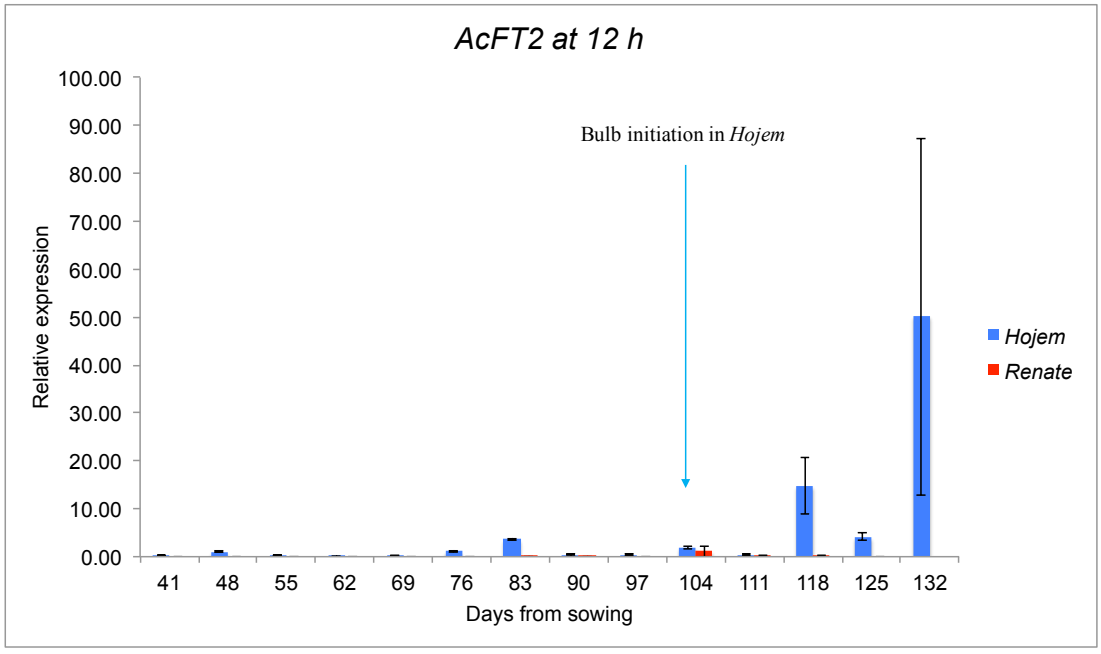


Figure 5.26 Expression of *AcFT2* in *Hojem* and *Renate F1* at 12 h, relative to *PP2A1, UBL* and *PP2AA3*. *AcFT2* was expressed in *Hojem* after bulb formation but not expressed in *Renate F1*. Error bars represent the SEM.

5.3.3.2.3 Expression of *AcFT4*

AcFT4 showed higher expression during the early stage of plant growth in *Hojem* during 12 h daylength (Figure 5.27). This result is also consistent with the previous study conducted by Lee et al. (2013), where they found that *AcFT4* was expressed at relatively high levels in leaves of young seedlings under both SD and LD contions. It was also observed that *AcFT4* was down-regulated just at the time of bulb formation (104 d) in *Hojem*. In *Renate F1* at 12 h, the *AcFT4* was expressed at the later part of the plant growth and development and the expression was up-regulated at the time when plants would generally bullb. This result is also consistent with the previous expeiment, where *AcFT4* was only expressed in SD conditions at *Renate F1* as described in section 5.3.2.2.2.

Two-way ANOVA confirmed that the difference in *AcFT4* expression between *Hojem* and *Renate F1* under 12 h daylength was significant (Table A11). It was also confirmed that the number of days from sowing had a significant affect on *AcFT4* expression (Table A11). Finally, the interaction between days from sowing and variety was also shown to be significant (Table A11), showing that the expression of the *AcFT4* over time is affected by daylength.

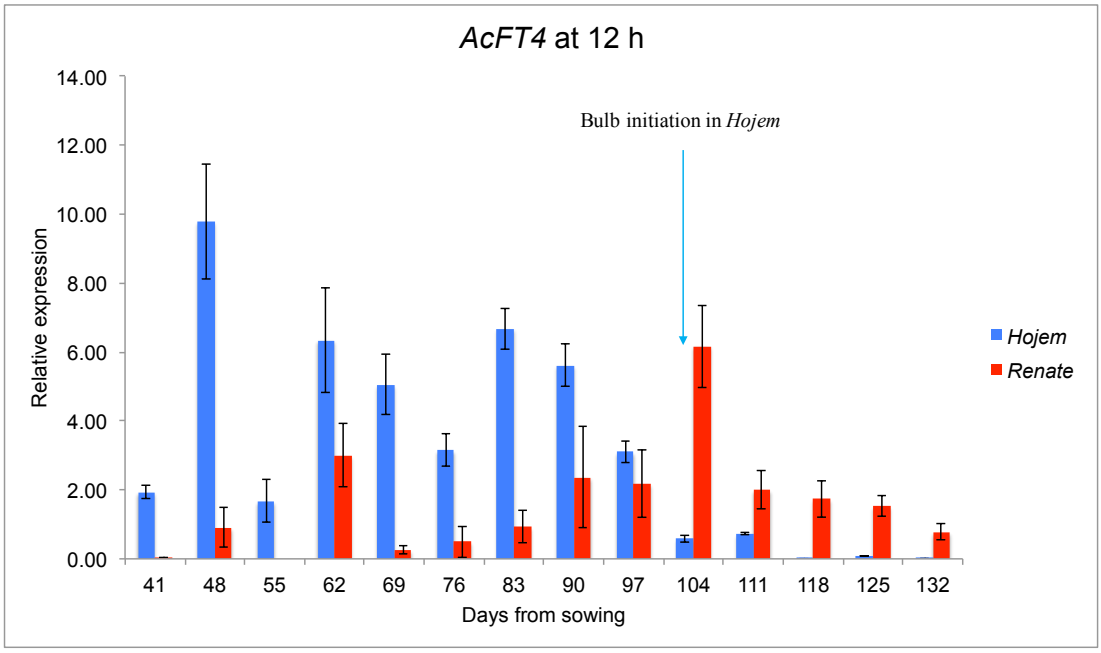


Figure 5.27 Expression of *AcFT4* in *Hojem* and *Renate F1* at 12 h, relative to *PP2A1, UBL* and *PP2AA3*. *AcFT4* was expressed in *Hojem* at the early stage of growth before bulb formation and was expressed in *Renate F1* at the later stage of growth and development. Error bars represent the SEM.

5.3.3.2.4 Expression of *AcFT5*

AcFT5 was expressed throughout the growth and development period in both *Hojem* and *Renate F1* at 12 h (Figure 5.28). However, it was observed that *AcFT5* was down-regulated at the time of bulb formation in *Hojem* and then up-regulated for the rest of the bulb development and maturity. In fact, *AcFT5* expression in *Hojem* at 12 h is consistent with the expression of *AcFT5* in *Renate F1* under different daylengths as described in section 5.3.2.2.3.

Two-way ANOVA confirmed that there was no significant difference found in *AcFT5* expression between the two varieties of onion (Table A12). However, it was confirmed that the number of days from sowing had a significant affect on *AcFT5* expression (Table A12). Finally, the interaction between days from sowing and variety was also shown to be significant (Table A12), showing that the expression of the *AcFT5* over time is affected by daylength.

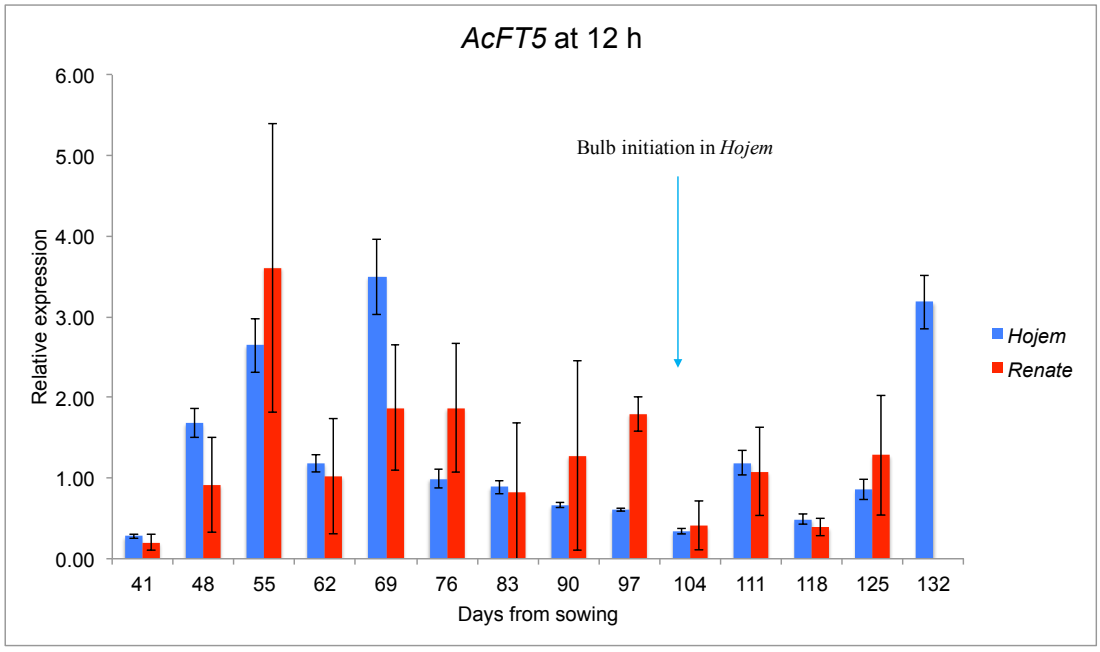


Figure 5.28 Expression of *AcFT5* in *Hojem* and *Renate F1* at 12 h, relative to *PP2A1, UBL* and *PP2AA3*. *AcFT5* was expressed in both *Hojem* and *Renate F1* throughout the development. Error bars represent the SEM.

5.3.3.2.5 Expression of *AcFT6*

AcFT6 was expressed at the early stage of plant growth in *Hojem*, while it was expressed at the middle stage of development in *Renate F1* at 12 h (Figure 5.29). It was observed that *AcFT6* was sharply down-regulated before bulb formation in *Hojem* (97 d) and was not expressed during the rest of the bulb development. This could be due to the positive correlation with other *FT* genes like *AcFT4* as described in section 5.3.3.2.3. While, *AcFT6* showed sporadic type of expression pattern in *Renate F1* which suggest that this gene might be involved during develoment at 12 h.

Two-way ANOVA confirmed that there was no significant difference found in *AcFT6* expression between the two varieties of onion (Table A13). However, it was confirmed that the number of days from sowing had a significant affect on *AcFT6* expression (Table A13). Finally, the interaction between days from sowing and variety was also shown to be significant (Table A13), showing that the expression of the *AcFT6* over time is affected by daylength.

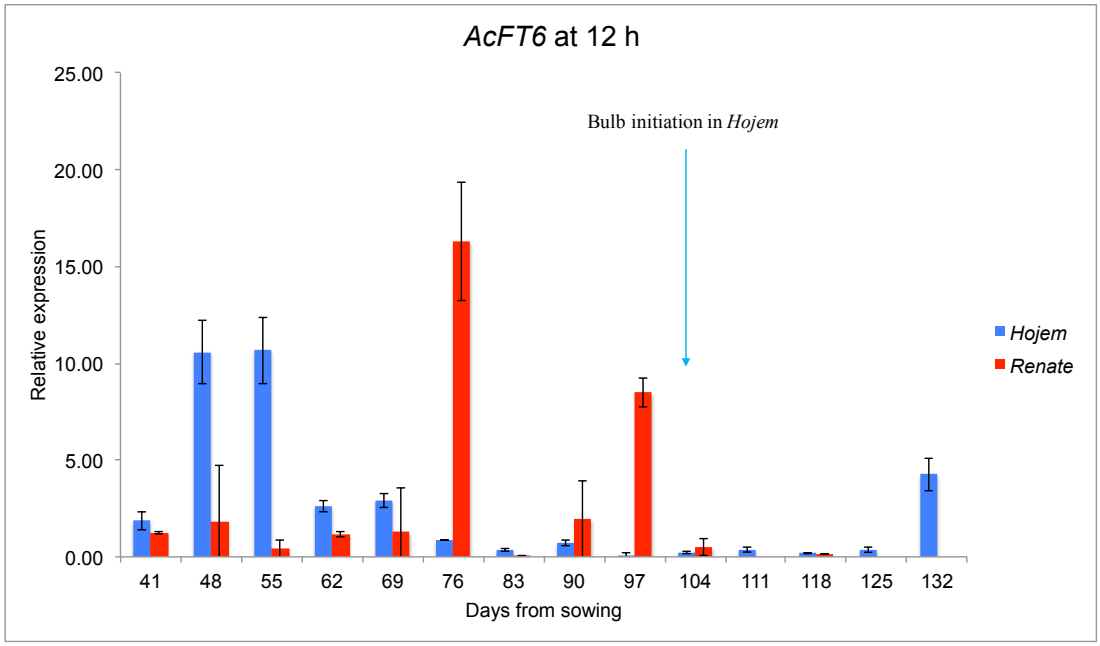


Figure 5.29 Expression of *AcFT6* in *Hojem* and *Renate F1* at 12 h, relative to *PP2A1, UBL* and *PP2AA3*. *AcFT6* was expressed in both *Hojem* and *Renate F1* at the early stage of the development. Error bars represent the SEM.

5.3.3.3 Expression of *AcLFY*

In this experiment we did not look at the bulb tissue. *AcLFY* showed higher expression in *Hojem* than *Renate F1* leaves throughout the early growth and development, while showing very limited expression in *Renate F1* at 62 and 69 DFS in 12 h daylength (Figure 5.30). This result could be supported by the previous experiment as described in 4.3.3, where it was found that *AcLFY* was only expressed in *Renate F1* bulb tissue but not in the leaf. However, the level of expression of this gene was lower at the early stage of plant growth in *Hojem* but increased during the period of bulb initiation before reducing with the bulb development and maturity.

Two-way ANOVA confirmed that the difference in *AcLFY* expression between *Hojem* and *Renate F1* under 12 h daylength was significant (Table A14). It was also confirmed that the number of days from sowing had a significant affect on *AcLFY* expression (Table A14). Finally, the interaction between days from sowing and variety was also shown to be significant (Table A14), showing that the expression of the *AcLFY* over time is affected by daylength.

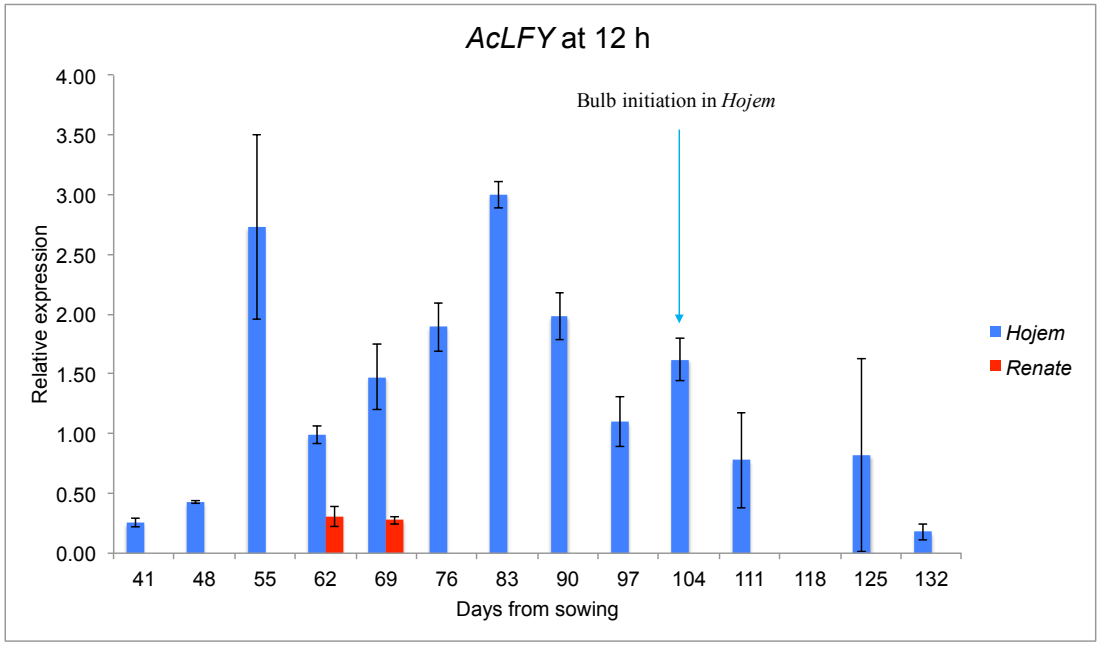


Figure 5.30 Expression of *AcLFY* in *Hojem* and *Renate F1* leaves at 12 h, relative to *PP2A1, UBL* and *PP2AA3*. *AcLFY* was expressed in *Hojem* throughout the development but showed very limited expression in *Renate F1* at the early part of the development. Error bars represent the SEM.

5.3.3.4 Expression of *GA3ox1*

GA3ox1 was expressed throughout the growth and development in both *Hojem* and *Renate F1* at 12 h (Figure 5.31). However, it was observed that the expression level was similar hroughout the growth and development in both varieties of onion.

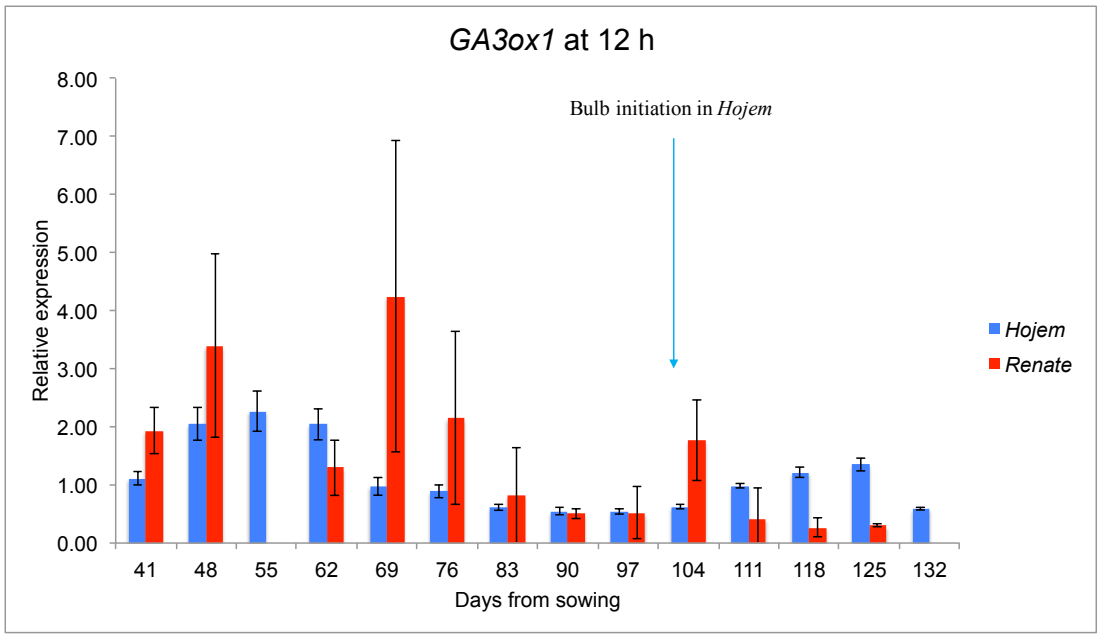


Figure 5.31 Expression of *GA3ox1* in *Hojem* and *Renate F1* at 12 h, relative to *PP2A1, UBL* and *PP2AA3*. *GA3ox1* was expressed in both *Hojem* and *Renate F1* throughout the development. Error bars represent the SEM.

5.3.4 Spatial gene expression in Renate F1

An experiment was performed to determine spatial expression of the genes of interest in onion. In particular, we were interested in the expression of these genes in the site of perception (green leaf) or response (basal tissues) under bulbing and non-bulbing daylegths. Study reported that perception of photoperiod in onion is localised as one might predict in the leaves especially green part (Heath and Holdsworth, 1948). This experiment was particularly focussed on the genes which are related to bulb formation and also linked to the circadian clock in onion.

5.3.4.1 Expression of *AcFKF1*

In *Renate F1*, *AcFKF1* showed more leaf specific expression in LD and bulb specific expression pattern in SD (Figure 5.32). However, the level of expression was significantly higher in LD than in SD especially in the green part of the leaf (site of perception), which suggests that this gene might be linked to circadian regulation which occurs in the leaf.

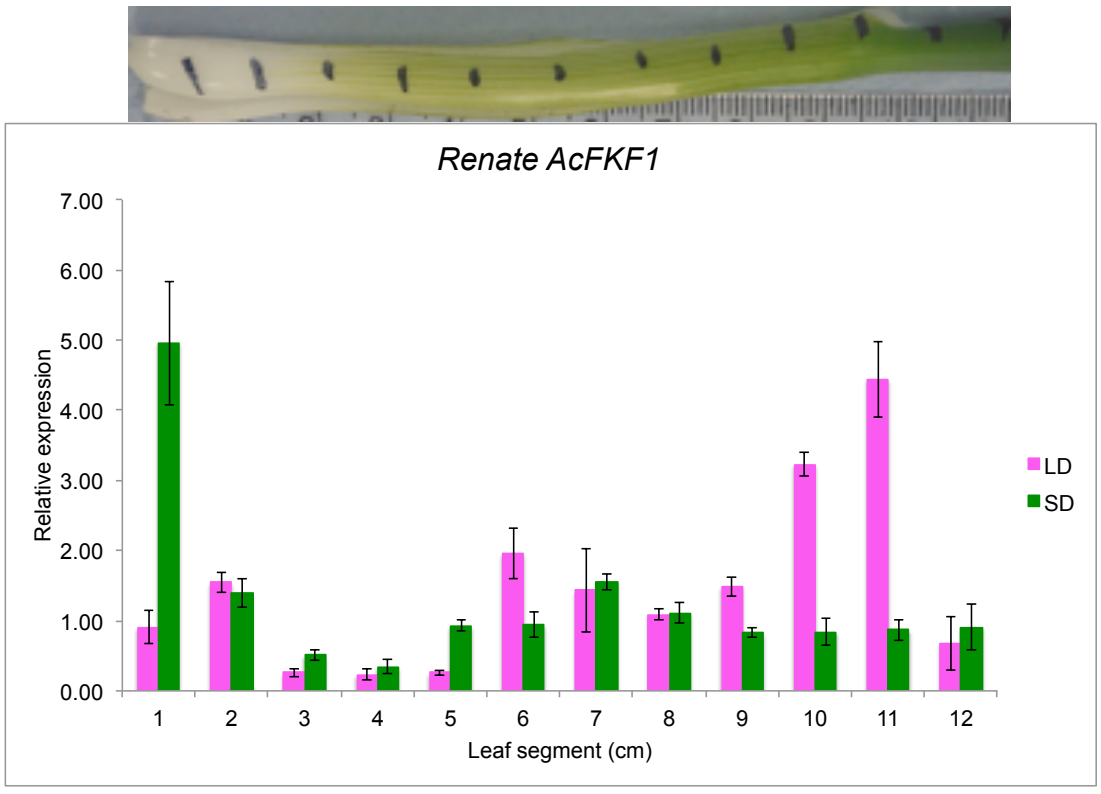


Figure 5.32 Expression of *AcFKF1* in onion leaf under LD and SD, relative to *PP2AA3, TIP41* and *UBL*. *AcFKF1* showed more leaf specific expression in LD and bulb specific expression pattern in SD. Error bars represent the SEM. Top panel shows greenness of the leaf.

5.3.4.2 Expression of *AcGI*

AcGI was present throughout the leaf of *Renate F1* and showed similar level of expression from the site of perception (green part) to the site of response (basal tissue) under LD and SD (Figure 5.33).

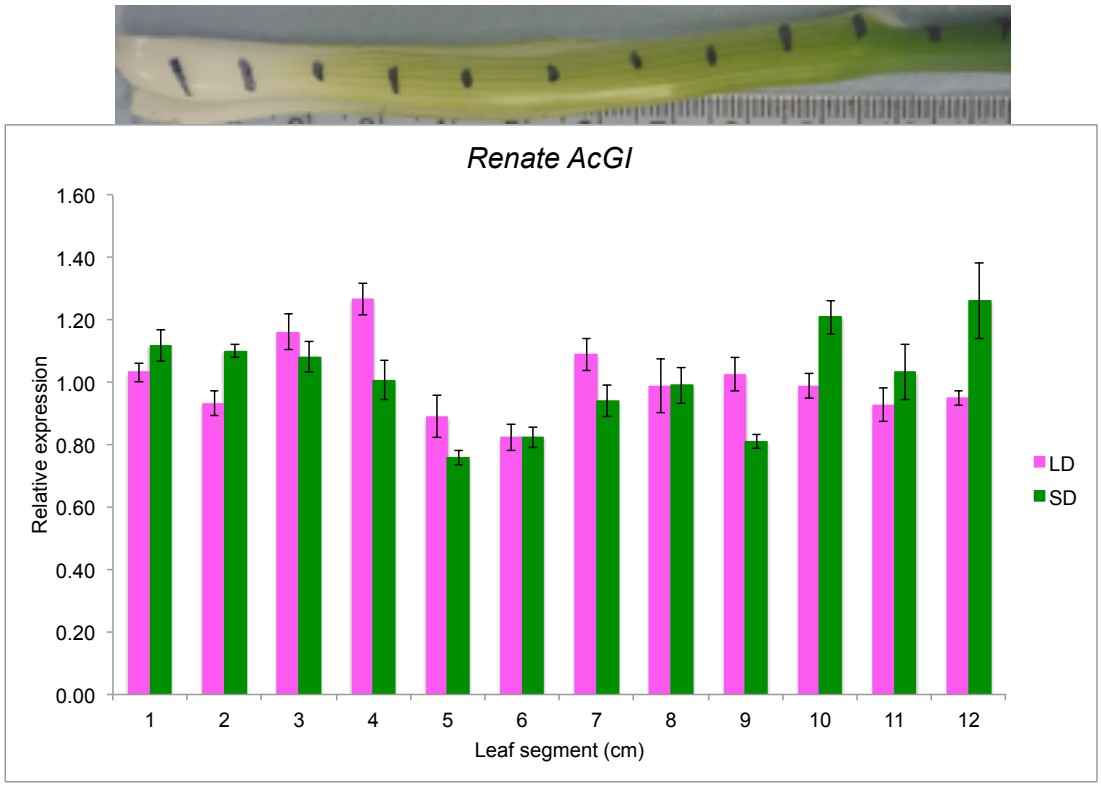


Figure 5.33 Expression of *AcGI* in onion leaf under LD and SD, relative to *PP2AA3, TIP41* and *UBL*. *AcGI* was expressed althrough the leaf from the site of perception (green part) to the site of response (basal tissue). Error bars represent the SEM. Top panel shows greenness of the leaf.

5.3.4.3 Expression of *COL* genes

AcCOL1 was expressed throughout the leaf tissue under both LD and SD conditions, although the level of expression was slowly decreased from the site of perception (green part) to the site of response (basal tissue) (Figure 5.34). This result suggests that this gene is not tissue specific in response to daylengths. On the other hand, *AcCOL2* and *AcCOL3* genes were not expressed in basal 2-3 cm under LD and SD conditions which suggesting that these genes could be tissue specific (Figure 5.35 and 5.36). However, *AcCOL2* showed slightly higher expression in green tissue in the LD leaf than in the SD, which is where daylength is perceived. In fact, there was significant difference found in expression of *AcCOL2* under LD and SD using two-way ANOVA (Table A15). *AcCOL3* also showed similar tissue specific expression pattern like *AcCOL2*, however, no significant difference was found between LD and SD conditions (Figure 5.36).

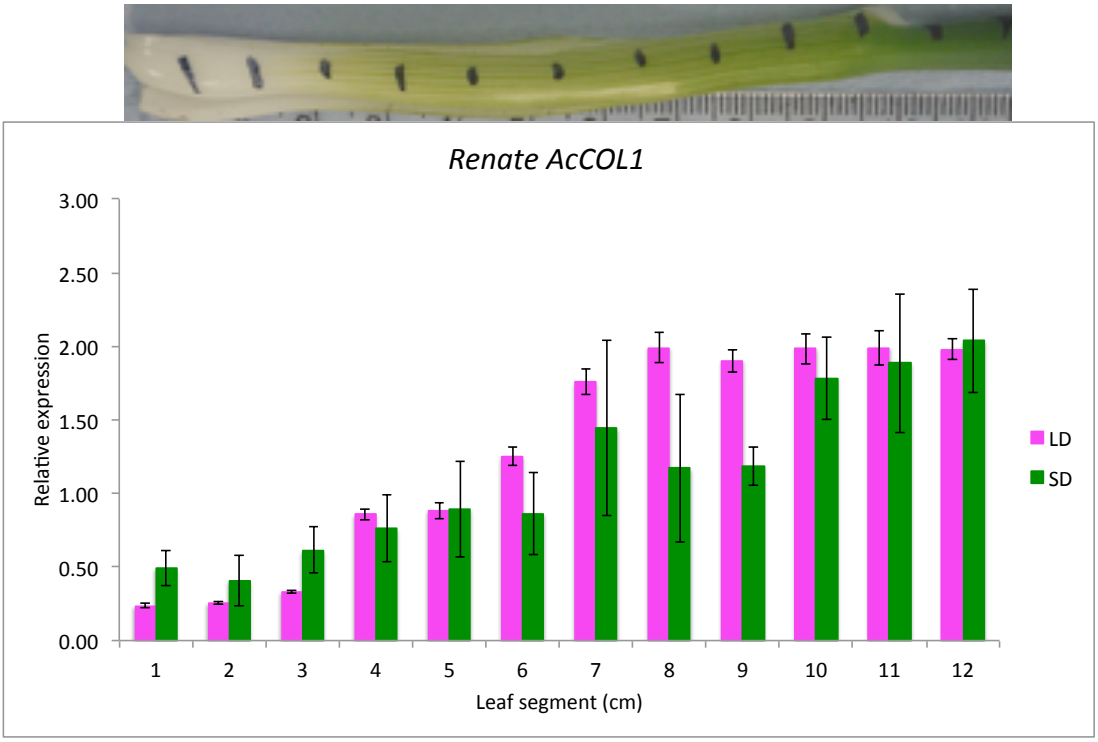


Figure 5.34 Expression of *AcCOL1* in onion leaf under LD and SD, relative to *PP2AA3, TIP41* and *UBL*. *AcCOL1* was expressed althrough the leaf in LD and SD leaf from the site of perception (green part) to the site of response (basal tissue). Error bars represent the SEM. Top panel shows greenness of the leaf.

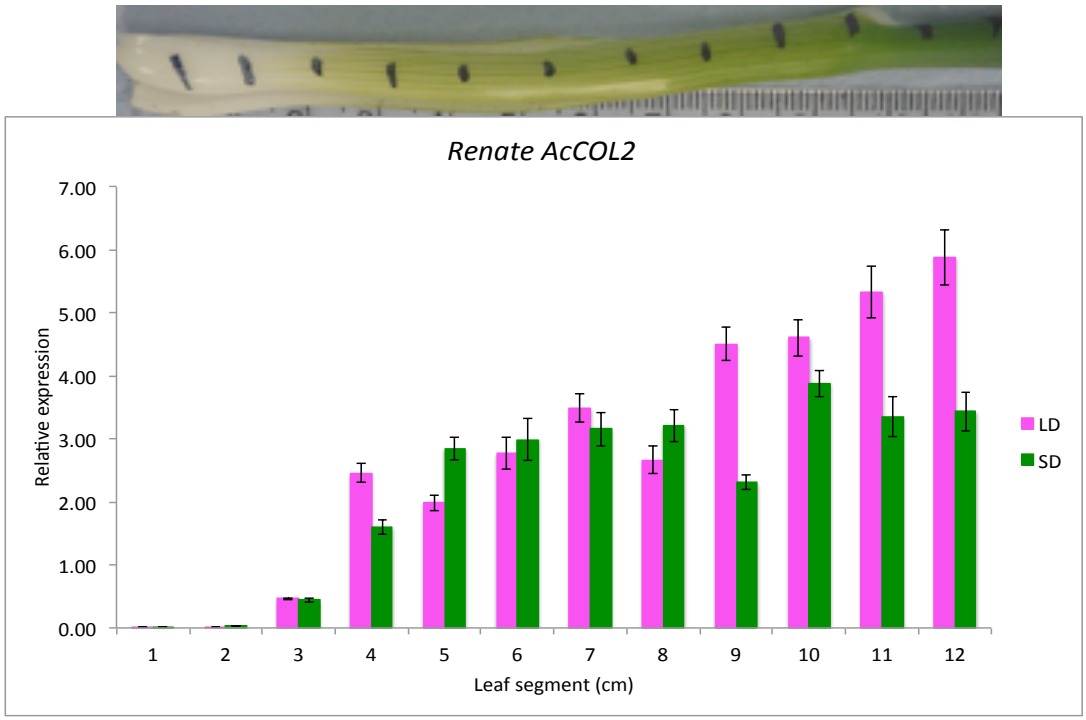


Figure 5.35 Expression of *AcCOL2* in onion leaf under LD and SD, relative to *PP2AA3, TIP41* and *UBL*. *AcCOL2* was mostly expressed in the green part of the leaf (site of perception) under LD and SD. Error bars represent the SEM. Top panel shows greenness of the leaf.

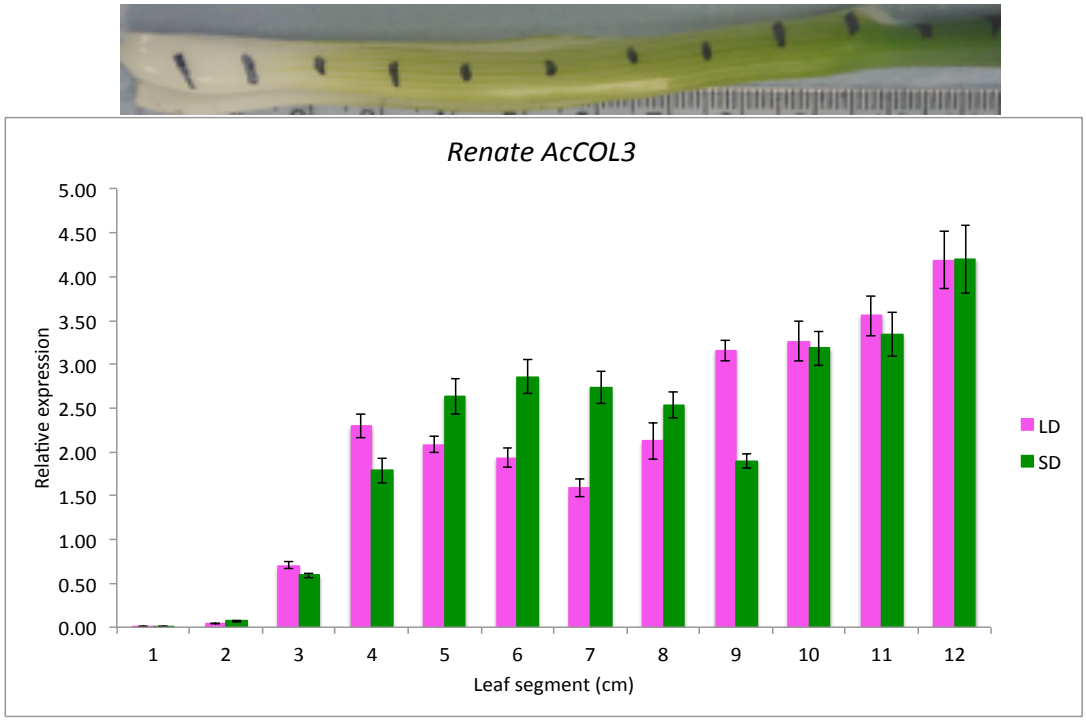


Figure 5.36 Expression of *AcCOL3* in onion leaf under LD and SD, relative to *PP2AA3, TIP41* and *UBL*. *AcCOL3* was mostly expressed in the green part of the leaf (site of perception) under LD and SD. Error bars represent the SEM. Top panel shows greenness of the leaf.

5.3.4.4 Expression of *FT* genes

5.3.4.4.1 Expression of *AcFT1*

AcFT1 showed differential expression in *Renate F1* leaf under LD and SD (Figure 5.37). In LD, it was expressed all throughout the green part of the leaf, although the expression was slowly increased from the transition to the site of perception. While, in SD, the expression of *AcFT1* was started from the the transitional part of the leaf and then sharply decreased at the site of perception (green part). This could be due to the quantity of light received by the plants under LD, where high levels of far-red light accelerate bulbing. However, it was observed that in both LD and SD conditions, *AcFT1* was not expressed in the basal tissue (site of response). Results suggest that the *AcFT1* was expressed differently under LD and SD but produced in the same tissue. Two-way ANOVA confirmed that the difference in *AcFT1* expression between LD and SD conditions was significant (Table A16). It was also confirmed that the leaf segments had significant affect on *AcFT1* expression (Table A16). Finally, the interaction between leaf segment and daylength was shown to be significant (Table A16), showing that the expression of the *AcFT1* on the leaf segments is affected by daylength.

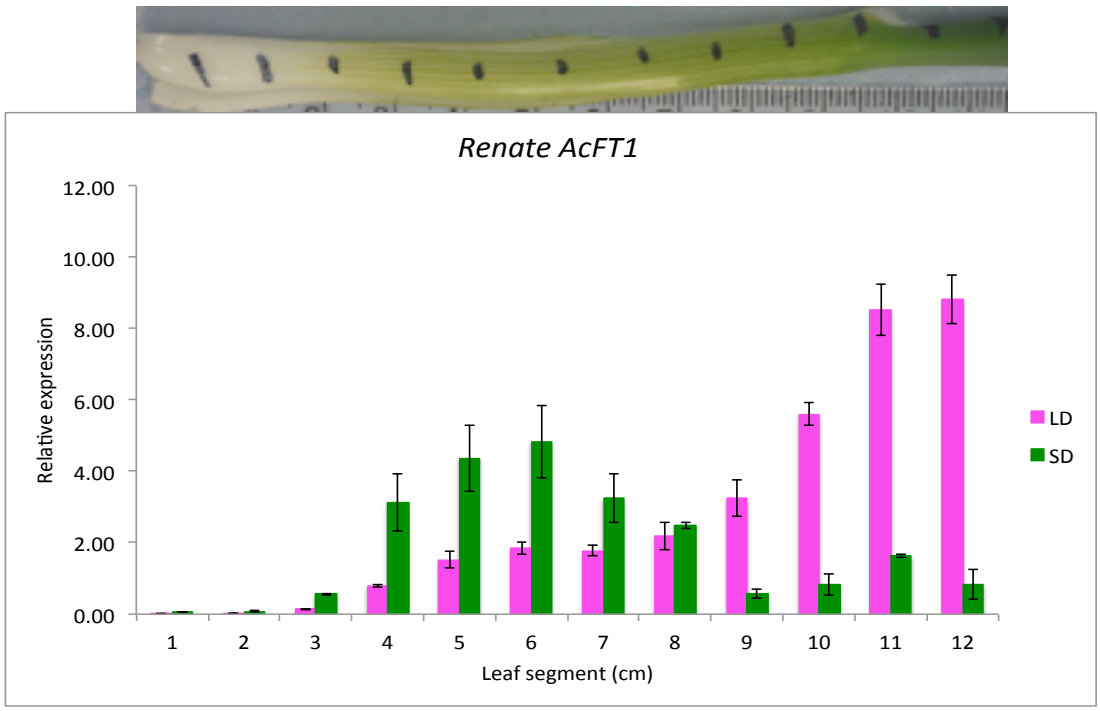


Figure 5.37 Expression of *AcFT1* in onion leaf under LD and SD, relative to *PP2AA3, TIP41* and *UBL*. *AcFT1* was expressed throughout the green part of the leaf (site of perception) but was not expressed in basal tissue (site of response) under LD and SD. Error bars represent the SEM. Top panel shows greenness of the leaf.

5.3.4.4.2 Expression of *AcFT4*

AcFT4 was expressed only in the green part of the leaf (site of perception) but was not expressed in the basal tissue (site of response) or transitional part of the leaf under both LD and SD conditions (Figure 5.38). However, the expression was significantly different between two photoperiods, where *AcFT4* showed very high level of expression in SD leaf but a very low level of expression in LD leaf. This result also suggests that the *AcFT4* was expressed differently under LD and SD but produced in the same tissue. In addition, no expression in the basal part (site of response) confirms that *AcFT4* is a tissue-specific gene. This tissue-specific expression pattern of *AcFT4* in SD indicates that this gene might inhibit bulb formation in *Renate F1* at SD which is consistent with the previous experimental results as described in section 5.3.2.2.2. Two-way ANOVA confirmed that the difference in *AcFT4* expression between LD and SD conditions was significant (Table A17). It was also confirmed that the leaf segments had significant affect on *AcFT4* expression (Table A17). Finally, the interaction between leaf segment and daylength was shown to be significant (Table A17), showing that the expression of *AcFT1* on the leaf segments is affected by daylength.

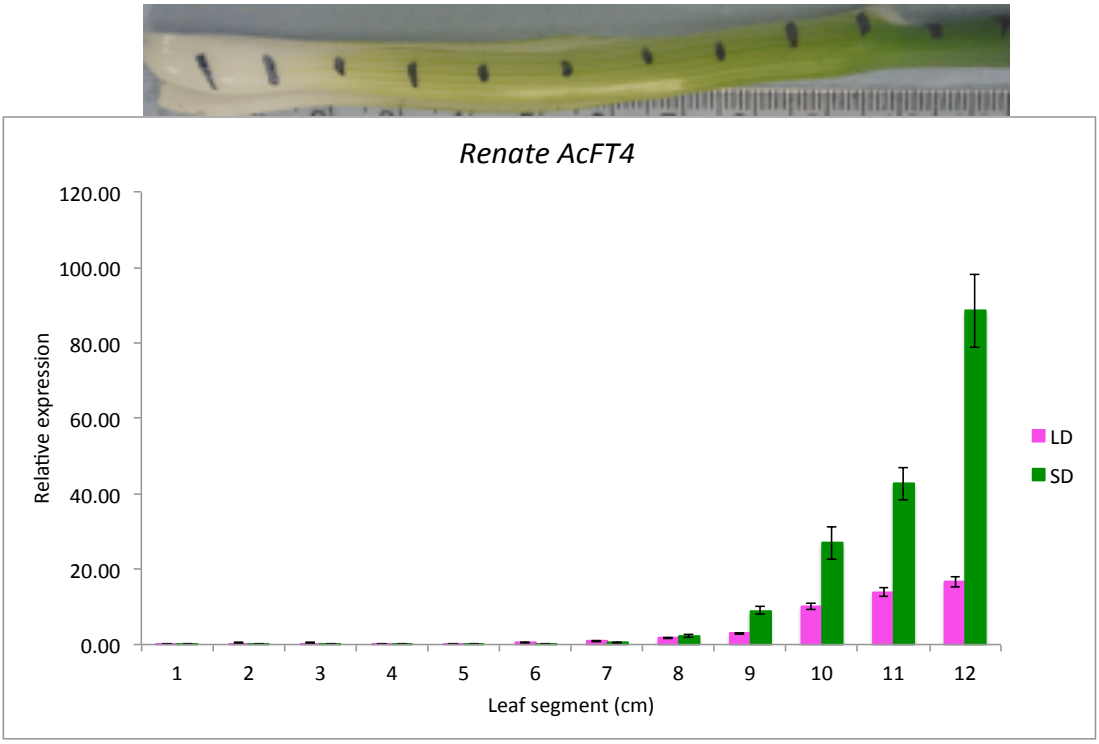


Figure 5.38 Expression of *AcFT4* in onion leaf under LD and SD, relative to *PP2AA3, TIP41* and *UBL*. *AcFT4* was only expressed in the green part of the leaf (site of perception) but was not expressed in basal tissue (site of response) under LD and SD. Error bars represent the SEM. Top panel shows greenness of the leaf.

5.3.4.4.3 Expression of *AcFT5*

In *Renate F1*, *AcFT5* was expressed throughout the leaf from the site of perception (green part) to the site of response (basal tissue) in both LD and SD, suggesting no tissue specific expression pattern of this gene (Figure 5.39). However, *AcFT5* showed higher level of expression in basal tissue to the transitional part than to green leaf tissue under LD, while, it showed lower level of expresssion in the basal tissue than to the green part under SD.

Two-way ANOVA confirmed that the difference in *AcFT5* expression between LD and SD conditions was not significant (Table A18). However, it was confirmed that the leaf segments had significant affect on *AcFT5* expression (Table A18). Finally, the interaction between leaf segment and daylength was also shown to be significant (Table A18), showing that the expression of *AcFT5* on the leaf segments is affected by daylength.

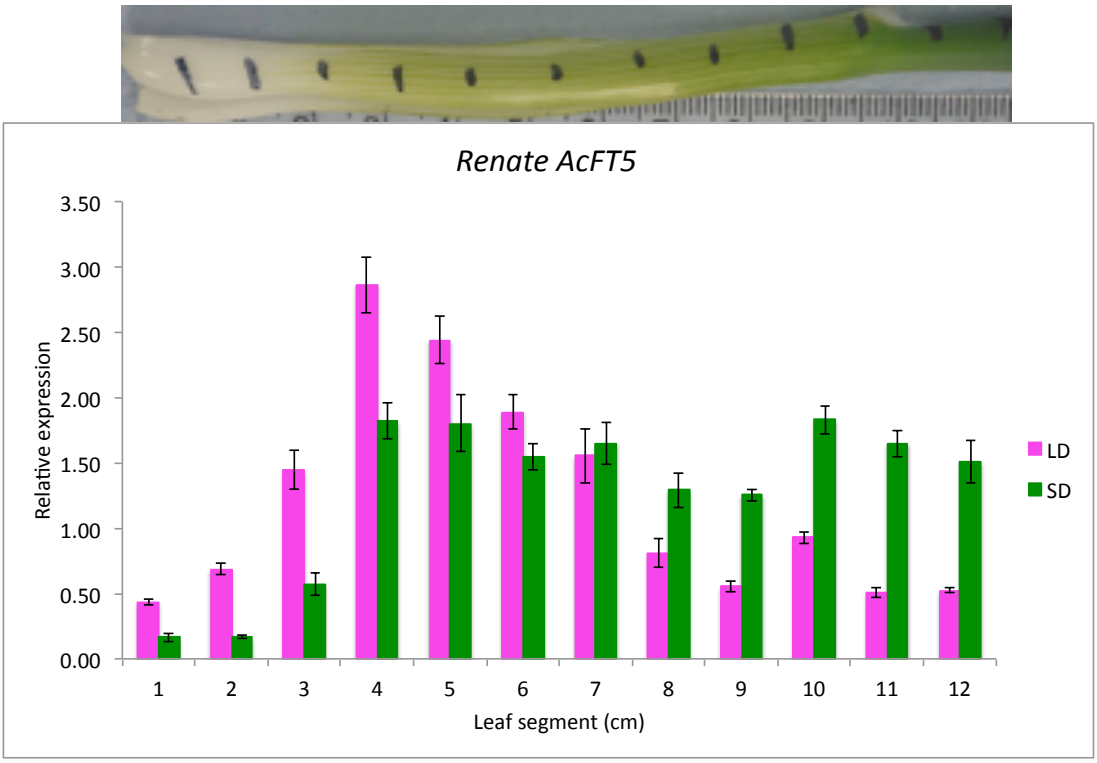


Figure 5.39 Expression of *AcFT5* in onion leaf under LD and SD, relative to *PP2AA3, TIP41* and *UBL*. *AcFT5* was expressed all throuhout the leaf from the site of perception (green part) to the site of response (basal tissue) under LD and SD. Error bars represent the SEM. Top panel shows greenness of the leaf.

5.3.4.4.4 Expression of *AcFT6*

In *Renate F1*, *AcFT6* expression was similar to the pattern of expression of *AcFT5* as described in section 5.3.4.4.3. *AcFT6* was expressed throughout the leaf from the site of perception (green part) to the site of response (basal tissue) in both LD and SD, suggesting no tissue specific expression pattern of this gene (Figure 5.40). However, *AcFT6* showed higher level of expression in basal tissue then sharply decreased in the rest part of the leaf under LD, but showed lower levels of expresssion in the basal tissue under SD. It then showed similar level of expression to the rest part of the leaf under SD.

Two-way ANOVA confirmed that the difference in *AcFT6* expression between LD and SD conditions was not significant (Table A19). However, it was confirmed that the leaf segments had significant affect on *AcFT6* expression (Table A19). Finally, the interaction between leaf segment and daylength was also shown to be significant (Table A19), showing that the expression of *AcFT6* on the leaf segments is affected by daylength.

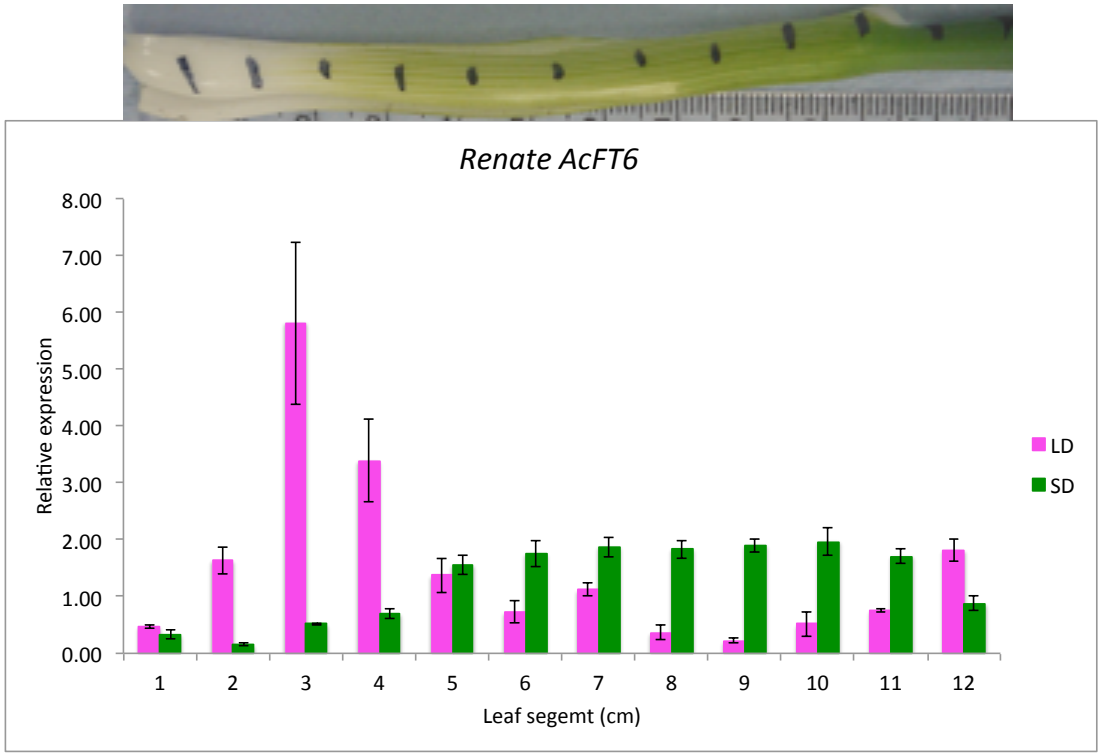


Figure 5.40 Expression of *AcFT6* in onion leaf under LD and SD, relative to *PP2AA3, TIP41* and *UBL*. throughout the leaf site of perception (green part) to the site of response (basal tissue) in both LD and SD. Error bars represent the SEM. Top panel shows greenness of the leaf.

5.3.4.5 Expression of *AcLFY*

AcLFY was mostly expressed in basal part of the leaf (site of response) under both LD and SD conditions (Figure 5.41). This result is consistent with results in the previous experiment as described in section 4.3.3, where *AcLFY* a mRNA band was only found in bulb tissue under LD and SD conditions through RT-PCR. Therefore, it could be confirmed that *AcLFY* is a bulb tissue specific gene. However, the qRT-PCR expression of *AcLFY* in *Renate F1* was significantly higher in bulb tissue under LD than to SD. This suggesting that *AcLFY* needs *AcFT1* to enhance bulbing under LD what we see in the leaf.

Two-way ANOVA confirmed that the difference in *AcLFY* expression between LD and SD conditions was significant (Table A20). It was also confirmed that the leaf segments had significant affect on *AcLFY* expression (Table A20). Finally, the interaction between leaf segment and daylength was shown to be significant (Table A20), showing that the expression of *AcLFY* on the leaf segments is affected by daylength.

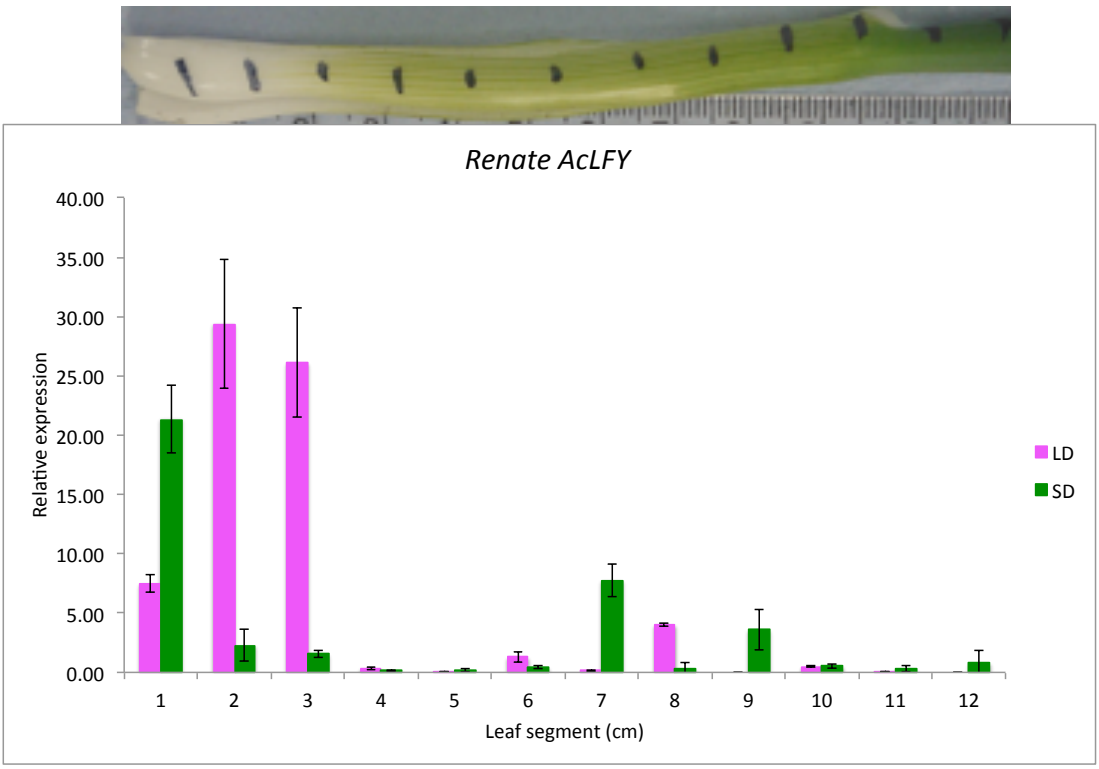


Figure 5.41 Expression of *AcLFY* in onion leaf under LD and SD, relative to *PP2AA3, TIP41* and *UBL*. *AcLFY* was mostly expressed in bulb tissue (site of response) under LD and SD. Error bars represent the SEM. Top panel shows greenness of the leaf.

5.3.4.6 Expression of *GA3ox1*

GA3ox1 was expressed throughout the leaf under both LD and SD conditions, although the level of expression was very low in the basal tissue (site of response) and slowly increased to top of the leaf (Figure 5.42). There was no significant difference found for *GA3ox1* expression in either leaf or bulb tissue under LD and SD. This result suggests that the tissue specificity of expression of this gene is not dependent on daylength.

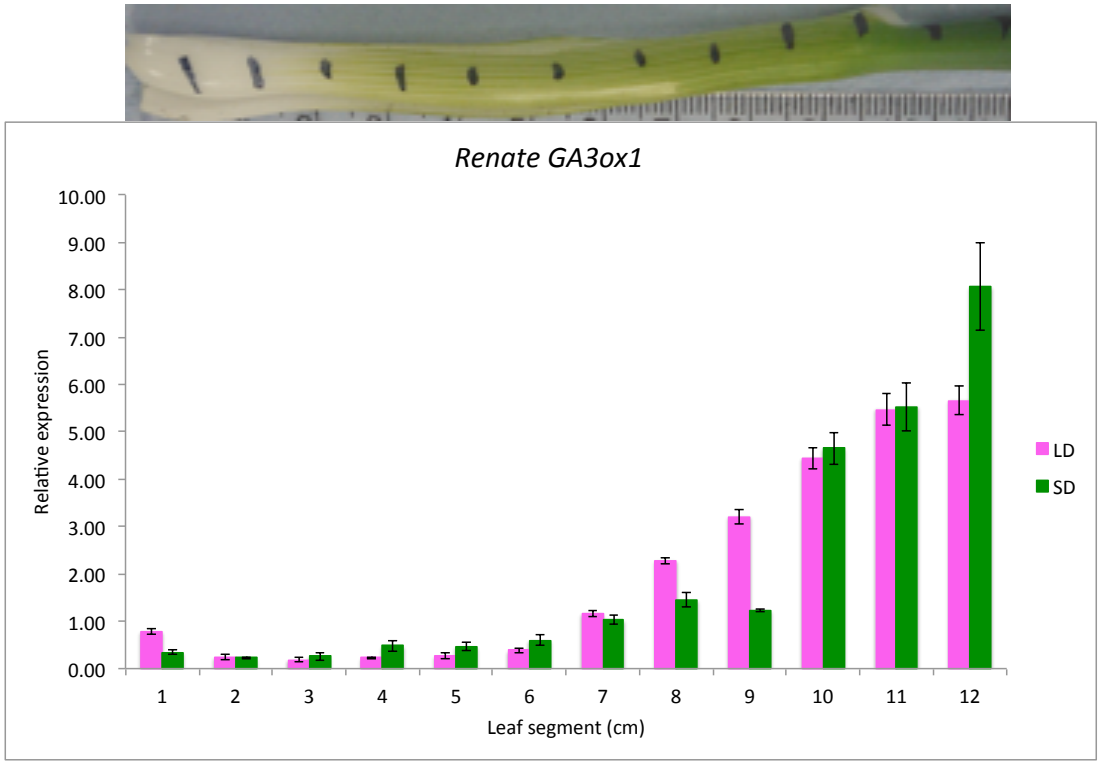


Figure 5.42 Expression of *GA3ox1* in onion leaf under LD and SD, relative to *PP2AA3, TIP41* and *UBL*. *GA3ox1* was expressed throughout the leaf under LD and SD. Error bars represent the SEM. Top panel shows greenness of the leaf.

5.4 Discussion and Conclusions

This chapter describes the developmental time-course expression of the genes in onion that are particularly involved in the photoperiodic regulation of flowering in Arabidopsis and other important pathways.

5.4.1 Preliminary RT-PCR expression

Preliminary RT-PCR results showed the genes under study, except *FT2*, were isolated in *Renate F1* using qRT-PCR primers. All detected sequences were shown to represent the expected gene through sequencing of PCR products. The inability to detect *FT2* might be due to the use of vegetative materials for this experiment, where it was found in flowering bulbs in previous study of Lee et al. (2013). *FT3* showed 83% identity with *FT5* and these sequences were not distinguished in the qRT-PCR analysis.

5.4.2 Daylength regulation during development

All three *COL* genes were expressed in *Renate F1* under all three daylength conditions of LD (NC), LD (CE) and SD. However, *AcCOL1* and *AcCOL3* showed higher expression in SD than to LD (NC) and LD (CE). These results might be supported by the previous study of Hayama et al. (2003) which revealed that the presence of *COL* genes in SD plants such as rice and Japanese morning glory (*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 SD inductive conditions and suggested that a broader role for *CO* than simply controlling flowering in LD and could be involved in bulb initiation (Martínez-García et al., 2002). In addition, *AcCOL2* was also expressed throughout the bulb initiation and development in onion and significant difference was found between LD (NC), LD (CE) and SD conditions. Statistical analysis also supports these results.

In *Renate F1, AcFT1* was expressed in LD (NC) and LD (CE) that might induce bulb formation but not expressed in SD. In addition, *AcFT1* showed significantly higher expression in LD (NC) than in LD (CE). This could be due to the much higher light level in LD (NC) during the summer compared to the LD (CE) chamber (Thomas

and Vince-Prue, 1997). In contrast, *AcFT4* was only expressed in SD which might inhibit bulb formation but was not expressed in either LD (NC) or LD (CE). These results are supported by the previous studies, where the authors proposed that *AcFT1* promotes bulb formation, while, *AcFT4* down-regulates the expression of *AcFT1*, hence inhibits bulb formation in onion (Lee et al., 2013; Manoharan et al., 2016). It could be confirmed that the expression of *AcFT1* is negatively correlated with *AcFT4* or vice versa. On the whole, the statistical analysis also supports these observations, which show different daylength responses.

AcFT5 showed a very interesting pattern of expression in *Renate F1* under different daylengths. Like *AcFT1*, statistical analysis also suggested that *AcFT5* showed a significantly higher level of expression in LD (NC) than in LD (CE). This could also be due to the sufficient light level in LD (NC) during the summer compared to the LD (CE) chamber (Thomas and Vince-Prue, 1997). *AcFT6* expression was different from that of the other *FTs* and only appears in LD (NC). It has been demonstrated that *FT* is rapidly upregulated in Arabidopsis and other plants, when plants are shifted from non-inductive conditions to an inductive photoperiod (Corbesier et al., 2007; Laurie et al., 2011). Therefore, this study suggests that *AcFT5* and *AcFT6* might be involved in the *Renate F1* bulb formation, although the function of these genes is still unknown.

In *Renate F1*, *AcLFY* was strongly expressed in bulb tissue under LD (NC) at the early stage of plant growth and at the later stage of bulb development but was not expressed in either LD (CE) or SD conditions. Statistical analysis also supports these results. Therefore, *AcLFY* might need *AcFT1* to correlate onion bulbing process in LD. This could be supported by the role of *LFY* in Arabidopsis, which triggers the expression of the floral homeotic genes at the floral apical meristem and causes flowering (Yoo et al., 2005), whereas, in onion, the apex is present at the base of plant (bulb). Disappearance of its expression after bulb formation in all three conditions could be due to the negative correlation or suppression by other genes like *FLOWERING LOCUS C* (*FLC*), which is a negative repressor for autonomous pathways in Arabidopsis (Thomas et al., 2006). While, re-emmergence of *AcLFY* in LD (NC) at bulb maturity suggests that this gene might not only be involved in the

bulb initiation process, but also plays an important role in apical meristem and bulb development in LD onion.

In *Renate F1*, *GA3ox1* was expressed in all three conditions throughout the bulb initiation and development. This suggests that *GA3ox1* might not be directly involved in bulb formation in *Renate F1*.

5.4.3 Comparison of Hojem and Renate F1 in intermediate daylengths

In the ID conditions, all three *COL* genes were expressed throughout the growth, bulb initiation and development in both *Hojem* and *Renate F1*. In addition, no significant difference found in expression between the two onion varieties at 12 h using two-way ANOVA (data not shown). Therefore, it could be concluded that three *COL* genes are not directly involved in the bulb initiation process and development.

AcFT1 showed very high expression in *Hojem* during the later stage of bulb development and maturity, but showed very limited, or no, expression in *Renate F1* at 12 h throughout development. This result could also be suppported by a previous study in *A. cepa*, where the authors proposed that *AcFT1* was down-regulated in the early maturity line under both SD and LD conditions (Manoharan et al., 2016). The expression of *AcFT2* was consistent with that of the *AcFT1* in ID conditions. Therefore, it could be speculated that both *AcFT1* and *AcFT2* might induce bulb formation and development in *Hojem* but not in *Renate F1* under ID conditions, because ID does not induce bulb formation in *Renate F1*.

AcFT4 was expressed at the early stage of plant growth in *Hojem*, but at the later stage of development in *Renate F1* at 12h. The data suggest that *AcFT4* might inhibit bulb formation in *Hojem* at the early stage of growth or during the juvenile phase by suppressing *AcFT1*. Down-regulation of *AcFT4* in *Hojem* during bulb initiation and development could be due to the suppression by, or strong expression of, *AcFT1*. On the other hand, up-regulation of *AcFT4* might down-regulate the expression of *AcFT1* in *Renate F1* under ID conditions. Therefore, it could be concluded that *AcFT4* is negatively correlated with *AcFT1* or vice versa in both varieties of onion.

AcFT5 was expressed all throughout the growth and development period in both *Hojem* and *Renate F1* at 12 h. The results suggest that *AcFT5* might not be involved in the bulb induction process itself in either *Hojem* or *Renate F1* under ID conditions. *AcFT6* was expressed at the early stage of plant growth in *Hojem*, while it was expressed at the middle stage of development in *Renate F1* at 12 h. However, these results are incosistent with the previous study of Lee et al. (2013), where the authors suggest that the expression of *AcFT5* and *AcFT6* were similar at the three stages (young, mature and bulb) of development and did not appear to be affected by daylength. Although the function of this gene is still unknown.

AcLFY showed strong expression in *Hojem* throughout the development, although the expression was highest at the middle stage just before bulb formation. On the contrary, it showed very limited expression in *Renate F1* and only apperas at 62 and 69 DFS in 12 h. Therefore, it could be confirmed that *AcLFY* might play a significant role in bulb initiation and development in *Hojem* but not in *Renate F1* under ID conditions. Statistical analysis also supports these results.

GA3ox1 was expressed all throughout the growth and development in both *Hojem* and *Renate F1* at 12 h. In addition, no significant difference found in expression level between two varieties of onion, suggests that *GA3ox1* might not be directly involved in bulb initiation in either *Hojem* or *Renate F1* at 12 h.

5.4.4 Spatial expression pattern in leaves

As monocotyledon leaves develop from a basal meristem, during leaf growth, cells move through the basal region into a transition zone and then into the green, photosynthesising part of the leaf. The photosynthetic and basal parts of the leaf can be considered as the sites of perception and response, respectively. By looking at the expression of genes at different points along the length of the leaf, it is possible to determine the extent to which their expression correlates with the different functional regions and also give a picture of how expression changes in cells of increasing age.

AcGI was present throughout the leaf from the site of perception (green tissue) to the site of response (basal tissue) under both LD and SD conditions suggesting no tissue specific expression pattern of this gene. This results are supported by previous

investigations as *AcGI* appears to be expressed in every organ, and is highly conserved in seed plants, including monocotyledons, such as rice, wheat and barley (Dunford et al., 2005; Hayama et al., 2003; Xiang et al., 2005) as well as dicots such as radish and *Medicago truncatula* (Curtis et al., 2002; Paltiel et al., 2006) and gymnosperms, such as loblolly pine (*Pinus taeda*) (Hayama et al., 2002; Mizoguchi et al., 2005). Furthermore, the expression of this gene in both leaf and bulb organs suggests that *AcGI* could have a function in diverse physiological processes in onion such as flowering time regulation, light signaling, control of circadian rhythm, sucrose signaling, starch accumulation, chlorophyll accumulation, various stresses tolerance, although the biochemical function of *GI* and its different domains are still unclear (Mishra and Panigrahi, 2015). *AcFKF1* showed a more leaf specific expression in LD and bulb specific expression pattern in SD. This supports the previous study of Taylor et al. (2010), where they found the expression of *AcFKF1* in onion leaf under LD conditions.

AcCOL1 was expressed throughout the leaf from the site of perception (green tissue) to the site of response (basal tissue) under both LD and SD conditions, suggesting no tissue specific expression pattern of this gene. On the other hand, the level of expression in both *AcCOL2* and *AcCOL3* was sharply increased from the basal part to the green part of the leaf, suggesting these genes are specifically expressed in photosynthetic but not storage tissues.

Both *AcFT1* and *AcFT4* genes showed tissue specific expression pattern under both LD and SD conditions. However, the results suggest that the both *AcFT1* and *AcFT4* genes were expressed differently under LD and SD but produced in the same tissue (leaf). This could be due to the quantity of light received by the plants under LD, where high levels of far-red light accelerate bulbing. Statistical analysis also supports these results. Furthermore, it was also observed that *AcFT4* expression in *Renate F1* leaf was opposite to that of the expression of *AcFT1* in both LD and SD conditions. Therefore, it could be confirmed that *AcFT4* in *Renate F1* leaf is negatively correlated with *AcFT1* or vice versa.

AcFT5 and *AcFT6* were expressed throughout the leaf from the site of perception (green tissue) to the site of response (basal tissue) in both LD and SD conditions,

suggesting no tissue specific expression pattern of these genes. Furthermore, statistical analysis revealed that the interaction effect of *AcFT5* and *AcFT6* expression in *Renate F1* leaf tissue was significantly different when grown under LD to that of the SD conditions. Therefore, these genes might be affected by different daylengths in relation to their expression in different parts of the leaf. These results could support a role for both genes in onion bulb formation in the developmental time-course experiment, but this would need to be confirmed by functional analysis of their roles.

AcLFY showed mostly bulb-specific expression pattern in both LD and SD conditions. Statistical analysis also supports these results. This is because *LFY* causes a group of undifferentiated cells named meristems to develop into flowers instead of leaves associated with shoots (Weigel et al., 1992).

GA3ox1 expression was present everywhere in the leaf tissue from the site of perception (green tissue) to the site of response (basal tissue) under both LD and SD conditions, suggesting no tissue specific expression pattern of this gene.

In conclusion, all three *COL* genes were expressed in LD, ID and SD conditions all throughout the growth and development irrespective of variety. *AcFT1* was expressed in LD, which might induce bulb formation, while *AcFT4* was expressed in SD, which might inhibit bulb formation. These two genes might negatively correlate with each other. *AcFT5* and *AcFT6* were expressed in LD and might be involved in bulb formation itself. *AcLFY* was potentially expressed in LD which might also be involved in bulb development. *FKF1*, *COL2*, *COL3*, *FT1*, *FT4*, *LFY* and *GA3ox1* genes showed distinctive patterns of tissue specific expression in onion.

CHAPTER 6: EFFECTS OF DAYLENGTH ON DIURNAL GENE EXPRESSION IN ONION

6.1 Introduction

Bulb initiation in LD onion at the physiological level is regulated in a similar way to the photoperiodic regulation of flowering in Arabidopsis (Taylor et al., 2010) as described in section 1.7. Both processes are photoperiodically driven and involve a homeotic conversion of leaves, though, in Arabidopsis, floral structures are the result, whereas, in onion it is a storage structure (Summerfield, 1991). Flowering in Arabidopsis has been characterised at the molecular and genetic level and is regulated by 6 major separate pathways viz., photoperiodic, convergent autonomous, sucrose, gibberellin, temperature and light quality pathway (Jack, 2004; Thomas et al., 2006). This study focuses on the photoperiodic pathway, which is mediated by the circadian clock, an autonomous mechanism that generates endogenous rhythms in a 24-hour period operated in the leaf (Hayama and Coupland, 2003). Numerous key genes are involved in circadian regulation, where the clock drives the rhythmic expression of key genes *FKF1*, *GI* and *CO* (Somers et al., 1998). *FKF1* and *GI* promote *CO* expression (Sawa et al., 2007) and this *CO* positively regulates *FT* (Jung et al., 2007). Then FT protein is translocated to the apical meristem through the phloem and causes flowering at the floral apical meristem in Arabidopsis (Greg et al., 2015; Nakamichi, 2011; Yoo et al., 2005). In a previous study, homologues of some major genes namely *GI*, *CO* and *FT* controlling photoperiodic flowering pathway in Arabidopsis are proposed to be conserved in onion (Taylor et al., 2010). Summary of interactions of the genes is shown in Figure 6.1.

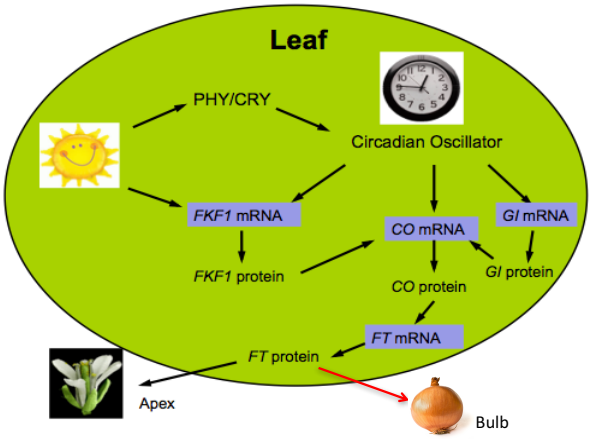


Figure 6.1 Summary of interactions of the genes involved in photoperiodic regulation of flowering in Arabidopsis and bulb initiation in onion.

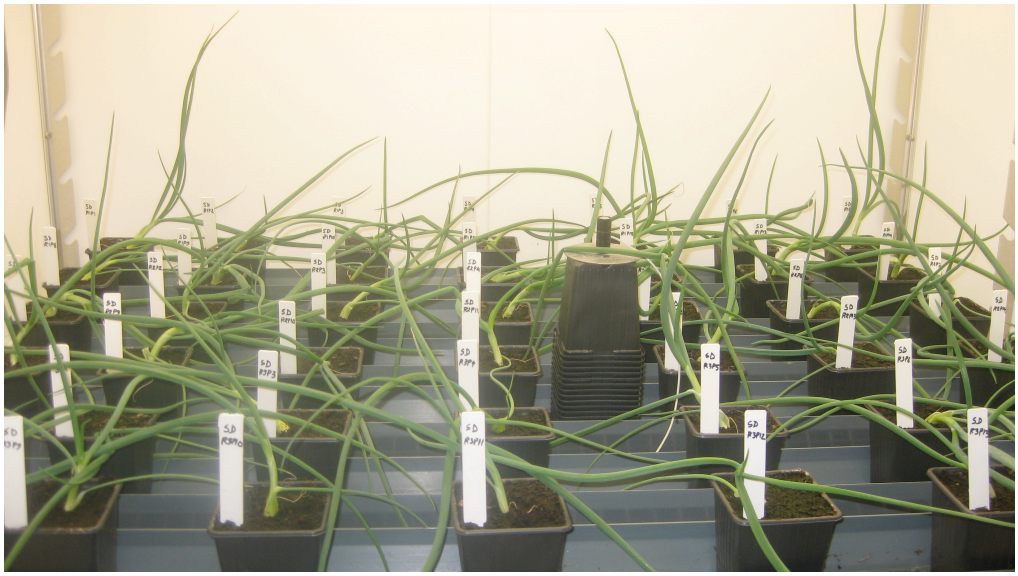
To test the main hypothesis of the study, this chapter describes experiments designed to quantify the diurnal expression in onion, of key genes identified as particularly linked to circadian regulation in Arabidopsis. The major objective of these experiments was to compare the circadian expression patterns of these genes in a LD type cv. *Renate F1* and a SD type cv. *Hojem* over a 48-hour period using qRT-PCR.

6.2 Materials and Methods

6.2.1 Diurnal time-course experiment to study gene expression in Renate F1

For the LD diurnal time-course, onion plants were grown under natural conditions in the Phytobiology Facility during the period from 26th July to 16th September 2013 when daylight was 15 h 42 min to 12 h 35 min, respectively. Initially, *Renate F1* seeds were sown in modular trays and after 4 weeks plants were potted up into 9 cm pots containing Levington M2 compost. At 52 d from sowing, all plants were transferred to two SANYO 2279 controlled environment cabinets for 10 d providing constant LD (16 h photoperiod including 8 h fluorescent followed by 8 h incandescent light) and other environmental conditions as described in section 3.2.1 for Experiment 1 except for a constant 22°C day/night temperature. Timing of the photoperiod was offset by 8 h in the two cabinets and harvesting of leaf materials was scheduled to provide samples at 2-h intervals to cover two consecutive 24-h day time periods. Three plants were harvested each time point and pooled together. Plants were selected for harvesting using a random number generator (Haahr, 2006). Sampling involved removing the middle part of the first newly expanded leaf, chopped into small pieces and freezing in liquid nitrogen before storing at -80°C. The harvested materials were used for molecular analysis.

For the SD diurnal time-course, onion plants were grown in natural conditions in the Phytobiology Facility during the period from 14th August to 13th October 2013 when daylight was 14 h 40 min to 10 h 50 min, respectively. Initially, *Renate F1* seeds were sown in modular trays and after 4 weeks; plants were potted up into 9 cm pots containing Levington M2 compost. At 61 d from sowing, all plants were transferred to two SANYO 2279 controlled environment cabinets for 10 d providing constant SD (8 h photoperiod with fluorescent light) and other environmental conditions as described in section 3.2.1 Experiment 1 except for a constant 22°C day/night temperature (Figure 6.2). Sampling, harvesting and storing were carried out as described for the LD diurnal time-course. The harvested materials were used for molecular analysis.



Renate F1 plants grown under 8 h light in SANYO Cabinet

Figure 6.2 Growth of *Renate F1* plants under SD conditions in the Controlled Environment SANYO Cabinet to generate material for molecular analyses in SD diurnal experiment. A similar design was employed for plants grown in LD and other diurnal experiments. White coloured labels represent different replications in CRD.

6.2.2 Diurnal time-course experiment to study gene expression in Hojem

For the LD diurnal time-course, onion plants were grown in a photoperiod controlled glasshouse compartment of Phytobiology Facility at 12 h daylight during the period from 17th March to 27th May 2014. Initially, *Hojem* seeds were sown in modular trays and after 4 weeks plants were potted up into 9 cm pots containing Levington M2 compost. At 71 d from sowing, all plants were transferred to two SANYO 2279 controlled environment cabinets for 10 d providing the same environmental conditions as described in section 6.2.1 for LD diurnal time-course. Sampling, harvesting and storing were also carried out as described in section 6.2.1. The harvested materials were used for molecular analysis.

For the SD diurnal time-course, onion plants were grown in a photoperiod controlled glasshouse compartment of Phytobiology Facility at 12 h daylight during the period from 16th May to 23rd July 2014. Initially, onion seeds were sown in modular trays and after 4 weeks plants were potted up. At 68 d from sowing, all plants were transferred to two SANYO 2279 controlled environment cabinets for 10 d providing same environmental conditions as described in 6.2.1 for SD diurnal time-course. Sampling, harvesting and storing were also carried out as described in section 6.2.1. The harvested materials were used for molecular analysis.

6.2.3 qRT-PCR analysis

The relative expression of the key genes was examined by qRT-PCR, which was carried out using the CFX384 Touch^{TM} Real-time PCR machine from BioRad (Bio-Rad Laboratories Ltd., UK), as described in section 2.2.5, and PCR conditions were followed as mentioned in Table 2.1. The methodology for designing (section 4.2.1) and testing of qRT-PCR primers (forward and reverse) was also carried out as mentioned in section 5.2.4. Extraction of total RNA, DNase treatment and synthesis of cDNA using 2 µg total RNA was followed by the protocol as described in section 2.2.1. At the end of PCR run, the qRT-PCR data were analysed and normalisation against expression levels of the house keeping genes such as *PP2AA3*, *PP2A1, TIP41* and *UBL* for each sample (Appendix XII) were achieved by using Biogazelle qBase+ software (www.biogazelle.com) as described in 5.2.4 (Hellemans et al., 2007; Vandesompele et al., 2002). Forty-eight hour averages of expression were calculated and standard errors included. Standard curves (using 10-fold serial dilutions) were plotted using cDNA synthesised from approximately 2 µg of toal RNA extracted from leaf material harvested at various time-points (0-48) in a 48-hour period as used for cDNA synthesis described in section 2.2.1.

6.3 Results

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

In this study, we looked at the genes (*FKF1*, *GI*, *COL* and *FT*) that are linked to the circadian clock. The expression of the genes was investigated in *Renate F1*, a LD onion variety (section 6.2.1) and in *Hojem*, a SD onion variety (section 6.2.2) under different daylengths, over a 48-hour period (0-48) using qRT-PCR.

6.3.1.1 Expression of *AcFKF1*

In *Renate F1*, *AcFKF1* showed a clear diurnal expression pattern peaking at around ZT8 in both LD and SD conditions (Figure 6.3). This result is slightly different to Arabidopsis *AcFKF1*, which showed peaks at around ZT10 in LD and ZT7 in SD (Imaizumi et al., 2003). In addition to that, expression pattern of *Renate F1 AcFKF1* in this experiment is inconsistent with the previous study of Taylor et al. (2010). We were unable to repeat the small difference of timing of peaking (data not shown). However, it is clearly evident that *AcFKF1* shows a diurnal rhythm of expression, similar to that of Arabidopsis *FKF1*. Taylor et al. (2010) examined the expression at various points over a 24-hour period and found that *AcFKF1* shows a peak of expression at ZT10 in both LD and SD grown plants.

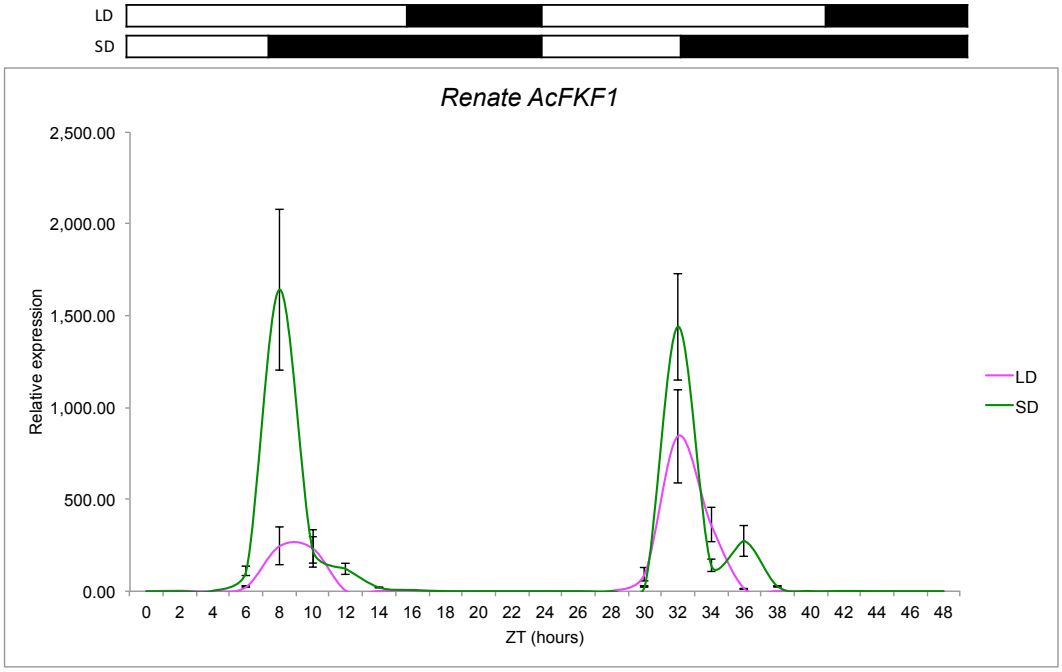


Figure 6.3 Expression of *AcFKF1* in *Renate F1* over a 48-hour period using qRT-PCR, relative to *PP2A1, TIP41* and *UBL*. *AcFKF1* showed clear diurnal expression pattern peaking at around ZT8 in both LD and SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

In *Hojem*, *AcFKF1* also showed clear diurnal expression pattern peaking at around ZT8 in both LD and SD conditions (Figure 6.4), which is consistent with the previous study of Taylor et al. (2010), where in *Agrifound Dark*, a SD onion variety, it peaked at around ZT8 in both LD and SD grown plants (Taylor et al., 2010). They reported that *AcFKF1* is seen to peak around ZT 7-8 in the SD variety (*Agrifound Dark*) compared with ZT 10 in the LD variety (*Renate F1*). These results showed that there is no distinct difference between the timing of the peak of expression in LD and SD varieties of onion used for this experiment grown under either 8 h or 16 h photoperiods. A summary of diurnal expression patterns of *AcFKF1* in onion is presented in Table 6.1.

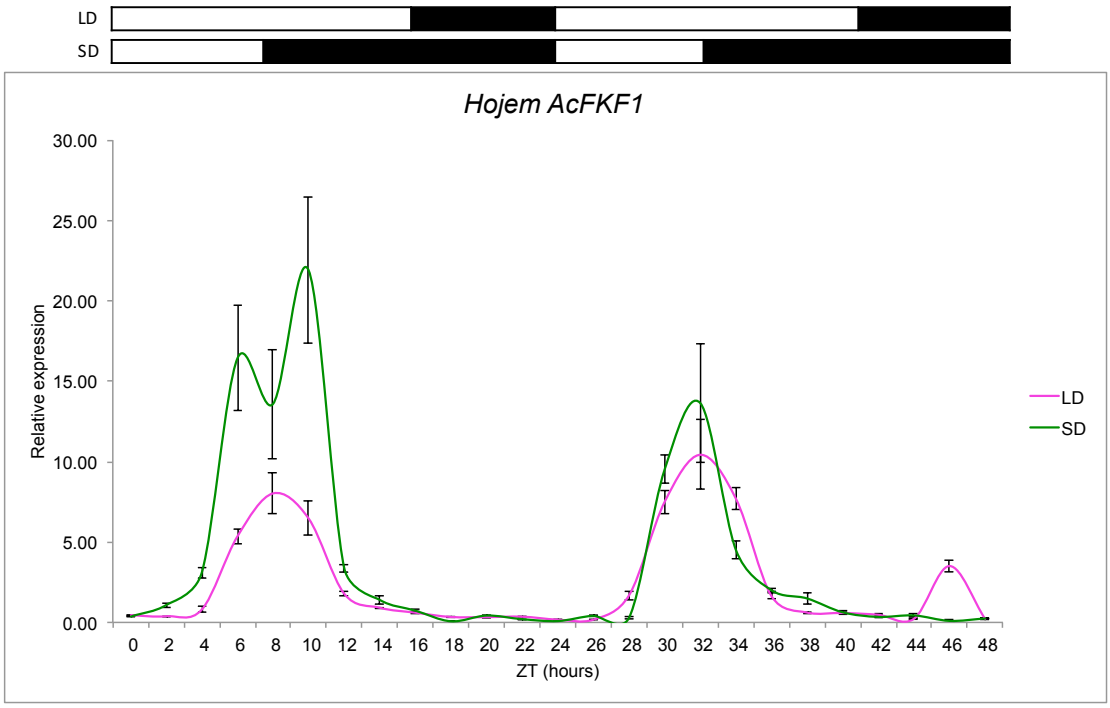


Figure 6.4 Expression of *AcFKF1* in *Hojem* over a 48-hour period using qRT-PCR, *PP2A1, UBL* and *PP2AA3*. *AcFKF1* showed clear diurnal expression pattern peaking at around ZT8 in both LD and SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

Table 6.1 Summary of diurnal expression patterns of LD (*Renate F1*) and SD (*Hojem*) varieties of onion under different conditions.

Gene	Summary of diurnal expression in onion			
	Renate F1		***Hojem***	
	LD	SD	LD	SD
AcFKF1	ZT8	ZT8	ZT8	ZT8
AcGI	ZT8	ZT8	ZT8	ZT8
AcCOL1	Continuous	Continuous	ZT4	ZT4
AcCOL2	ZT10	ZT20	ZT12	ZT16
AcCOL3	Continuous	Continuous	ZT12	Continuous
AcFT1	ZT22	No expression	ZT22	No detectable expression
AcFT2	-	-	ZT4	Continuous
AcFT4	ZT2-4	ZT22-24	ZT2-4	ZT22-24
AcFT5	ZT10-12	No detectable expression	ZT8	ZT10
AcFT6	ZT6	ZT6	ZT8	ZT8

6.3.1.2 Expression of *AcGI*

In *Renate F1*, *AcGI* showed a clear diurnal expression pattern peaking at around ZT8 in both LD and SD (Figure 6.5), which is quite similar to the expression of Arabidopsis *AcGI*, where it peaks at ZT10 in LDs and ZT8 in SDs (Fowler et al., 1999). However, expression pattern of *Renate F1 AcGI* is inconsistent with the previous study of Taylor et al. (2010), where it peaked at around ZT10 in LD and ZT7 in SD (Taylor et al., 2010).

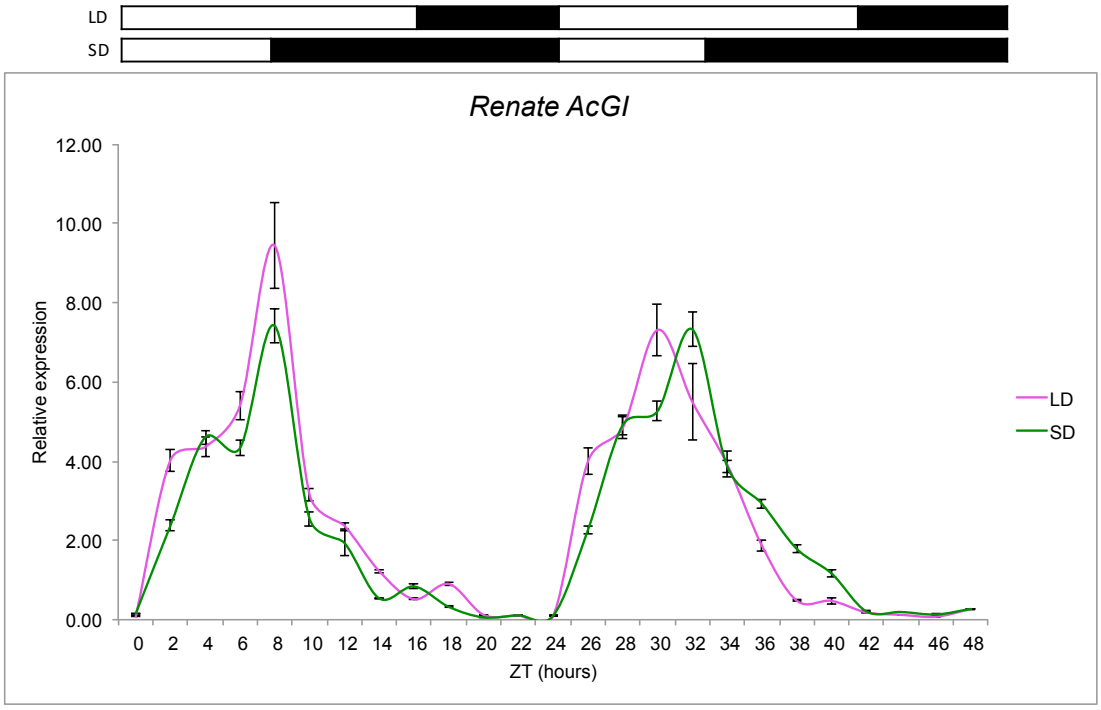


Figure 6.5 Expression of *AcGI* in *Renate F1* over a 48-hour period using qRT-PCR, relative to *PP2A1, TIP41* and *UBL*. *AcGI* showed clear diurnal expression pattern peaking at around ZT8 in both LD and SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

In *Hojem*, *AcGI* showed a clear diurnal expression pattern peaking at around ZT8 in both LD and SD conditions (Figure 6.6). The result was also inconsistent with the previous study of Taylor et al. (2010), where in *Agrifound Dark*, it peaked at around ZT10 in LD and ZT7-8 in SD (Taylor et al., 2010). However, it suggests that the circadian rhythm component of the photoperiod pathway is active in LD and SD varieties of onion. A summary of diurnal expression patterns of *AcGI* in onion is presented in Table 6.1.

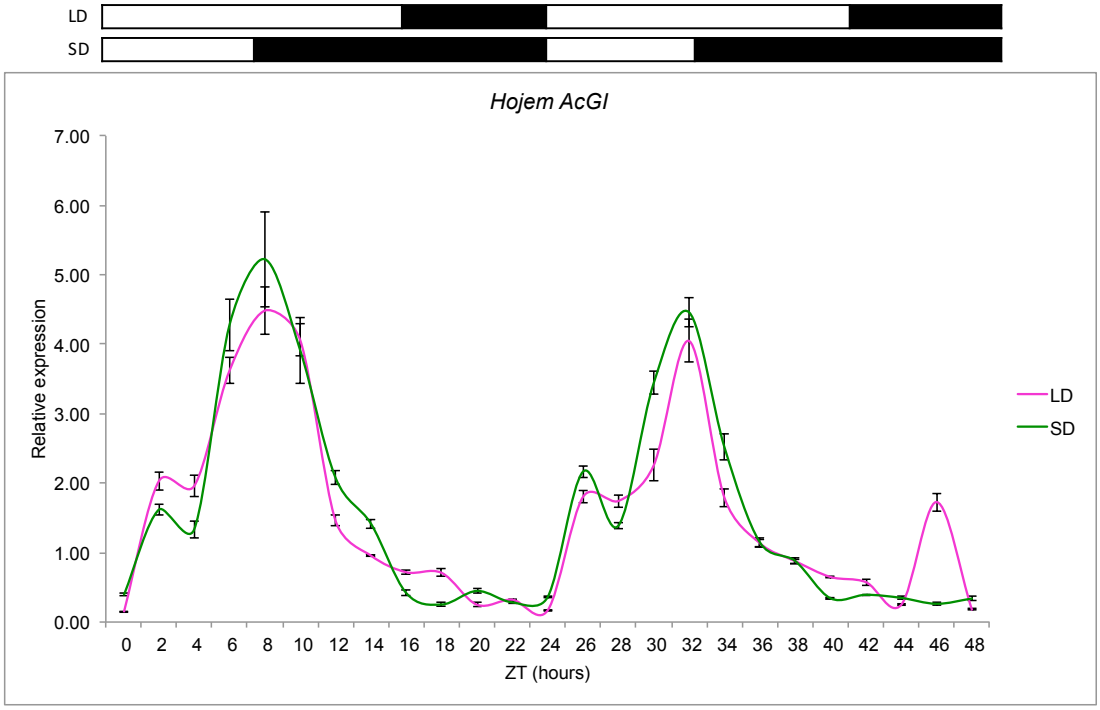


Figure 6.6 Expression of *AcGI* in *Hojem* over a 48-hour period using qRT-PCR, *PP2A1, UBL* and *PP2AA3*. *AcGI* showed clear diurnal expression pattern peaking at around ZT8 in both LD and SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

6.3.1.3 Expression of *COL* genes

6.3.1.3.1 Expression of *AcCOL1*

In *Renate F1*, *AcCOL1* showed no distinct peak or obvious trend of expression and various peaks are seen in both LD and SD (Figure 6.7). The expression pattern in the first cycle was also different from that of the second cycle under both daylength conditions, which suggests that the expression of this gene is not diurnally regulated.

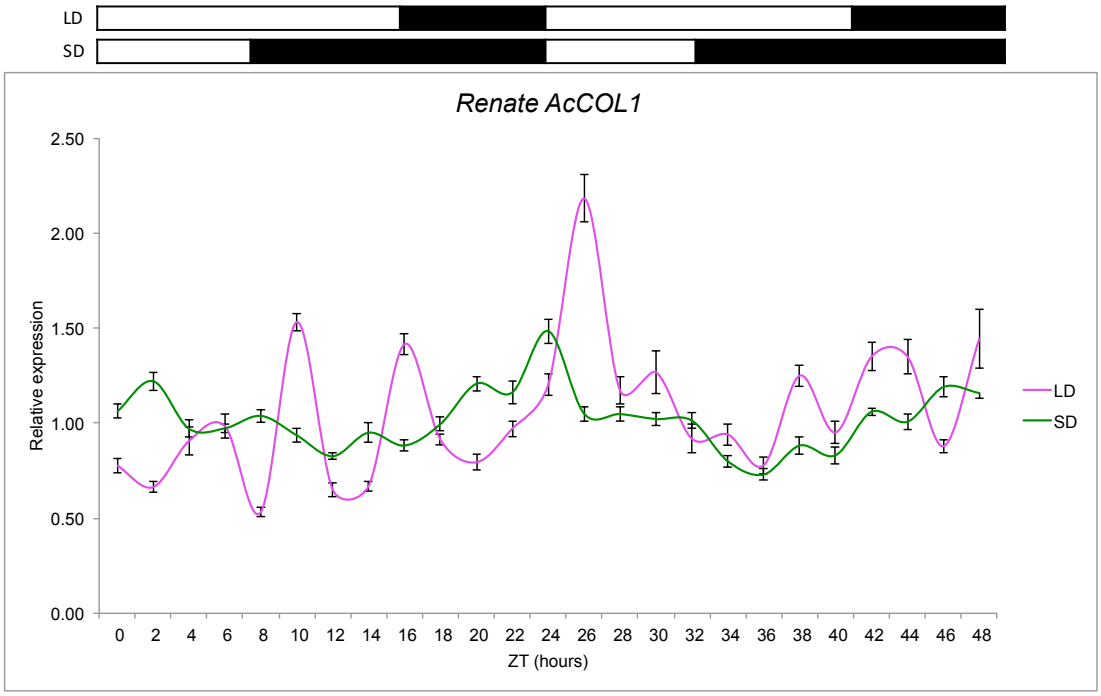


Figure 6.7 Expression of *AcCOL1* in *Renate F1* over a 48-hour period using qRT-PCR, relative to *PP2A1, TIP41* and *UBL*. *AcCOL1* showed no obvious trend and various peaks are seen in both LD and SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

In *Hojem*, *AcCOL1* showed some weak expression in very early morning at around ZT4 in both LD and SD conditions (Figure 6.8), although lots of noise was present in both the cycles. However, sequence and expression data together suggest that *AcCOL1* could be a CO-like gene but it is not fully under circadian or diurnal regulation.

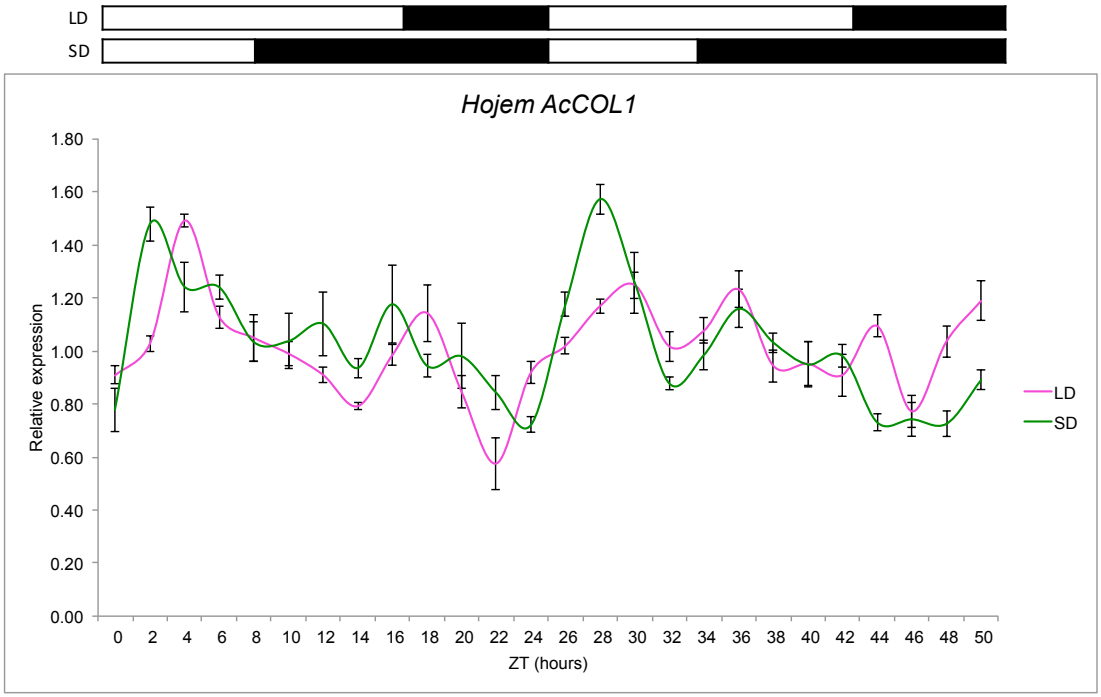


Figure 6.8 Expression of *AcCOL1* in *Hojem* over a 48-hour period using qRT-PCR, *PP2A1, UBL* and *PP2AA3*. *AcCOL1* was expressed in very early morning peaking at around ZT4 in LD and around ZT2 in SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

6.3.1.3.2 Expression of *AcCOL2*

In *Renate F1*, *AcCOL2* showed a diurnal expression pattern, peaking at around ZT10 in LD and around ZT20 in SD (Figure 6.9). The expression pattern of *AcCOL2* is quite similar to the expression of Arabidopsis *CO*, which shows clear expression peaks around ZT16 in LD and ZT20 in SD (Suarez-Lopez et al., 2001).

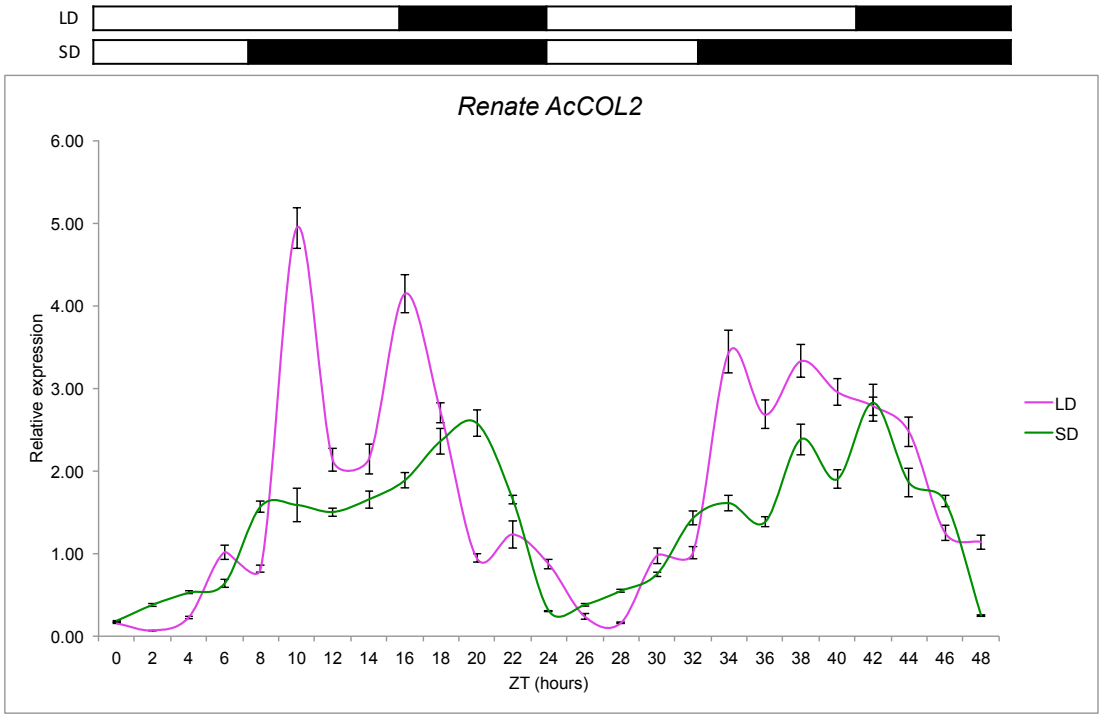


Figure 6.9 Expression of *AcCOL2* in *Renate F1* over a 48-hour period using qRT-PCR, relative to *PP2A1, TIP41* and *UBL*. *AcCOL2* showed a diurnal expression pattern peaking at around ZT10 in LD and ZT20 in SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

In *Hojem*, *AcCOL2* also showed a diurnal expression pattern peaking at around ZT12 in LD and around ZT16 in SD (Figure 6.10), though, the timing of the peaks is slightly different to that of the LD variety. However, it showed a diurnal regulation of expression in both LD and SD varieties of onion. The expression and sequence data suggest that *AcCOL2* is a CO-like gene that is under circadian regulation and which has a diurnal expression pattern consistent with a role in daylength regulation of bulb initiation.

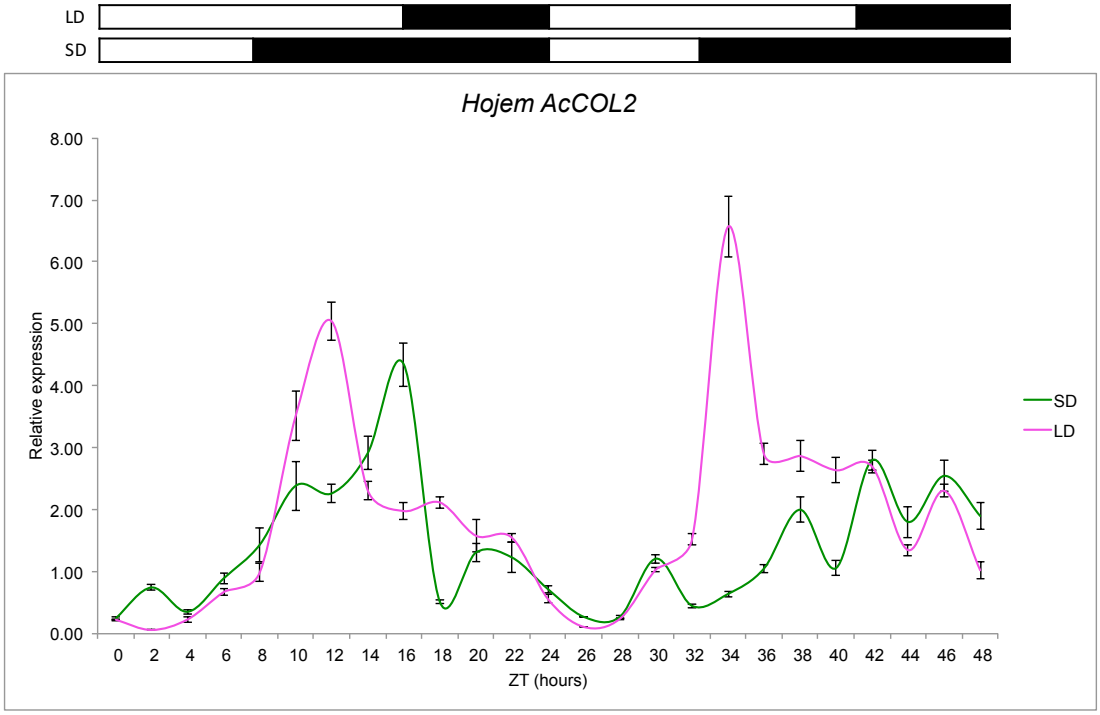


Figure 6.10 Expression of *AcCOL2* in *Hojem* over a 48-hour period using qRT-PCR, *PP2A1, UBL* and *PP2AA3*. *AcCOL2* showed a diurnal expression pattern peaking at around ZT12 in LD and ZT16 in SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

6.3.1.3.3 Expression of *AcCOL3*

The expression of *AcCOL3* showed no obvious trend and no consistent peaks of activity either between LD and SD treatments or between first and second cycles. This was similar to the expression pattern of *AcCOL1* in *Renate F1* (Figure 6.11), suggesting that the expression of this gene is also not diurnally regulated.

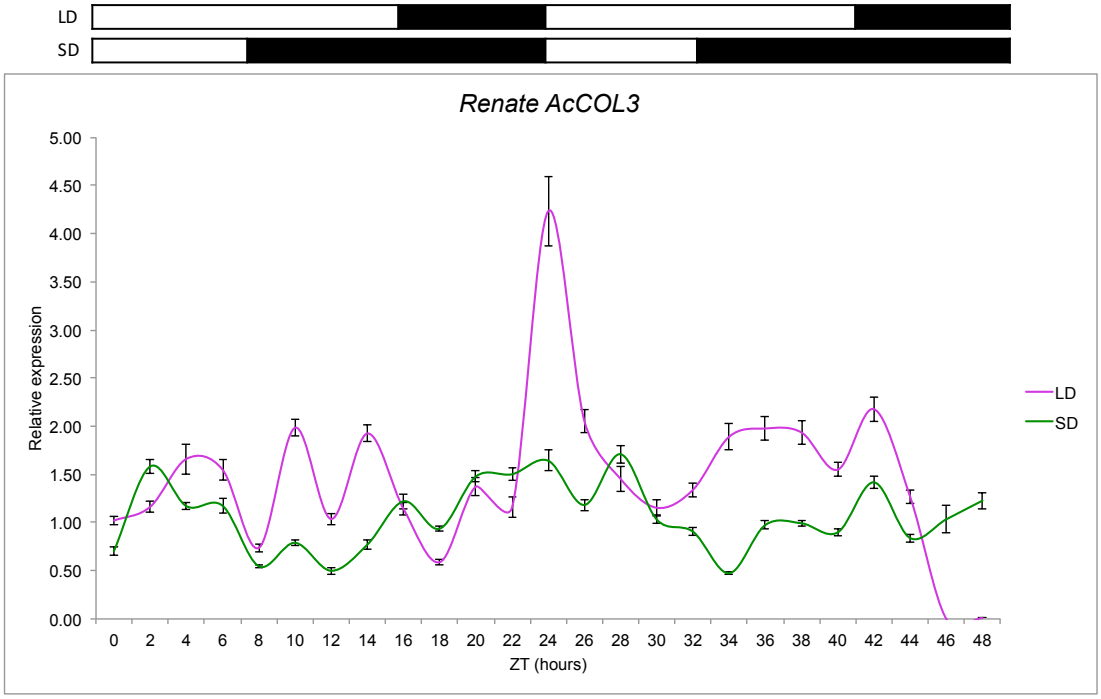


Figure 6.11 Expression of *AcCOL3* in *Renate F1* over a 48-hour period using qRT-PCR, relative to *PP2A1, TIP41* and *UBL*. *AcCOL3* showed no obvious trend and various peaks were seen in both LD and SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

In *Hojem*, *AcCOL3* did show some pattern of expression peaking at around ZT12 in LD in both first and second cycles, but there was no obvious pattern in SD (Figure 6.12). Sequence and expression data taken together indicate that *AcCOL3* could be a CO-like gene but not fully under circadian or diurnal regulation. A summary of the diurnal expression patterns of all three *COL* genes in onion is presented in Table 6.1.

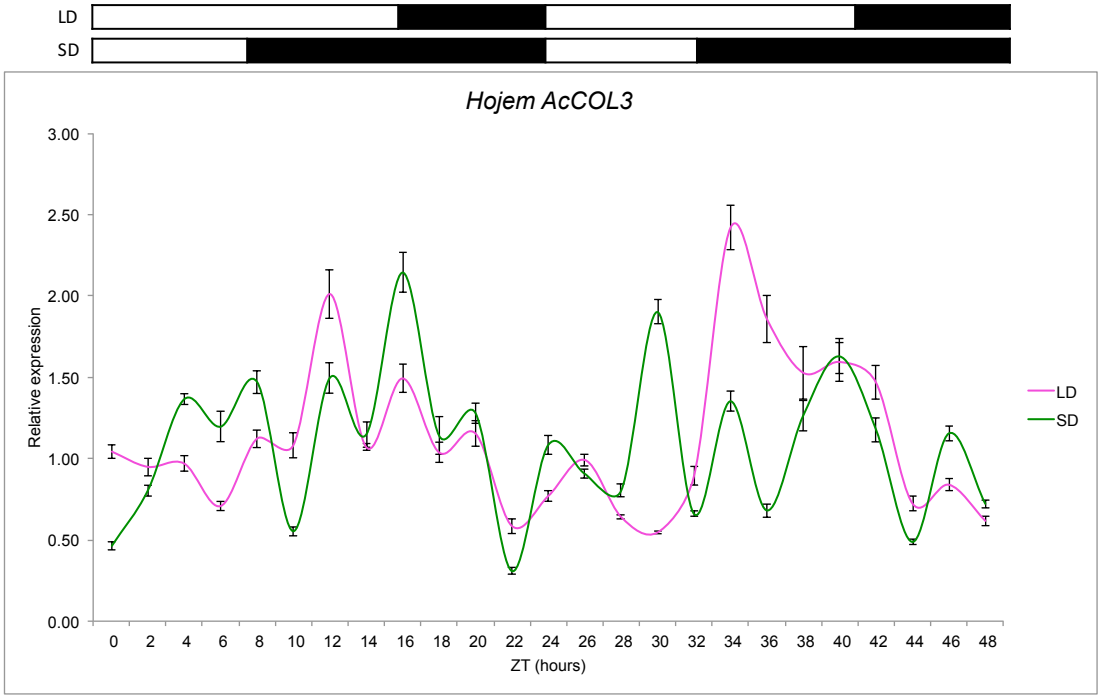


Figure 6.12 Expression of *AcCOL3* in *Hojem* over a 48-hour period using qRT-PCR, *PP2A1, UBL* and *PP2AA3*. *AcCOL3* showed some distinct pattern of expression peaking at around ZT12 in LD but no obvious pattern in SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

6.3.1.4 Expression of *FT* genes

6.3.1.4.1 Expression of *AcFT1*

In *Renate F1*, *AcFT1* showed a distict and repeatable (data not shown) diurnal pattern of expression in LD, being expressed in the later part of the day and during the dark period in both cycles. In contrast, there was no expression of *AcFT1* in SD (Figure 6.13). This pattern is very similar to the expression of Arabidopsis *FT*, which shows one distinct peak at the end of the LD and very low-level expression in SD (Kardailsky et al., 1999).

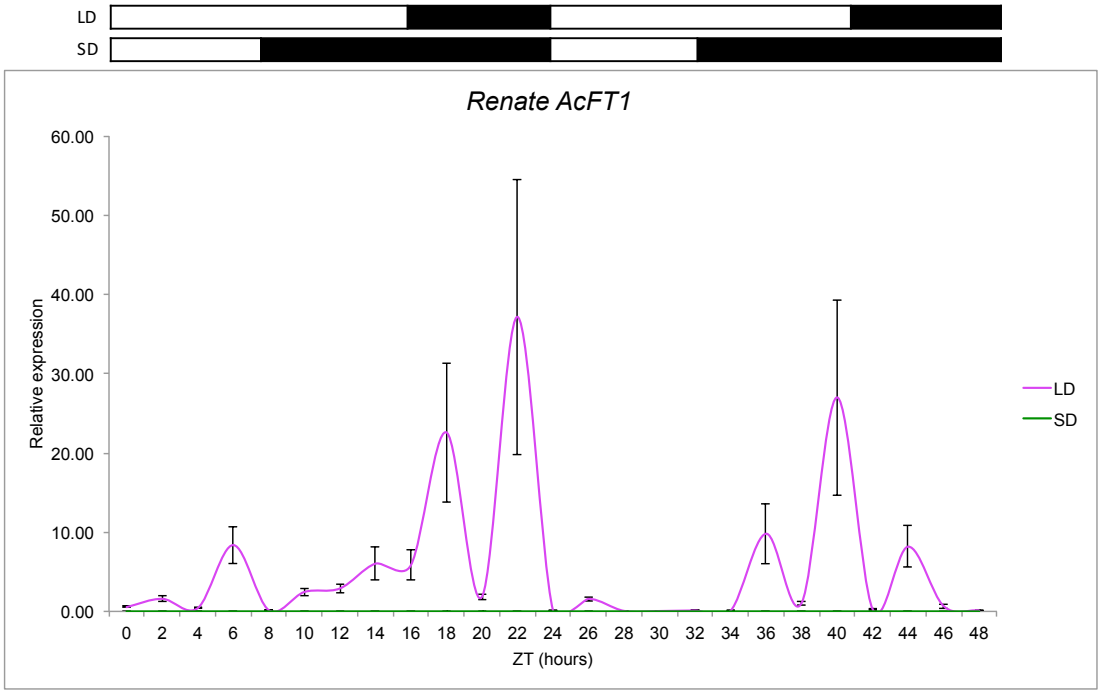


Figure 6.13 Expression of *AcFT1* in *Renate F1* over a 48-hour period using qRT-PCR, relative to *PP2A1, TIP41* and *UBL*. *AcFT1* is expressed in the later part of the day at both cycles in LD but was not expressed in SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

The pattern of expression of *AcFT1* in *Hojem* was similar to that of the expression in *Renate F1*, peaking in the later part of the day during the dark period at both cycles in LD but showing no detectable expression in SD (Figure 6.14). Although *Hojem* is a SD variety, the 8 h SD treatment is below its CDL and would not promote bulbing. This consistent pattern of expression of this gene in LD, which might induce bulbing, but not in SD, which might not induce bulbing in both daylength types of onion strongly suggests that *AcFT1* could have the equivalent role as Arabidopsis *FT* in mediating the response to daylength.

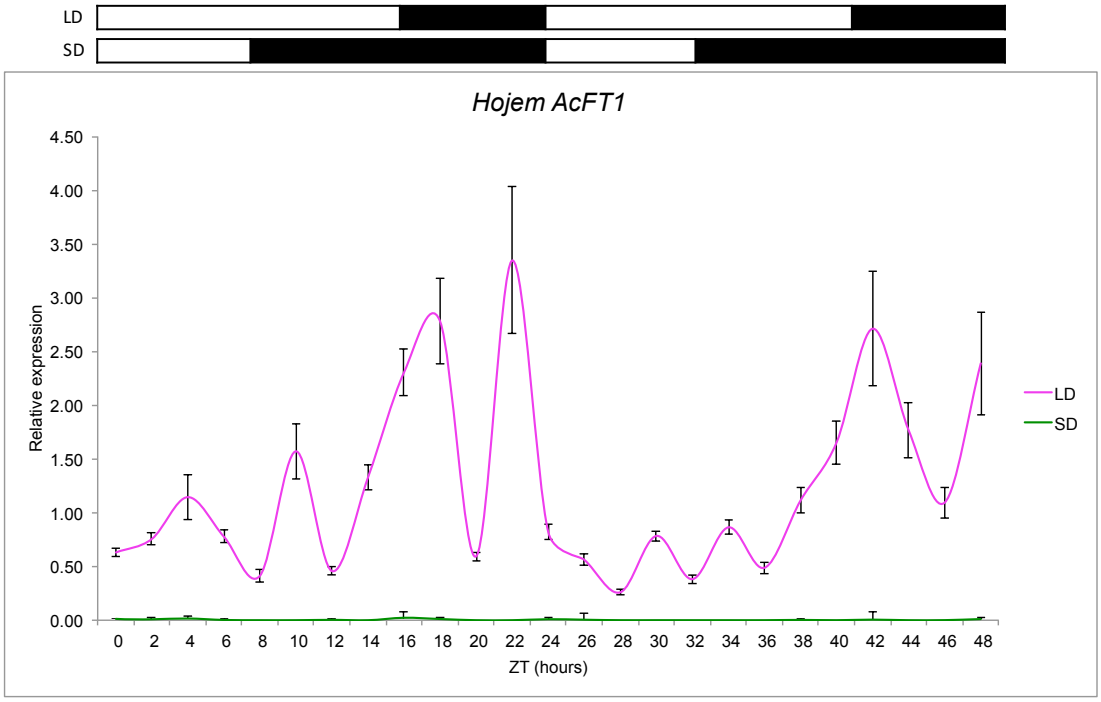


Figure 6.14 Expression of *AcFT1* in *Hojem* over a 48-hour period using qRT-PCR, *PP2A1, UBL* and *PP2AA3*. *AcFT1* is expressed in the later part of the day at both cycles in LD but showed no or very limited expression in SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

6.3.1.4.2 Expression of *AcFT2*

The expression of *AcFT2* was initially investigated in *Renate F1*, a LD onion variety but it was not expressed in either LD or SD conditions, which may not be surprising as its expression is proposed to be linked to flowering in onion (Lee et al., 2013), and vegetative material was used for this experiment.

The expression of *AcFT2* was further investigated in *Hojem*, a SD onion variety. Expression was detected in these plants with some indication of a diurnal pattern, at least in LD. It was expressed in the early part of the day peaking at about ZT2-4 during the light period in both cycles in LD but otherwise showed no obvious pattern in SD (Figure 6.15).

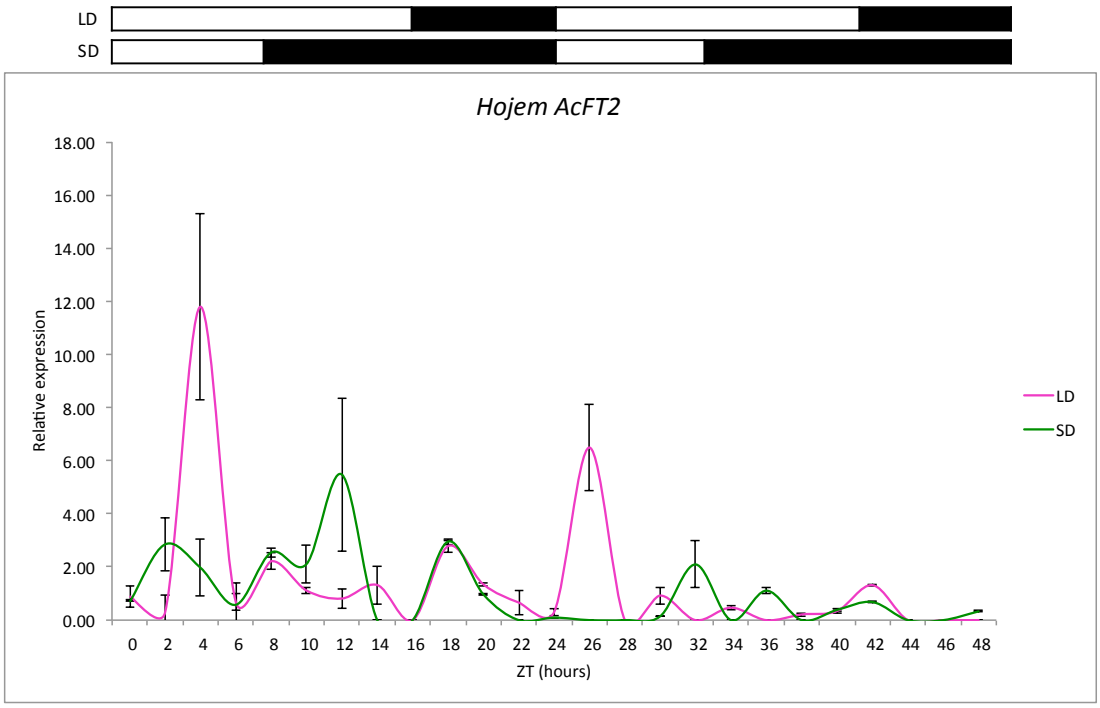


Figure 6.15 Expression of *AcFT2* in *Hojem* over a 48-hour period using qRT-PCR, *PP2A1, UBL* and *PP2AA3*. *AcFT2* was expressed in the early part of the day at both cycles in LD but no obvious pattern in SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

6.3.1.4.3 Expression of *AcFT4*

In *Renate F1*, *AcFT4* showed a clear diurnal expression pattern peaking at the end of the dark period and in the early part of the day in SD, but, in contrast, showed limited expression with no obvious trend in LD (Figure 6.16). The high expression in the early part of the day only in SD is consistent with the proposal that *AcFT4* is inhibitory for bulbing.

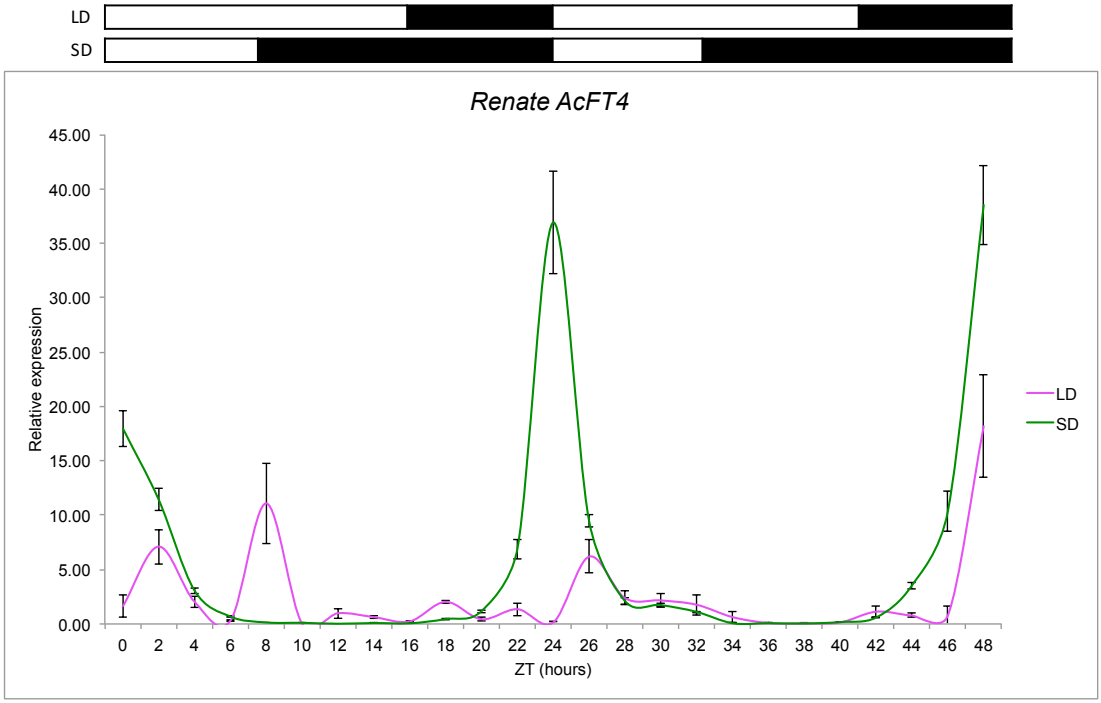


Figure 6.16 Expression of *AcFT4* in *Renate F1* over a 48-hour period, relative to *PP2A1, TIP41* and *UBL*. *AcFT4* showed a clear diurnal expression pattern peaking in the early part of the day in SD, while, showed limited expression with no obvious trend in LD. White and black bars denote light/dark cycles. Error bars represent the SEM.

In *Hojem*, *AcFT4* was expressed under both LD and SD conditions. It showed a clear diurnal expression pattern, peaking at the end of the dark period and in the early part of the day in SD, as seen in *Renate F1*. The expression in LD was higher in *Hojem* than seen in *Renate F1* but there was a less obvious pattern in LD than in SD for *Hojem* although expression tended to be higher in the early part of the day compared to the later period (Figure 6.17). However, *AcFT4* showed a consistent pattern of expression in both LD (*Renate F1*) and SD (*Hojem*) varieties of onion in SD conditions. Therefore, it was confirmed that *AcFT4* shows distinct circadian or diurnal regulation under SD conditions.

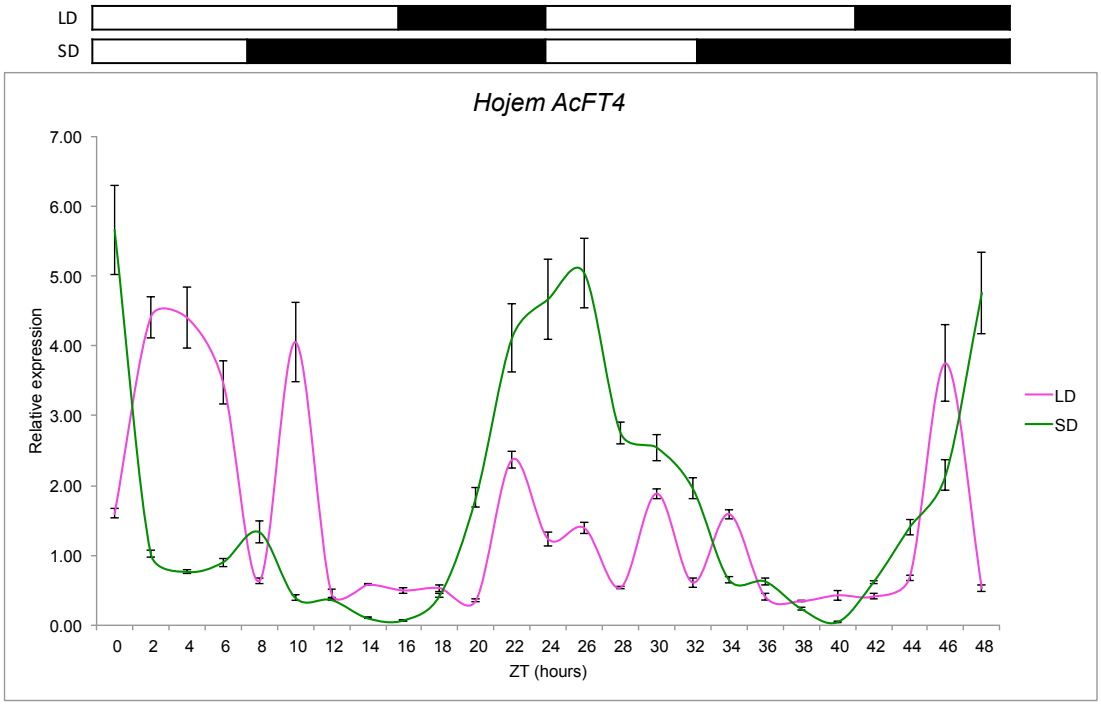


Figure 6.17 Expression of *AcFT4* in *Hojem* over a 48-hour period, *PP2A1, UBL* and *PP2AA3*. *AcFT4* showed a clear diurnal expression pattern peaking at early part of the day in SD, while, shows no obvious trend in LD. White and black bars denote light/dark cycles. Error bars represent the SEM.

6.3.1.4.4 Expression of *AcFT5*

In *Renate F1*, *AcFT5* was expressed throughout the day in LD, although the expression patterns were variable between the first and second 24 h cycles, while, showed very limited expression in SD (Figure 6.18). It was difficult to explain the variable expression patterns of *Renate F1 AcFT5* in LD, as repeating the qPCR revealed the same results. In addition to that, the same samples were used as for the other genes, including *AcFKF1* and *AcGI*, which show consistent patterns of expression in both LD and SD conditions and between first and second cycles (section 6.3.1.1 and 6.3.1.2). Therefore, while no circadian pattern of expression could be confirmed for *AcFT5* expression did seem higher in LD than in SD in *Renate F1*.

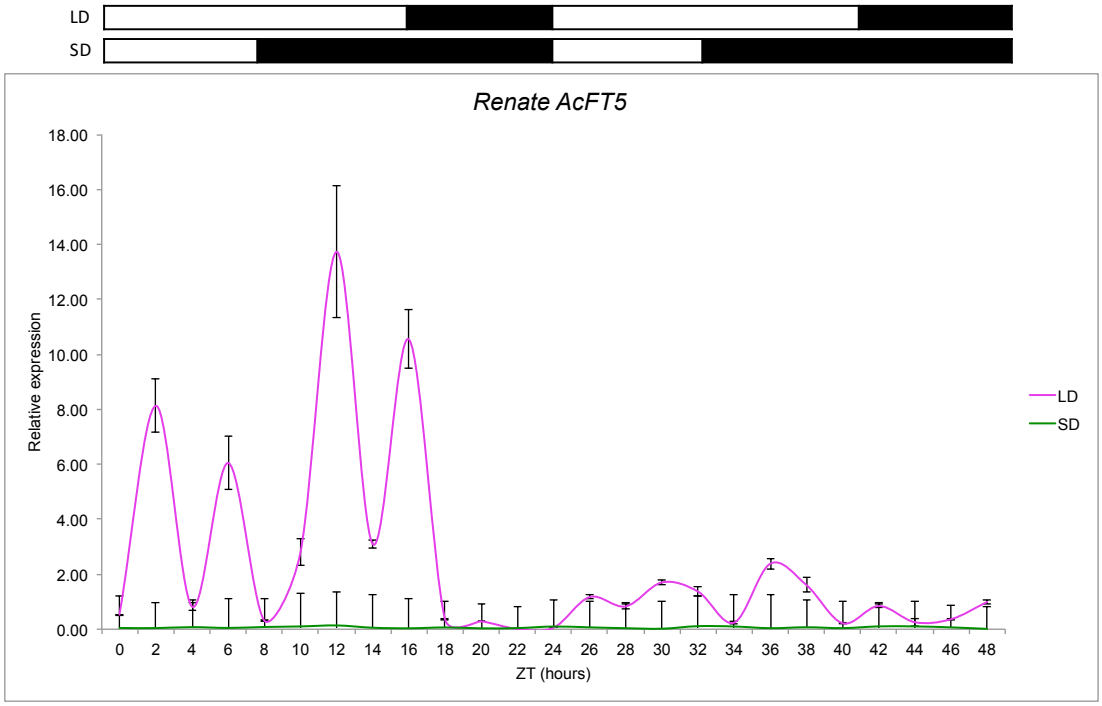


Figure 6.18 Expression of *AcFT5* in *Renate F1* over a 48-hour period, relative to *PP2A1, TIP41* and *UBL*. *AcFT5* was expressed throughout the day in LD, while showed very limited expression in SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

In *Hojem*, *AcFT5* showed a clear diurnal rhythm peaking at the middle part of the day and around ZT8 during light period in LD, while, showed no obvious diurnal expression in SD where various peaks were seen between the first and second 24 h cycles (Figure 6.19). The timing of the peaks and expression pattern in both *Renate F1* and *Hojem* suggesting that *AcFT5* might have active components present for circadian or diurnal regulation under LD conditions.

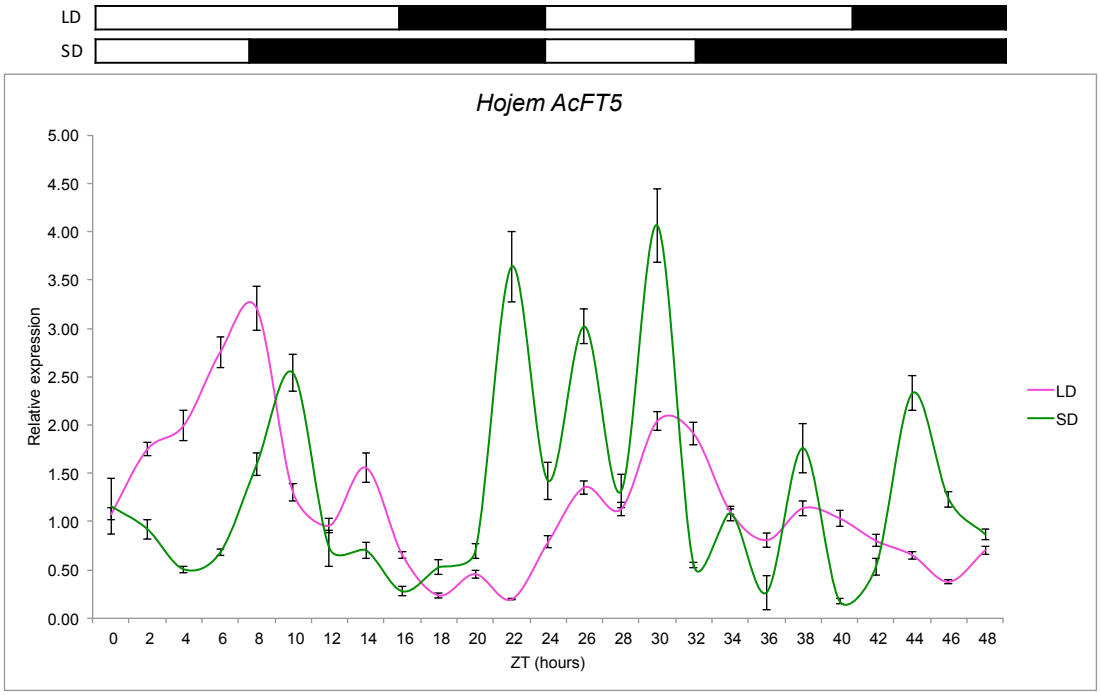


Figure 6.19 Expression of *AcFT5* in *Hojem* over a 48-hour period, *PP2A1, UBL* and *PP2AA3*. *AcFT5* showed a clear diurnal expression pattern peaking at around ZT8 in LD, while, no obvious trend in SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

6.3.1.4.5 Expression of *AcFT6*

In *Renate F1*, *AcFT6* showed a clear diurnal expression pattern peaking at the early part of the day and during the light period in both LD and SD conditions (Figure 6.20).

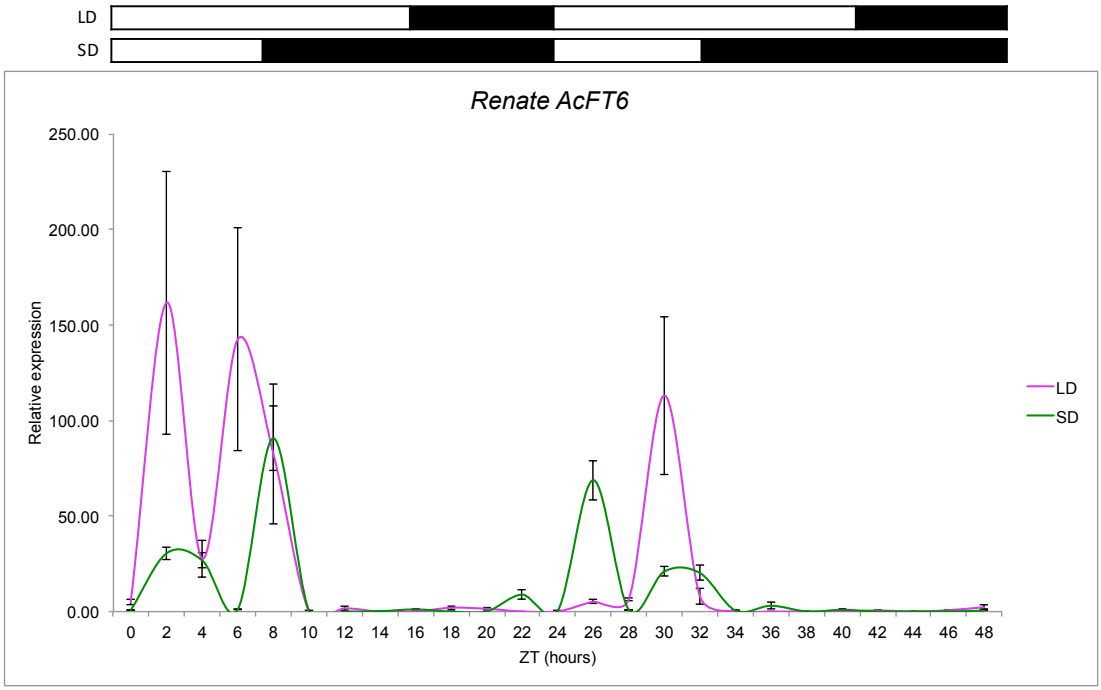


Figure 6.20 Expression of *AcFT6* in onion over a 48-hour period, relative to *PP2A1, TIP41* and *UBL*. *AcFT6* was expressed in the early part of the day in both LD and SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

In *Hojem*, *AcFT6* showed a clear diurnal expression pattern peaking at around ZT8 during light period in both LD and SD (Figure 6.21). Though, the function of this gene is still unknown. However, considering the timing of the peaks and expression pattern in both LD and SD varieties of onion, it could be confirmed that this gene might be circadian or diurnally regulated. A summary of diurnal expression patterns of all *AcFT* genes in onion is presented in Table 6.1.

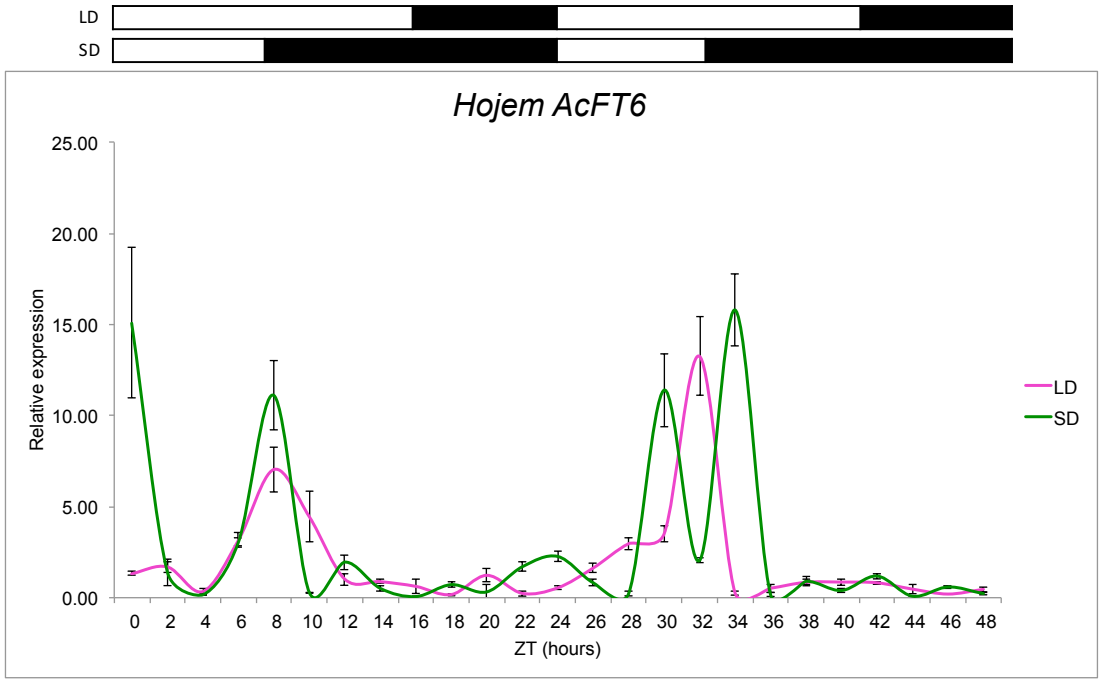


Figure 6.21 Expression of *AcFT6* in *Hojem* over a 48-hour period, *PP2A1, UBL* and *PP2AA3*. *AcFT6* showed a clear diurnal expression pattern peaking at around ZT8 during light period in both LD and SD. White and black bars denote light/dark cycles. Error bars represent the SEM.

6.4 Discussion and Conclusions

This chapter details the diurnal time-course expression of genes in onion that are linked to circadian regulation in Arabidopsis. At the physiological level, bulb initiatio in LD onion is regulated in the similar way to the photoperiodic regulation of flowring in Arabidopsis. Therefore, the hypothesis is that the the clock genes in Arabidopsis would be circadian regulated in onion.

Under both LD (*Renate F1*) and SD (*Hojem*) varieties of onion *AcFKF1* showed a clear diurnal expression patterns in LD and SD and can therefore be considered as internal controls for assessing diurnal rhythmicity for the other genes assayed in the experiment. The diurnal expression pattern of *AcFKF1* also showed no distinct difference between the timing of expression in LD and SD conditions in onion varieties under study. We were unable to repeat the small difference of timing of peaking (data not shown). This could be due to the harvesting of the samples from this experiment at 2 h intervals, whereas, they sampled at 4 h intervals (Taylor et al., 2010). However, it is clearly evident that *AcFKF1* shows a diurnal rhythm of expression, similar to that of Arabidopsis *FKF1*. Taylor et al. (2010) examined the expression of *FKF1* at various points over a 24-hour period and there was a gap of around two and a half hours with no data point between ZT 7.5 and the perceived expression peak at ZT10.

The data showed that *AcGI* has a clear diurnal expression pattern, characteristic of genes involved in the photoperiod response (Jackson, 2009). This result is also supported by earlier studies, where it was shown that in Arabidopsis, the timing of *AcGI* expression is an essential component of a circadian clock–controlled flowering pathway (Mizoguchi et al., 2005; Sawa et al., 2007).

AcCOL2 showed a diurnal expression in both LD and SD in both *Renate F1* and *Hojem*, peaking towards the end of the LD and slightly later, into darkness, in SD. This is very similar to the expression pattern of *CO*, reponsible for daylength regulation of flowering in Arabidopsis. This suggests that *AcCOL2* is a candidate for being a homologue of Arabidopsis *CO*. This could be tested by the functional complementation of onion *AcCOL2* in the Arabidopsis *CO* mutant (Taylor et al., 2010).

In contrast, *AcCOL1* showed no distinct diurnal expression in *Renate F1* and the expression pattern of this gene is not similar to the expression pattern of Arabidopsis *CO,* which shows clear expression peaks around ZT16 in LD and ZT20 in SD (Suarez-Lopez et al., 2001). This result is also consistent with the earlier study conducted in *Renate F1*, where the authors did not find a diurnal expression pattern for this gene (Taylor et al., 2010). They also found that the expression profile of *AcCOL* shows no obvious trend and various peaks in LD and SD. The expression pattern in the first day was also different from that of the second day. However, this study shows some evidence for higher expression in the morning in *Hojem* in both LD and SD conditions. This suggests that it is likely to be a CO-like gene and could still be a *CO* homologue but not the orthologue. *AcCOL3* showed similar type of expression pattern to *AcCOL1* in *Renate F1* but showed some distinct pattern of expression in *Hojem*, suggesting that *AcCOL1* and *AcCOL3* might not be under circadian or diurnal regulation. However, they could be CO-like genes regulating a member of *FT* genes (Cheng and Wang, 2005) and/or correlating with other members of *COL* and involved in bulbing and/or flowering in SD varieties of onion (Taylor et al., 2010).

The literature reports the presence of CO-like genes in SD plants such as rice and *Pharbitis nil*, which suggests a conserved pathway that regulates flowering during an inductive daylength. Also overexpression of Arabidopsis *CO* in potato, impairs tuberisation in SD inductive conditions, indicating a wider role for *CO* in daylength regulation than just the control of flowering (Martínez-García et al., 2002).

All *FTs* showed different diurnal expression patterns peaking at different times of the day. In both *Renate F1* and *Hojem*, *AcFT1* was expressed in the later part of the day during the dark period in LD but very limited or no expression in SD, which is very similar to the expression of Arabidopsis *FT* (Kardailsky et al., 1999). The previous study of Lee et al. (2013) proposed that *AcFT1* promoted bulb formation in onion and the data here are consistent with *AcFT1* being responsible for the correlation of bulbing under LD conditions. The diurnal expression pattern of *AcFT1* suggests that this gene could be a homologue of Arabidopsis *FT*, and might be positively regulated by *AcCOL2* and have an important role in the daylength regulation of bulb formation in onion (Lee et al., 2013).

AcFT2 was not expressed in *Renate F1* but was expressed in *Hojem*. Lee et al. (2013) reported that the flowering is promoted by vernalization and correlates with the upregulation of *AcFT2* and the expression of this gene was either not detected or at very low levels in seedlings and older plants before or after bulb formation. The precise timing of the peaks was not distinct, or consistent in the first cycle with that of the second cycle confirming that this gene is not fully under circadian or diurnal regulation under these non-flowering conditions. Even though *Hojem* is a SD variety and vegetative material was also used in this experiment, some evidence showed that SD varietes are more likely to flower under SD in the glasshouse conditions.

In *Renate F1* and *Hojem*, *AcFT4* showed a clear diurnal expression peaking in the early part of the day in SD, but was expressed at a lower level in LD, particularly in *Renate F1*. Lee et al. (2013) proposed that *FT4* inhibited bulb formation. The higher expression in SD is consistent with that proposal.

The expression of *FT1*, which might induce bulbing and *FT4*, which might inhibit bulb formation show evidence of negative correlation. For example, *AcFT1* is expressed in the later part of the day in LD but shows very limited, or no, expression in SD, whereas *AcFT4* is expressed at the end of the dark period and in the early part of the day in SD but has more limited expression in LD. It is therefore possible that *AcFT1* may be negatively regulating *AcFT4* or vice versa. The timing of the expression during development, as described in chapter 5, also suggests that *AcFT4* could be responsible for juvenile phase by inhibiting bulb formation at early stages of growth.

For the other two *FT* genes, *FT5* and *FT6*, there was no obvious pattern that could be easily linked to the bulbing response to daylength. In *Renate F1*, *AcFT5* was expressed throughout the day in LD but showed no or very limited expression in SD. In *Hojem*, *AcFT5* showed a clear diurnal expression pattern in LD but no obvious trend in SD. *AcFT6* showed distinct expression pattern in the early (*Renate F1*) to middle (*Hojem*) part of the day in both LD and SD, which suggsting that *AcFT6* might be circadian or diurnally regulated. However, further work is required to understand the roles of these genes.

Statistical analysis using the method reported by Smieszek et al. (2014) could be carried out to assess the significance of the differences in diurnal gene expression for time-series data. The authors proposed that progressive combinations of multiple promoter elements acting in concert may be responsible for the full range of phases observed in plant circadian output genes. Prior to application of dimensionality reduction, data could be pre-processed. The gene expression data might represent as an $n \times m$ matrix X with n genes (rows) under m conditions (columns). The pre-processing procedure (Holter et al. 2001) will involve log2 transformation, centring of the columns by subtracting the average, column normalization, centring the rows by subtracting the average and then row normalization should be applied (Smieszek et al. 2014). From the resultant data, a covariation matrix should be constructed and then independent component analysis (ICA) will be performed. The resulting gene transcriptional responses will have a mean of 0 and unit standard deviation.

The FastICA package for Matlab (http://research.ics.aalto.fi/ica/fastica/) could be used to carry out ICA on the circadian gene regulation data. As the FastICA algorithm relies on random initialisations for its maximisation and faces the problem of convergence to local optima, FastICA could iterate for 100 times and the average can be taken in order to alleviate the instability of the slightly different results in each iteration. ICA measures the interestingness of a linear combination $a^T x$ in terms of the size of its absolute kurtosis. After pre-processing and normalization, the ICA model for gene expression data can be expressed as: $X = AS$. Each column of A is associated with a specific gene expression mode. S contains the $m \times m$ gene signatures where the rows of S are statistically independent of each other. The gene profiles in X are considered to be a linear mixture of statistically independent components S combined by an unknown mixing matrix A. Once latent variable matrix A was obtained, the corresponding elementary modes could be identified to extract information for classification. Empirical significance test could also be carried out for ICA. However, due to the time limits, we were not able to carry out actual statistical analysis using the methods of Smieszek et al. (2014) which would consider as future work.

CHAPTER 7: FUNCTIONAL COMPLEMENTATION OF ONION *COL2* IN ARABIDOPSIS *CO*

7.1 Introduction

The diurnal gene expression in onion in response to daylength is described in chapter 6. It was observed that onion *AcCOL2* showed a clear diurnal expression pattern, which is very similar to the expression pattern of *CO*, reponsible for daylength regulation of flowering in Arabidopsis (Suarez-Lopez et al., 2001). This suggests that *AcCOL2* is a good candidate for being a homologue of Arabidopsis *CO* (Taylor et al., 2010). Therefore, the main objective of this study is to isolate, characterise and test the function of onion *COL2* by complementing Arabidopsis *CO*.

Genetic transformation has been successfully applied to most of the Allium species including onion, shallot, garlic and leek (Eady et al., 2003a; Eady, 2002; Eady et al., 2005; Eady et al., 2003b; Zheng et al., 2004). Genetic transformation refers to the genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous genetic material (exogenous DNA) from its surroundings through the cell membrane(s) (Joshua, 1994). Generally two major methods are used for the genetic transformation of onion namely microprojectile bombardment (Klein et al., 1987) and *Agrobacterium*-mediated transformation (Dommisse et al., 1990). The *Agrobacterium*-mediated transformation where the gene is inserted into the bacterial vector *Agrobacterium* has been most widely used (Brewster, 2008). In this genetic transformation of onion, reporters, which have herbicide or antibiotic resistance genes, are used to select the transformed cell cultures since, all producing antibiotics can survive in growth media (Eady et al., 2003a; Eady et al., 2003b). 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. Further studies also reported that transformations carried out using callus induced from mature embryos have been successfully applied in regeneration of transgenic plants (Zheng et al., 2001).

7.2 Materials and Methods

7.2.1 Plant materials

Renate F1 onion leaf material harvested from LD conditions (section 3.2.1) was used for extracting RNA and synthesising cDNA as described in section 2.2.1. Seeds of *A. thaliana* Landsberg wild type (*Ler*) as well as mutant (*co-2*) were used for complementation studies. The original source of all Arabidopsis seed was the Nottingham Arabidopsis Stock Centre (NASC).

7.2.2 Plant growth conditions

Arabidopsis seeds (both wild type and mutant) were sown into Plantpak P40 trays containing Levington F2 modular compost (Figure 7.1). Immediately after sowing, the compost containing seeds was heavily watered and the trays were covered with a plastic 634x413 mm clear autoclave bag and then with aluminum foil. To get synchronised germination of seeds, the trays were left at 4°C in fridge for about 48 h and then placed in a LD photoperiod of 16 h of light and 8 h of dark conditions at 22°C within a Phytobiology Facility compartment. After two weeks, one healthy seedling was sown in each cell into Plantpak P24 trays. At 28 d, four plants were potted up into each 9 cm pot containing the same compost as mentioned above. The inflorescences at the early stage were cut into the base a couple of times. to get more flowers on the inflorescences, which were used for dipping. Cutting helps to produce more branching in the inflorescence.

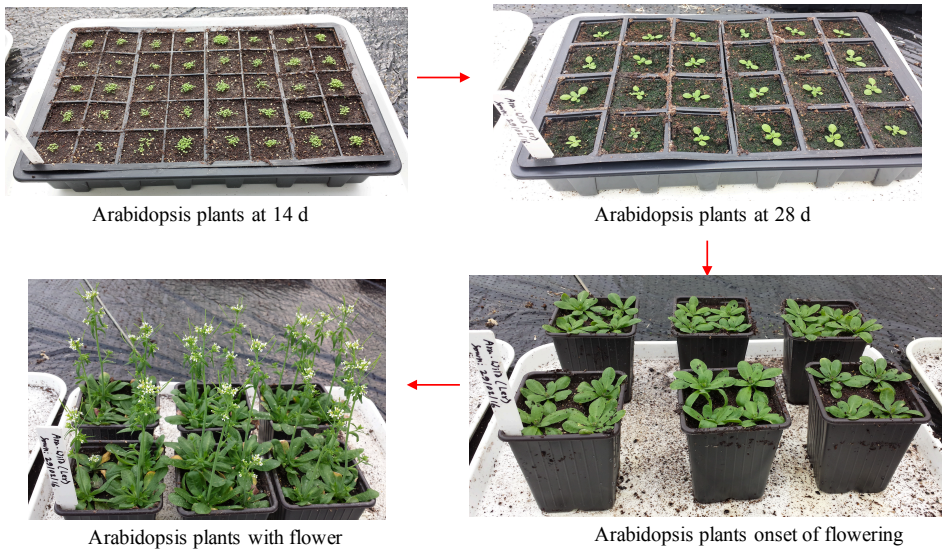


Figure 7.1 Growth of Arabidopsis wild type (*Ler*) plants under LD in Phytobiology Facility for floral dipping. The same growth steps were followed for Arabidopsis mutant (*co-2*) plants.

7.2.3 Expression of AcCOL2 using RT-PCR

The expression of *AcCOL2* in *Renate F1* leaf was examined using gradient PCR. The primers used for gradient PCR were RT-FOR and RT-REV (see Appendix XIV for sequences) with an annealing temperature ranging from 50-60 ºC, a cycle number of 39 and other conditions as mentioned in section 2.2.4. The PCR products were purified and sequenced using both forward and reverse primers to confirm the identity of the gene, as described in section 2.2.6 and 2.2.7.

7.2.4 Gateway cloning

Cloning was carried out using the cloning kit supplied by the manufacturer as described in section 2.2.8. Cloning products were generated using primers (Appendix XIV) with ATT sites of a pDONR207 vector (Invitrogen Ltd.) (Figure 7.2) specific to the Gateway® cloning system, and amplified using PCR from pGEM-T easy. Each PCR product was quantified using a NanoDrop®ND-1000 Spectrophotometer and diluted to 150 ng/µl. Two microliters of Gateway® BP Clonase® II Enzyme (Invitrogen) was mixed with 1 µl of 150 ng/µl PCR product (containing att sites), 1 µl of 150 ng/µl pDONR207 vector and 6 µl of TE buffer. The reaction mixture was then incubated overnight at 25°C. The ligation product was added to *E. coli* EC100 electrocompetent cells (Cambio Ltd) and electroporated. After electroporation, LB (tryptone:yeast extract:NaCl=10:5:10)/1.5% agar plus 25 µg/ml GENT plates were used to select for insert-containing colonies. Colonies were screened by PCR (see section 2.2.4 and 4.2.2.3 for PCR conditions) for expected inserts using M13 (For & Rev) and gene specific primers as presented in Appendix XIV. The plasmid DNA was isolated from positive clones (see section 2.2.9) and their identity was confirmed by sequencing with gene specific primers. The colonies containing the correct sequence were used with the Gateway® LR clonase® II (Invitrogen, Cat. No. 11791). 2 µl of enzyme was added to 1 µl 150 ng/µl plasmid DNA from the correct sequence carrying vector from the BP reaction, 1 µl 150 ng/µl pB2GW7 vector (Karimi et al., 2002) and 6 µl TE buffer. The reaction was then incubated overnight at 25°C. 1 µl product was added to 20 µl *E. coli* EC100 electrocompetent cells (Cambio Ltd) and electroporated. LB (tryptone:yeast extract:NaCl=10:5:1)/1.5% (w/v) agar plus 100 µg/ml SPEC plates were used to recover insert-containing colonies. Selected colonies were screened by PCR followed by plasmid DNA extraction (section 2.2.9) and positive clones were then sequenced.

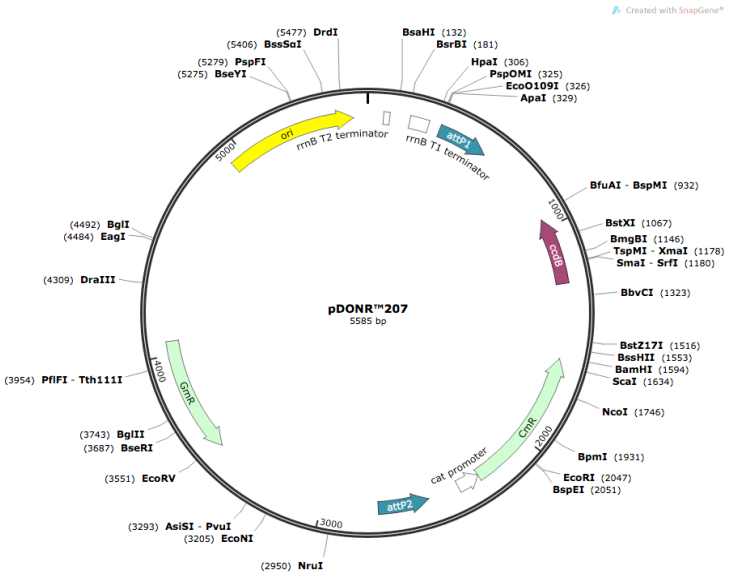


Figure 7.2 Map of the pDONR207 vector (Landy, 1989)

7.2.5 Functional complementation with AcCOL2 using Arabidopsis transformations

AcCOL2 was cloned into a pB2GW7 vector (Figure 7.3) using Gateway® LR clonase® II (Invitrogen, Cat. No. 11791) following manufacturer's guidelines. The pB2GW7 vector contains a CaMV 35S promoter and confers resistance to Glufosinate-ammonium via the *bar* gene. Using electroporation, the plasmid DNA containing cDNAs representing the CDS of *AcCOL2* was transformed into *Agrobacterium tumefaciens* strain c58pGV3101. 500 µl of SOC buffer was added and the cell culture was incubated at 28°C for 2-3 hours. After incubation, this was plated onto LB (tryptone:yeast extract:NaCl=10:5:10)/1.5% (w/v) agar plus 20 µg/ml GENT, 50 µg/ml SPEC and 50 µg/ml RIF. The plates were then incubated again at 28°C for 48 hours. The clones were checked for the correct plasmid insertion by inoculating 10 ml LB (tryptone:yeast extract:NaCl=10:5:10) plus 20 µg/ml GENT, 50 µg/ml SPEC and 50 µg/ml RIF and incubated overnight at 28°C before extracting the DNA using a Plasmid DNA Extraction kit as described in section 2.2.9. The plasmid DNA was sequenced using gene specific primers (Appendix XIV) and after getting positive clones, 500 ml LB (tryptone:yeast extract:NaCl=10:5:10) plus 20 µg/ml GENT, 50 µg/ml SPEC and 50 µg/ml RIF was inoculated using 5 ml cell culture of *A. tumefaciens* strain c58pGV3101 transformed with the plasmid. This was incubated overnight at 28°C. After incubation, the culture was centrifuged and the supernatant was removed. 500 ml 5% (w/v) sucrose solution was then used to re-suspend the cells and 100 µl Silwet L-77 added before dipping the inflorescences of

the selected plants (Figure 7.4) (Clough and Bent, 1998). The plants were sealed in a bag for 48 hours before putting them in 16 h daylight at 22°C for flowering and seed setting. When the pods became mature, T1 seeds were harvested and sown onto Arabidopsis mix soil (Levington F2s: sand: vermiculite fine grade 6:1:1). BASTA (Glufosinate-ammonium (150 g/L)) soil soaking method was used at 1:1000 as the selection of transformed seedlings. The resistant plants will flower. The first treatment was given and the trays were covered and placed at 4°C for three days. These were removed and put under a propagator lid in 16 h of light at 22°C.

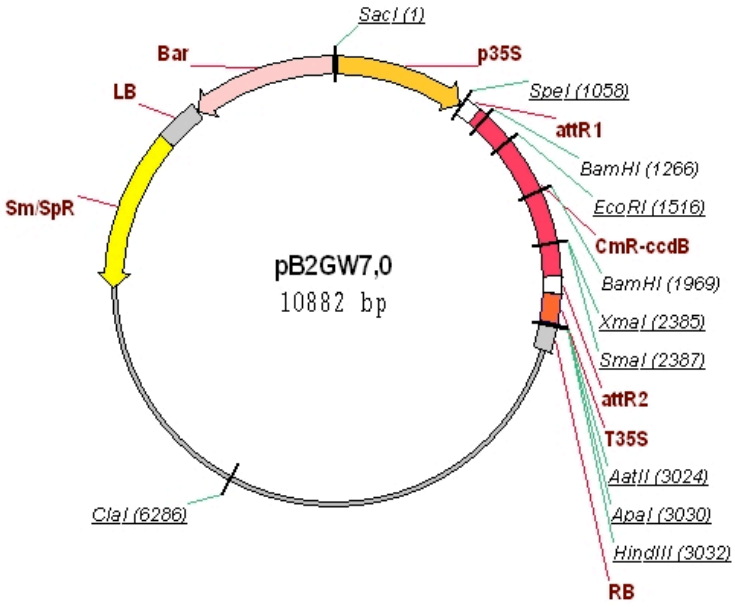
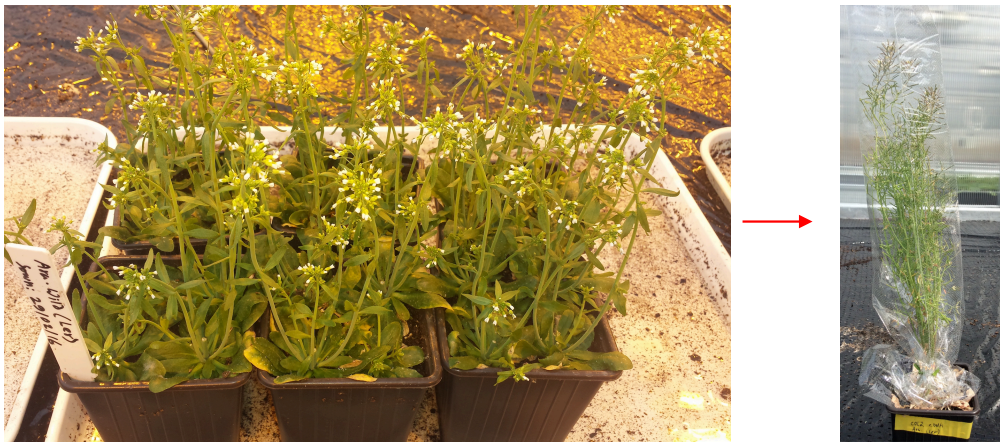


Figure 7.3 Map of the pB2GW7 transformation vector (Dubin et al. 2008)



Arabidopsis plants at the stage of floral dipping

Production of T1 seeds

Figure 7.4 Arabidopsis plants used for floral dip transformation and production of T1 seeds.

7.3 Results

7.3.1 Cloning of AcCOL2

Gradient PCR results showed that *AcCOL2* was amplified from both cDNA and genomic DNA samples under all annealing temperatures (Figure 7.5). However, 58.5 ºC was selected as the optimum annealing temperature as it showed most intensive cDNA and genomic DNA bands.

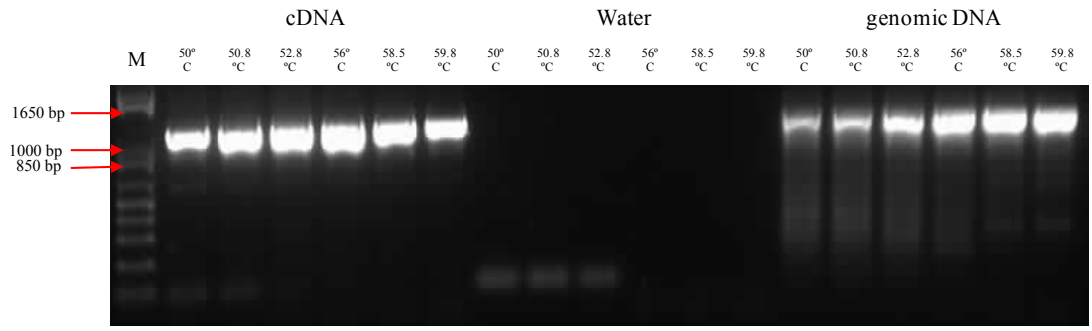


Figure 7.5 Amplification of *AcCOL2* in *Renate F1* leaf grown under LD. M= marker.

The gene was then cloned as described in section 7.2.5 to get the full-length cDNA (Figure 7.6) and sequenced. The template used for the cloning reaction was 1 µl of purified PCR product obtained under annealing temperature of 58.5 ºC. Contigs were constructed in order to obtain information on the entire gene sequences of the gene. Sequencing analysis showed that the *AcCOL2* clone obtained covered the entire coding region of this gene, which spanned 1077 bp (359 amino acids). This is quite similar to Arabidopsis *CO,* which has a coding region spans 1122 bp (374 amino acids). The sequence of *AcCOL2* in onion is presented in Appendix V. Further sequence analysis of *AcCOL2* revealed that this gene contains both a B-Box and CCT domain, which are found in all *CO* and *CO*-like genes (Robson et al., 2001; Taylor et al., 2010).

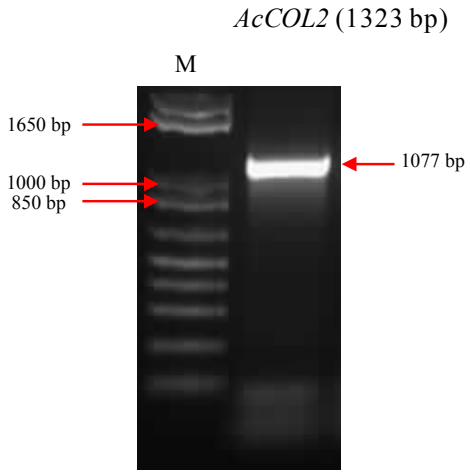


Figure 7.6 Amplification of full length *AcCOL2* cDNA. M= 1 kb Plus DNA ladder. 1077 bp product representing the full coding sequence.

7.3.2 Expression of AcCOL2 in Arabidopsis plants

Transformations were carried out in order to investigate the effect of *AcCOL2* on flowering time in Arabidopsis *co-2* mutant and *Ler* wild-type plants. This followed the method described in section 7.2.4 and 7.2.5. BASTA treatment was used for the selection of transformed seedlings. None of transformed mutant T1 plants survived the BASTA selection which could be due to the contamination with fungi or bacteria (Figure 7.7). However, due to time limits, it was not possible to repeat the BASTA treatment.

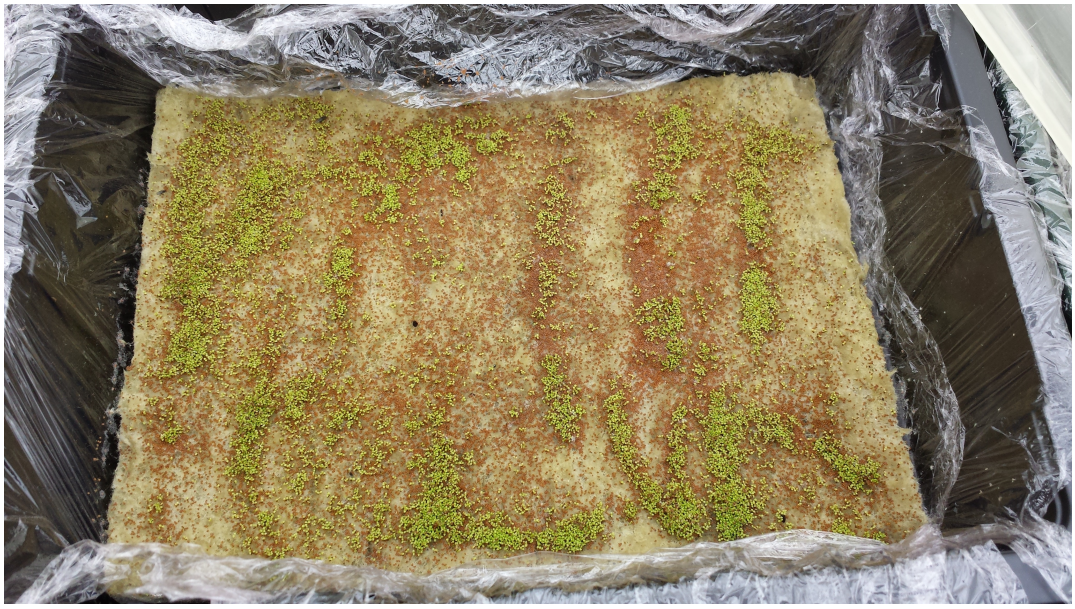


Figure 7.7 Seedlings from *A. thaliana Ler* T1 seed obtained from floral dip transformation using *A. tumefaciens* strain c58pGV3101.

7.4 Discussion

Although the cloning of *AcCOL2* and its incorporation into the transformation vector by Gateway cloning was successful, the first attempt to obtain transformants by floral dip was unsuccessful and there was insufficient time in the project to repeat the floral dip and BASTA selection. The failure could have been due to the contamination with fungi or bacteria. To counteract this, in future experiments, seed should be sterilised before treatment to reduce the contamination.

Previous studies have suggested that DNA gel blot analysis is useful for screening T1 plants, which survived the BASTA herbicide selection and demonstrated that the surviving plants are indeed transgenic (Logemann et al., 2006). In this study, BASTA selection was carried out in soil, however, it could be conducted on agar. It was found that placing of Arabidopsis T1 seeds obtained from floral dip transformation on 1% agar containing MS medium with appropriate antibiotics allows quicker identification of transformed seedlings (Harrison et al., 2006). However, due to the time limits, we were not able to redo the BASTA selection using any of those methods to get the transformed seedlings.

CHAPTER 8: GENERAL DISCUSSION AND CONCLUSIONS

8.1 General discussion

The main aim of this work was to gain a better understanding of the molecular regulation of bulbing in response to daylength and specifically to test whether the molecular regulation was similar to the regulation of flowering by daylength in Arabidopsis. To test this hypothesis, a series of physiological and molecular experiments were carried out throughout the project. The results obtained suggest that a few genes identified and isolated in onion such as *AcFT1*, *AcFT4*, *AcFT5*, *AcFT6* and *AcLFY* were potentially involved in daylength control of bulb formation. In addition, the expression patterns of some of the genes of interest namely *AcFKF1*, *AcGI*, *AcFT1* and *AcCOL2* support the hypothesis that bulb formation in response to daylength is similar to the daylength regulation of flowering. A discussion of the specific project aims in support of the main objectives of the research is presented below.

8.1.1 Daylength regulation of bulbing in onion

Bulb formation in temperate onions is daylength-dependent and LD of at least 14 h of light are required to stimulate bulb initiation (Lancaster et al., 1996; Mettananda and Fordham, 1997; Okporie and Ekpe, 2008). Therefore, the objectives of this study were to optimise the experimental conditions for daylength-dependent bulb initiation in onion by a comprehensive set of developmental time-course experiments and to generate materials for molecular analyses. In an initial time-course experiment, it was observed that onions form bulbs (measured by bulbing ratio) around 48 DFS when daylength extends beyond 14 h. The leaf and bulb materials obtained from this experiment were used for molecular analyses. Based on the timing of the bulb response, in the later experiment in *Renate F1* shown that the bulbing ratio increased in plants kept in LD (NC) or transferred to LD (CE) at the time that bulbing was just beginning, but not in those transferred to SD at that time (Section 3.3.1/Figure 3.3 and 3.4). Statistical analysis also supports this result (Section 3.3.1/Table 3.1 and Appendix I). Therefore, it could be confirmed that bulb initiation was controlled by daylength and LD conditions with fourteen to sixteen hours of light stimulating the bulbing process in *Renate F1* (Taylor, 2009).

In ID (12 h daylength) conditions, it was observed that the SD variety *Hojem* forms bulbs at around 104 DFS, while *Renate F1* did not form any bulbs even at 132 DFS (Section 3.3.3/Figure 3.6 and 3.7). Statistical analysis also supports this result (Section 3.3.3/Table 3.3 and Appendix II). This is an example of how the daylength requirement for onion bulb formation varies with the type of cultivar, ranging from 10 to 16 hours (van den Berg et al., 1997). The adaptation of onion cultivars to a certain production area depends largely on adaptation to daylength through the daylength requirement of the specific cultivar (Wiles, 1989). This CDL for bulb formation indicates the adaptability of different onions in different latitudes in the world (Thomas et al., 2006). Therefore, the daylength at a specific production area or latitude at the time of bulb initiation will influence on the selection of onion cultivars. Even though a 12 h daylength would be long enough to promote bulbing in SD varieties of onion, there was a long period of time (104 DFS) to bulb initiation in *Hojem*, compared to the time to bulbing in *Renate F1* plants grown initially in NC. This delay could be due to the reduced light integral during the early stage of plant growth in the 12 h daylength chamber compared to NC (Thomas and Vince-Prue, 1997). In addition, the slow increase of bulbing ratio in *Renate F1* under SD or delay in bulb initiation in *Hojem* at 12 h could be due the accumulation of an inhibitor of bulb formation in SD conditions or at constant 12 h.

8.1.2 Gene identification and isolation

A major objective of the project was to identify and isolate a range of key genes hypothesised to be involved in bulbing in response to daylength. A number of approaches were used, including identifying genes from EST databases, sequences from published work and through a transcriptome assembly. The study focused on the following genes –

8.1.2.1 *FKF1* and *GI*

It was observed that *Renate F1 FKF1* showed high percentage of sequence similarity with Arabidopsis *FKF1* (Table 8.1), suggesting that *AcFKF1* might be homologous to Arabidopsis *FKF1* (Nelson et al., 2000; Somers et al., 2000; Taylor et al., 2010). Homology is the existence of shared common ancestry between a pair of structures, or genes, in different taxa (Pearson, 2013). A common rule of thumb is that two sequences are homologous if they are more than 30% identical over their entire

lengths. Studies have shown that sequences that share more than 40% identity are very likely to be considered as high homology or functional similarity as judged by Enzyme Commission (E.C.) numbers (Pearson, 2013). In addition to percent identity, E-value is also very useful which reflect the evolutionary distance of the two aligned sequences, the length of the sequences, and the scoring matrix used for the alignment. The similarity scores for two sequences are always be statistically significant when E-value is <0.001 (Pearson, 2013). *Renate F1 GI* also showed high percentage of sequence similarity with Arabidopsis *GI* (Table 8.1), suggesting that *AcGI* might be homologous to Arabidopsis *GI.*

Table 8.1 Summary of the genes of interest in onion and their degree of homology to Arabidopsis gene sequences (NCBI, 2016) at the nucleotide and amino acid levels. The homology was compared over the entire region of the genes. The similarities of the sequences are statistically significant when E-value is <0.001.

Gene name	GeneBank ID for Arabidopsis	Degree of homology to Arabidopsis (%)		E-value
		Nucleotide level	Amino acid level	
AcFKF1	NM_105475.3	66.1	66.7	<0.001
AcGI	NM_102124.3	67	60.9	<0.001
AcCOL1	X94937.1	47.9	41.6	<0.001
AcCOL2	X94937.1	52.5	23.1	<0.001
AcCOL3	X94937.1	46.5	30.9	<0.001
FT-LIKE PROTEIN 1	AB027504.1	60.2	84.9	<0.001
FT-LIKE PROTEIN 2	AB027504.1	61.1	65.7	<0.001
AcFT1	AB027504.1	90.1	72.4	<0.001
AcFT2	AB027504.1	64.7	49.1	<0.001
AcFT3	AB027504.1	69.7	67.4	<0.001
AcFT4	AB027504.1	65.3	58.5	<0.001
AcFT5	AB027504.1	69.7	67.4	<0.001
AcFT6	AB027504.1	56.5	55.2	<0.001
AcLFY	KF051022.1	61	62.4	<0.001
GA3ox1	NM_101424	77.7	72.8	<0.001

8.1.2.2 *COL* genes

At the beginning of this study, only one CO-like gene (*AcCOL*) had been identified in onion (Section 4.3.1) (Taylor et al., 2010). Sequence analysis revealed that this gene contains both a B-Box and CCT domain, which are found in all *CO* and *CO*-like genes (Robson et al., 2001; Taylor et al., 2010). This suggested that *AcCOL* could be homologous to Arabidopsis *CO*. RNA-Seq analysis revealed 3 *COL* genes including two new sequences (*AcCOL2* & *AcCOL3*) and also supports the previous results. *AcCOL2* and *AcCOL3* showed high percentage of nucleotide sequences similarity with Arabidopsis *CO* (Table 8.1). Therefore, both genes might be homologous to Arabidopsis *CO*. Further analysis of *AcCOL2* and *AcCOL3* transcriptome sequences revealed that both genes contain B-Box and CCT domain regions, the conserved domains, which are present in all *CO* and *CO*-like genes.

8.1.2.3 *FT* genes

At the beginning of this study, only two FT-like genes (*FT-LIKE PROTEIN 1* and *FT-LIKE PROTEIN 2*) had been identified in onion (Section 4.3.2.6 /Figure 4.6) (Taylor, 2009) that show high percentage of nucleotide and amino acid identities with Arabidopsis sequences (Table 8.1), suggesting that these genes could be homologues of Arabidopsis *FT*. During the project, Lee et al. (2013) published a paper in which they identified 6 FT-like genes (*FT1-6*). We were able to identity 5 of them in *Renate F1*, with the exception of *FT2,* which was, however, detected in *Hojem* (Section 4.3.4/Figure 4.13). Sequencing of PCR products confirmed the identity of the *FT* genes. Further analysis revealed that *FT5* is identical to the previously identified *FT-LIKE PROTEIN 2* and *FT6* is identical to *FT-LIKE PROTEIN 1*. RNA-Seq analysis also supports those results.

8.1.2.4 *LFY* and *GA3ox1*

It was shown that *AcLFY* shows high percentage of nucleotide and amino acid similarity with Arabidopsis *LFY* (Table 8.1), suggesting that this gene might be homologous to Arabidopsis *LFY*. Another important Arabidopsis flowering pathway gene *GA3ox1* was found in onion (Section 4.3.2.5 /Figure 4.6). It was shown that *Renate F1 GA3ox1* showed high percentage of amino acid similarity with Arabidopsis *GA3ox1* (Table 8.1), suggesting that this gene might be homologous to Arabidopsis *GA3ox1*.

8.1.3 Spatial and temporal gene expression in onion in response to daylength

Onion is a biennial plant, where bulb formation, being an overwintering stage (Brewster, 2008) occurs in the first year and flowering occurs following a period of vernalisation (Brewster, 1997) in the second year (Chapter 1/Figure 1.4). Therefore, a question arises of which genes are involved in the photoperiodic flowering pathway and which are involved in onion bulbing (Taylor, 2009). It was clearly observed that onions initiate bulbing under inductive daylengths when flowering is inhibited (Chapter 3), suggesting that bulb formation and not floral initiation is the daylength response (Brewster, 2008; Lancaster et al., 1996; Taylor et al., 2010). Previous reports also revealed that *FT* is a target of *CO* (Suarez-Lopez et al., 2001) and has been shown to be the major component of the floral signal molecule, florigen and thus can induce flowering by long-distance transportation to the apical meristem with the help of other floral homeotic genes like *LFY* (Andres and Coupland, 2012; Corbesier et al., 2007; Purwestri et al., 2009; Thomas et al., 2006). At the physiological level, bulb initiation in onion is very similar to the floral initiation in Arabidopsis (Thomas et al., 2006). Thus, FT, which is the mobile signal controlling flowering in Arabidopsis (Corbesier et al., 2007; Jaeger and Wigge, 2007) would control bulb initiation in onion (Taylor, 2009). Therefore, the experimental hypothesis is that, if *FT* genes regulate bulbing, their expression should be correlated with bulb formation under a range of conditions and in different response types. For genes involved in daylength sensing, they should be related to the bulbing response e.g. present in the sites of daylength perception, but independent of the bulbing process. To test this hypothesis, this study focused on the quantitative gene expression analysis in different response types of onion under a range of bulbing and non-bulbing conditions.

8.1.3.1 Clock genes

In Arabidopsis, the circadian clock regulates *FKF1* and *GI* genes, which can mediate the *CO* stability regulation for the precise control of flowering time (Song et al., 2014). Therefore, we should expect them to be expressed in the green part of the leaf, which will be the site of perception (Heath and Holdsworth, 1948). In addition, the expression of *AcFKF1* and *AcGI* genes would not necessarily be correlated with bulb initiation and development, however, they should show a diurnal rhythm of expression.

In *Renate F1*, expression of *AcFKF1* and *AcGI* was detectable all throughout the leaf from the site of perception (green part) to the site of response (basal tissue) under both LD and SD conditions (Chapter 5/Figure 5.32 and 5.33), suggesting their expression is daylength insensitive and is not tissue-specific (Taylor et al., 2010). This is not surprising as clock genes are expected to be expressed everywhere to be able to detect and mediate the response to natural light/ dark cycles (Redei, 1962). Reports show that *GI* is highly conserved in seed plants, including monocotyledons (Dunford et al., 2005; Hayama et al., 2003; Xiang et al., 2005) as well as dicotyledons (Curtis et al., 2002; Paltiel et al., 2006) and gymnosperms (Hayama et al., 2002; Mizoguchi et al., 2005). In addition, both *AcFKF1* and *AcGI* show a clear diurnal expression pattern peaking at ZT8 under both LD and SD in both varieties of onion (Chapter 6/ section 6.3.1.1 & 6.3.1.2). Therefore, *AcFKF1* and *AcGI* genes provide good internal controls for assessing diurnal rhythmicity for the other genes assayed in the experiment. The distinct peak timing clearly suggested that these genes are diurnally regulated in onion. This is consistent with previous studies in Arabidopsis, where, the authors found that *AcFKF1* interacts with *AcGI* through the LOV domain to form a complex in a blue-light dependent manner in the late afternoon and regulates the expression of *CO* and induction of flowering specifically under LD conditions (Mizoguchi et al., 2005; Sawa et al., 2007). In the LD conditions, sufficient *FKF1-GI* complex is formed to activate *CO* transcription during the daytime, and which is stabilized by light at the end of the day. However, the consistent diurnal patterns are different from the previous results of Taylor et al. (2010), because we were unable to repeat the small difference of timing of peaking with them (data not shown). This could be due to the harvesting of the samples from this experiment at 2 h intervals, whereas, they sampled at 4 h intervals (Taylor et al., 2010). The authors examined the expression of *FKF1* and *GI* at various points over a 24-hour period and there was a gap of around two and a half hours with no data point between ZT 7.5 and the perceived expression peak at ZT10. Statistical analysis using the method reported by Smieszek et al. (2014) could be carried out to assess the significance of the differences in diurnal gene expression for time-series data.

8.1.3.2 *CO* and *COL* genes

In Arabidopsis, *CO* is a direct output from the clock and functions at the site of perception in leaf (Thomas et al., 2006). It plays a central role in the mechanism of

photoperiod measurement, integrating clock and light signals to provide photoperiod-specific induction of the mobile floral integrator, *FT* and thus controls flowering in Arabidopsis (Andres and Coupland, 2012; Song et al., 2013; Thomas, 2006). Therefore, the experimental hypothesis is that, the *COL* genes might be involved in onion bulb initiation and development but their spatial and developmental expression pattern will not necessarily be changed in either LD or SD conditions. They will be expressed in the site of perception (leaf) and the right *COL* gene involved in daylength response will be diurnally regulated.

All three *COL* genes identified in onion were present in all types of daylengths irrespective of varieties (Chapter 5/section 5.3.2.1 & 5.3.3.1). In addition, all three *COL* genes were strongly expressed in the green part of *Renate F1* leaf, which is the site of perception (Chapter 5/section 5.3.4.3) but in addition, *AcCOL2* and *AcCOL3* showed increasing expression in both LD and SD conditions from the base to apex. *AcCOL1* and *AcCOL3* showed no distinct diurnal expression in both LD and SD in *Renate F1*, although they show some noisy peaks in *Hojem* especially under LD (Chapter 6/section 6.3.1.3.1 & 6.3.1.3.3). This suggests that they are likely to be a CO-like gene and could still be a *CO* homologue but not the orthologue. However, we would not consider them as fully circadian regulated.

In contrast, *AcCOL2* showed a clear diurnal expression pattern in response to daylength and onion varieties (Section 6.3.1.3.2), which is a pattern of expression consistent with a role in daylength perception for bulbing. This is very similar to the expression pattern of Arabidopsis *CO*. Therefore, it could be confirmed that *AcCOL2* is diurnally regulated and would be a good candidate for being a homologue of Arabidopsis *CO*. The timing of the peak and expression pattern suggests that *AcCOL2* might regulate one or more of the *FT* genes (Cheng and Wang, 2005; Taylor et al., 2010). We have demonstrated three *COL* genes in onion, where the previous one (*AcCOL1*) does not have the property expected for *CO* involved in daylength sensing. *AcCOL2*, a new sequence identified from the transcriptomic assembly is a good candidate, has the right sequence elements and appropriate spatial and diurnal pattern of expression. This hypothesis could be further tested by the functional complementation of onion *AcCOL2* in the Arabidopsis *CO* mutant. Preliminary work towards the complementation carried out during the PhD study in

order to investigate the effect of *AcCOL2* on flowering time in Arabidopsis *co-2* mutant and *Ler* wild-type plants. Although the cloning of *AcCOL2* and its incorporation into the transformation vector by Gateway cloning was successful, the first attempt to obtain transformants by floral dip was unsuccessful and there was insufficient time in the project to repeat the floral dip and BASTA selection.

8.1.3.3 *FT* genes

FT genes have been shown to act as long distance signals mediating resposnses to daylength in a range of species. In *Renate F1*, we were able to confirm the presence of six sequences as published by Lee et al. (2013). They proposed that *FT1* promoted bulb formation, whereas *FT4* inhibited it. We tested this hypothesis by looking at *FT* gene expression in a range of conditions. If *FT1* was correlated bulbing in response to daylength, we would expect that it would be expressed at higher levels in LD than SD in the site of perception, and show a diurnal pattern of expression similar to *FT* in Arabidopsis. The data we obtained by quantitative PCR was consistent with this prediction. Statistical analysis also supports these observations (Table A4 and A5).

During development, *FT1* was expressed in LD at all stages, including the early stages, prior to the onset of bulbing (Chapter 5/Figure 5.16). However, transfer of plants to SD when bulbing initiated resulted in expression being repressed. The difference in expression between LD and SD was confirmed in the diurnal expression experiments (Chapter 6/section 6.3.1.4.1). Some expression of *FT1* was detected in the transition zone in SD (Section 5.3.4.4.1/Figure 5.37). *FT1* is proposed to travel from the leaf to the apex as part of the supply of assimilates from photosynthesising, .i.e. exporting tissues. It is likely that the transition tissue acts as a sink and does not supply assimilates to the apex. *FT1* expressed in these tissues may thus be inactive for bulbing. *FT1* was also expressed in *Hojem* at the time it began to bulb in ID, but showed no appreciable expression in *Renate F1* (Chapter 5/Figure 5.25), which did not bulb under those conditions. Overall, the expression characteristics are consistent with *FT1* correlating bulbing in onions (Lee et al., 2013).

Lee et al. (2013) proposed that *AcFT4* inhibited the bulbing process. The data shown in this thesis, is overall, in support of that idea. When *Renate F1* plants were

transferred from LD to SD during development, *FT4* expression was upregulated at the same time that bulbing was inhibited (Chapter 5/Figure 5.17). This differential expression, showing upregulation in SD and repression in LD was confirmed in the diurnal and spatial expression experiments (Kardailsky et al., 1999). One new and interesting observation was that unlike *FT1*, *FT4* is expressed at the end of the night and in the early part of the day (Chapter 6/Figure 6.16 & 6.17). The expression pattern in *Hojem* differed from that in *Renate F1* in that *AcFT4* was expressed during early development in ID, and decreased during bulbing (Chapter 5/Figure 5.27), which may indicate that the function of this gene has diverged in different daylength types. Thus in *Hojem*, it might be involved in maintaining the juvenile phase, whereas in *Renate F1*, it is not expressed in early development. Therefore, the spatial and temporal expression patterns of *FT1* and *FT4* genes in two different onion cultivars in response to daylengths suggest that these genes might be negatively co-ordinated with each other. Lee et al. (2013) proposed that two antagonistic FT-like genes regulate bulb initiation, where *AcFT1* promotes bulb formation, while *AcFT4* prevents upregulation of *AcFT1* and inhibits bulbing in transgenic onions. These results could also be supported by the previous studies in sugar beet, where a similar regulatory pathway has evolved and was found that two *FT* genes with opposite expression profiles functions antagonistically for control of flowering (Pin et al., 2010; Pin and Nilsson, 2012). The negative co-ordination of *FT* with *DORMANCY ASSOCIATED MADS-BOX* (*DAM*) was observed in leafy spurge (*Euphorbia esula* L.), where it was found that DAM proteins potentially controls dormancy transition and maintenance by negatively regulating the expression of *FT* (Hao et al., 2015). *FT* also shows further inhibitory role in plants, where it controls seed dormancy through the inhibition of proanthocyanidin synthesis in fruits and thus altered the seed coat tannin content (Chen et al., 2014).

The expression of *AcFT2* in *Hojem* at 12 h showed a pattern of expression similar to that of the *AcFT1* (Chapter 5/section 5.3.3.2.2). Therefore, it could be speculated that *AcFT2* might induce bulb formation in *Hojem* but not in *Renate F1* under ID. Although, there is no evidence that *AcFT2* has a role in bulb formation in onion. *AcFT2* a flower promoting gene in onion (Lee et al., 2013) was also expressed in *Hojem* peaking in the early part of the day in LD but showed no obvious expression in SD (Chapter 6/section 6.3.1.4.2). Therefore, we would not consider it fully under

circadian regulation, although it could have an effect on *Hojem* bulb formation in LD. This could be supported by the previous report, which mentioned that *FT* is involved in the SD induction of tuberisation in potato (Navarro et al., 2011).

AcFT5 and AcFT6 showed different types of expression pattern from those of the other *FTs* and were mostly expressed under LD in *Renate F1* (Chapter 5/sections 5.3.3.2.4 & 5.3.3.2.5), however, they were expressed throughout the development in both *Hojem* and *Renate F1* under ID conditions (Chapter 5/Figure 5.28). Thus suggesting that these genes may not directly relate to bulbing but may be in other ways. Furthermore, *AcFT5* and *AcFT6* expression was present everywhere in *Renate F1* leaf, which is the site of perception, although they are also strongly expressed in LD in the basal tissue, which would be the site of response. *AcFT5* did not show any obvious pattern in *Renate F1* or *Hojem* (Chapter 6/Figure 6.18 & 6.19) that could easily be linked to the bulbing response to daylength. *AcFT6* showed a distinct expression in terms of daylengths and varieties (Chapter 6/Figure 6.20 & 6.21) from which we would consider it as diurnally regulated. However, further work is required to understand the roles of these genes.

8.1.3.4 *LFY* and *GA3ox1*

In this study, our hypothesis was that if *LFY* or *GA3ox1* were involved in bulb formation in response to daylength it would be reflected in their patterns of expression. In *Renate F1*, the strong presence of *AcLFY* in bulb tissue under LD at the early stage of plant growth (Chapter 5/Figure 5.20) suggested that this gene causes the plant to be less sensitive to environmental signals at this time (Weigel and Nilsson, 1995). This result is also supported by its bulb-specific expression pattern in *Renate F1* in the spatial distribution experiment (Chapter 5/Figure 5.41). In addition, the strong expression of *AcLFY* in *Hojem* leaf tissue under ID throughout development (Chapter 5/Figure 5.30) suggests that it might play a significant role in bulb development irrespective of onion varieties. This is supported by previous studies that reported *LFY* is expressed widely in both vegetative and reproductive tissues in a range of higher plants, and plays an important role in promoting flower formation by mutual interaction and coordination in a complex network with other genes such as *TFL, AP1, AP2, FT, AP3, CO,* and *GA1* (Siriwardana and Lamb, 2012; Wang et al., 2004).

GA3ox1, on the other hand was expressed in *Renate F1* under all three daylengths as well as all throughout the development in both *Hojem* and *Renate F1* at 12 h (Chapter 5/section 5.3.2.4 & 5.3.3.4), suggesting that this is daylength insensitive irrespective of onion cultivars and might not directly be involved in onion bulb formation. Gibberellin was successful to promote flowering in Arabidopsis (*A. thaliana*) through the activation of the promoter of the floral integrator gene *LFY* (Blazquez et al., 1998) and is also involved in onion flowering (Rabinowitch, 1990). However, gibberellins could affect bulb formation by acting as a promoter or inhibitor. Previous studies shows that an inhibitor of gibberellin biosynthesis promotes bulbing in non-inductive photoperiods which suggests an inhibitory role in bulb initiation (Mita and Shibaoka, 1984; Rabinowitch, 1990). Therefore, it can be speculated that there is a crossover between the genetic control of flowering and bulbing in onion. Although the role of GA has not been fully characterised in onion, it was clearly shown that *GA3ox1* was strongly expressed in the green part of the leaf (Chapter 5/section 5.3.4.6), which is the site of perception. It may be that there are other members of the gene family in onion, one or more of which might be related to the bulb response. This could be supported by previous studies, where four *GA3ox* genes were identified in Arabidopsis, each of which exhibits a unique organ-specific expression pattern; suggesting individual *AtGA3ox* member played a distinct developmental roles (Williams et al., 1998; Yamaguchi et al., 1998).

8.2 Recommendations for future work

Firstly, it would be useful to look at diurnal expression in a range of daylengths in *Renate F1* and *Hojem* to understand the relative contributions of the whole range of *COL* and *FT* genes in mediating the daylength response.

Bioinformatic analyses could be carried out using transcriptome data to provide more bulb-specific genes, such as those for quality traits such as carbohydrates (e.g., *TREHALOSE-PHOSPHATE PHOSPHATASE*) and organosulphur content (e.g., *LACHRYMATORY FACTOR SYNTHASE*).

This is also the first report on the diurnal and spatial expression patterns of the *COL* and *FT* genes that are potentially involved in the onion bulb formation process. Spatial and temporal expression experiment could also be conducted using quality-related genes to better understanding their functions in onion.

Further bioinformatic analyses by blasting Arabidopsis *FLC* homologues against onion transcriptome sequences might help to find this gene in onion. As *FLC* functions in the vernalisation pathway, blocking the function or knocking out this gene would help onion plants to flower annually, which could be useful in breeding lines.

It would be useful to sample younger materials from *Hojem* to better understand the function of *AcFT4* in relation to juvenility.

Transformation and complementation studies for onion *COL2*, *FT1*, *FT4*, *FT5*, *FT6* and *LFY* genes using respective Arabidopsis mutants would help to functionally characterise those genes including their potential interactions and/or correlations between them.

Molecular interaction studies should be carried out, possibly using Chip-seq or a Yeast Two-Hybrid system (Gietz et al., 1997) for different *COL* and *FT* genes such as *FT5* and *FT6*. While we know the *FT* interactor for flowering, what is the equivalent for bulbing. This would also help to know whether a particular *COL* gene activates transcription of different *FT* genes.

8.3 Conclusions

Day length dependent bulb initiation and development were characterised in LD and SD varieties of onion namely *Renate F1* and *Hojem*, respectively. The results obtained from this study suggested that the model for bulb formation in onion in response to daylength is similar to the photoperiodic regulation of flowering in Arabidopsis. It was also clearly observed that immediately after bulb formation, bulbs grow more rapidly in older plants than younger ones. RNA-Seq analysis identified a large number of differentially expressed transcripts on a day length and tissue basis. Five *FT* and three *COL* genes were identified including two novel *COL* sequences. A new CO-like gene namely *AcCOL2* identified in onion shows a pattern of expression consistent with Arabidopsis *CO*. Therefore, it could be concluded that *AcCOL2* might be homologous to Arabidopsis *CO*. However, the expression of previously identified *COL* genes viz. *AcCOL1* and another new *COL* gene, *AcCOL3* is not consistent with that model (Taylor et al., 2010).

In both *Renate F1* and *Hojem, AcFT1* might induce bulb formation, while *AcFT4* might inhibit bulb formation, possibly by negatively correlating *AcFT1* (Lee et al., 2013). *AcFT2* might also induce bulb formation and development in *Hojem* under ID conditions, although no previous evidence has been found for its bulb induction role. *AcFT5* and *AcFT6* have unclear roles and might be involved in the daylength regulation of bulbing in onion. Statistical analysis using two-way ANOVA also supports these results. However, further work is needed to confirm the function of these genes. *AcLFY* might be involved in the bulb development in onion; however, *GA3ox1* did not show any evidence of bulb formation in onion.

AcFT1, AcFT4 and *GA3ox1* show leaf specific expression pattern, while *AcLFY* show bulb specific expression in *Renate F1*. Other genes under study did not show any tissue specific expression pattern.

FKF1, GI and *COL2* show clear diurnal expression patterns in onion consistent with photoperiod sensing and regulation of *FT1*. It was found that all *FT* genes have a particular expression patterns peaking at different times of the day. Notably, *FT1*, which promotes bulbing is expressed in the later part of the day, while *FT4* is

expressed late in the night and the early morning. In LD and SD varieties of onion, no difference was found for the expression of *FT* genes. However, the only difference we found is that *AcFT4* is under less stringent daylength control in *Hojem* than in *Renate F1*.

Therefore, it can be concluded that the developmental, spatial and diurnal expression patterns of the genes under study in LD and SD conditions provide the molecular tools to investigate the basis of difference between LD- and SD- onion varieties, and understand the adaptability of different onions to different latitudes in the world.

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APPENDICES

Appendix I. Multiple Comparisons between DFS in *Renate F1*.

(I) Time	(J) Time	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
1	2	0.027	0.118	1.000	-0.664	0.718
	3	0.093	0.119	1.000	-0.599	0.786
	4	-0.160	0.103	1.000	-0.759	0.439
	5	-0.911	0.196	0.157	-2.055	.232
	6	-1.340*	0.163	0.008	-2.293	-0.387
	7	-1.322*	0.182	0.015	-2.383	-0.261
	8	-1.610*	0.190	0.007	-2.717	-0.503
	9	-1.402*	0.211	0.025	-2.632	-0.173
	10	-1.427*	0.144	0.003	-2.265	-0.588
2	1	-0.027	0.118	1.000	-0.718	0.664
	3	0.067	0.017	0.360	-0.033	0.167
	4	-0.187*	0.016	0.001	-0.279	-0.095
	5	-0.938	0.200	0.150	-2.104	0.228
	6	-1.367*	0.081	0.000	-1.837	-0.896
	7	-1.349*	0.145	0.004	-2.194	-0.504
	8	-1.637*	0.090	0.000	-2.160	-1.114
	9	-1.429*	0.115	0.001	-2.099	-0.759
	10	-1.453*	0.182	0.009	-2.513	-0.393
3	1	-0.093	0.119	1.000	-0.786	0.599
	2	-0.067	0.017	0.360	-0.167	0.033
	4	-0.253*	0.023	0.002	-0.390	-0.117
	5	-1.004	0.205	0.123	-2.203	0.195
	6	-1.433*	0.083	0.000	-1.916	-0.951
	7	-1.416*	0.142	0.003	-2.245	-0.586
	8	-1.703*	0.084	0.000	-2.191	-1.215
	9	-1.496*	0.112	0.001	-2.152	-0.839
	10	-1.520*	0.180	0.007	-2.570	-0.470
4	1	0.160	0.103	1.000	-0.439	0.759
	2	0.187*	0.016	0.001	0.095	0.279
	3	0.253*	0.023	0.002	0.117	0.390
	5	-0.751	0.195	0.377	-1.888	0.386
	6	-1.180*	0.087	0.000	-1.688	-0.672
	7	-1.162*	0.145	0.009	-2.008	-0.316
	8	-1.450*	0.101	0.000	-2.040	-0.860
	9	-1.242*	0.125	0.003	-1.975	-0.509
	10	-1.267*	0.172	0.015	-2.274	-0.260

5	1	0.911	0.196	0.157	0-.232	2.055
	2	0.938	0.200	0.150	0-.228	2.104
	3	1.004	0.205	0.123	0-.195	2.203
	4	0.751	0.195	0.377	0-.386	1.888
	6	-0.429	0.200	1.000	-1.598	0.740
	7	-0.411	0.165	1.000	-1.374	0.552
	8	-0.699	0.232	1.000	-2.054	0.657
	9	-0.491	0.261	1.000	-2.018	1.036
	10	-0.516	0.274	1.000	-2.116	1.085
6	1	1.340*	0.163	0.008	0.387	2.293
	2	1.367*	0.081	0.000	0.896	1.837
	3	1.433*	0.083	0.000	0.951	1.916
	4	1.180*	0.087	0.000	0.672	1.688
	5	0.429	0.200	1.000	-0.740	1.598
	7	0.018	0.151	1.000	-0.866	0.902
	8	-0.270	0.059	0.169	-0.614	0.074
	9	-0.062	0.142	1.000	-0.892	0.768
	10	-0.087	0.181	1.000	-1.144	0.971
7	1	1.322*	0.182	0.015	0.261	2.383
	2	1.349*	0.145	0.004	0.504	2.194
	3	1.416*	0.142	0.003	0.586	2.245
	4	1.162*	0.145	0.009	0.316	2.008
	5	0.411	0.165	1.000	-0.552	1.374
	6	-0.018	0.151	1.000	-0.902	0.866
	8	-0.288	0.159	1.000	-1.219	0.644
	9	-0.080	0.191	1.000	-1.194	1.034
	10	-0.104	0.243	1.000	-1.526	1.317
8	1	1.610*	0.190	0.007	0.503	2.717
	2	1.637*	0.090	0.000	1.114	2.160
	3	1.703*	0.084	0.000	1.215	2.191
	4	1.450*	0.101	0.000	0.860	2.040
	5	0.699	0.232	1.000	-0.657	2.054
	6	0.270	0.059	0.169	-0.074	0.614
	7	0.288	0.159	1.000	-0.644	1.219
	9	0.208	0.112	1.000	-0.449	0.864
	10	0.183	0.209	1.000	-1.037	1.404
9	1	1.402*	0.211	0.025	0.173	2.632
	2	1.429*	0.115	0.001	0.759	2.099
	3	1.496*	0.112	0.001	0.839	2.152
	4	1.242*	0.125	0.003	0.509	1.975
	5	0.491	0.261	1.000	-1.036	2.018
	6	0.062	0.142	1.000	-0.768	0.892
	7	0.080	0.191	1.000	-1.034	1.194
	8	-0.208	0.112	1.000	-0.864	0.449
	10	-0.024	0.229	1.000	-1.360	1.311

10	1	1.427*	0.144	0.003	0.588	2.265
	2	1.453*	0.182	0.009	0.393	2.513
	3	1.520*	0.180	0.007	0.470	2.570
	4	1.267*	0.172	0.015	0.260	2.274
	5	0.516	0.274	1.000	-1.085	2.116
	6	0.087	0.181	1.000	-0.971	1.144
	7	0.104	0.243	1.000	-1.317	1.526
	8	-0.183	0.209	1.000	-1.404	1.037
	9	0.024	0.229	1.000	-1.311	1.360

Based on estimated marginal means

*. The mean difference is significant at the 95% confidence interval

b. Adjustment for multiple comparisons: Bonferroni.

Appendix II. Multiple Comparisons in DFS between varieties.

					95% Confidence Interval for Difference[b]	
(I) Time	(J) Time	Mean Difference (I-J)	Std. Error	Sig.[b]	Lower Bound	Upper Bound
1	2	0.058	0.050	1.000	-0.442	0.558
	3	-0.020	0.022	1.000	-0.239	0.199
	4	0.118	0.027	1.000	-0.149	0.386
	5	0.097	0.048	1.000	-0.390	0.584
	6	0.068	0.058	1.000	-0.513	0.650
	7	-0.018	0.026	1.000	-0.282	0.245
	8	-0.113	0.043	1.000	-0.547	0.320
	9	-0.193	0.036	0.527	-0.555	0.169
	10	-0.373	0.050	0.162	-0.881	0.134
	11	-0.543*	0.040	0.015	-0.941	-0.146
	12	-0.705*	0.030	0.002	-1.006	-0.404
	13	-0.743	0.099	0.155	-1.743	0.256
	14	-1.335*	0.107	0.022	-2.416	-0.254
2	1	-0.058	0.050	1.000	-0.558	0.442
	3	-0.078	0.032	1.000	-0.396	0.239
	4	0.060	0.039	1.000	-0.330	0.450
	5	0.038	0.039	1.000	-0.357	0.434
	6	0.010	0.037	1.000	-0.361	0.381
	7	-0.077	0.044	1.000	-0.520	0.367
	8	-0.172	0.047	1.000	-0.646	0.302
	9	-0.252	0.047	0.535	-0.725	0.221
	10	-0.432	0.066	0.263	-1.100	0.236
	11	-0.602*	0.048	0.021	-1.083	-0.120

	12	-0.763*	0.036	0.003	-1.123	-0.404
	13	-0.802	0.083	0.059	-1.637	0.034
	14	-1.393*	0.070	0.003	-2.096	-0.691
3	1	0.020	0.022	1.000	-0.199	0.239
	2	0.078	0.032	1.000	-0.239	0.396
	4	0.138	0.022	0.281	-0.080	0.356
	5	0.117	0.044	1.000	-0.324	0.558
	6	0.088	0.052	1.000	-0.436	0.613
	7	0.002	0.028	1.000	-0.284	0.287
	8	-0.093	0.039	1.000	-0.481	0.294
	9	-0.173	0.032	0.526	-0.498	0.151
	10	-0.353	0.058	0.339	-0.939	0.232
	11	-0.523*	0.044	0.027	-0.970	-0.077
	12	-0.685*	0.028	0.002	-0.967	-0.403
	13	-0.723	0.091	0.125	-1.642	0.195
	14	-1.315*	0.096	0.015	-2.284	-0.346
4	1	-0.118	0.027	1.000	-0.386	0.149
	2	-0.060	0.039	1.000	-0.450	0.330
	3	-0.138	0.022	0.281	-0.356	0.080
	5	-0.022	0.035	1.000	-0.378	0.334
	6	-0.050	0.048	1.000	-0.535	0.435
	7	-0.137	0.021	0.247	-0.345	0.071
	8	-0.232*	0.019	0.023	-0.421	-0.042
	9	-0.312*	0.014	0.002	-0.455	-0.168
	10	-0.492	0.067	0.168	-1.167	0.183
	11	-0.662*	0.049	0.015	-1.150	-0.174
	12	-0.823*	0.040	0.003	-1.225	-0.422
	13	-0.862*	0.074	0.028	-1.602	-0.122
	14	-1.453*	0.095	0.010	-2.406	-0.500
5	1	-0.097	0.048	1.000	-0.584	0.390
	2	-0.038	0.039	1.000	-0.434	0.357
	3	-0.117	0.044	1.000	-0.558	0.324
	4	0.022	0.035	1.000	-0.334	0.378
	6	-0.028	0.028	1.000	-0.308	0.251
	7	-0.115	0.046	1.000	-0.574	0.344
	8	-0.210	0.032	0.261	-0.534	0.114
	9	-0.290	0.045	0.282	-0.747	0.167
	10	-0.470	0.073	0.267	-1.200	0.260
	11	-0.640*	0.044	0.012	-1.086	-0.194
	12	-0.802*	0.040	0.003	-1.207	-0.397
	13	-0.840*	0.071	0.026	-1.550	-0.130
	14	-1.432*	0.082	0.006	-2.255	-0.608
6	1	-0.068	0.058	1.000	-0.650	0.513
	2	-0.010	0.037	1.000	-0.381	0.361
	3	-0.088	0.052	1.000	-0.613	0.436

	4	0.050	0.048	1.000	-0.435	0.535
	5	0.028	0.028	1.000	-0.251	0.308
	7	-0.087	0.048	1.000	-0.565	0.392
	8	-0.182	0.051	1.000	-0.692	0.328
	9	-0.262	0.056	0.850	-0.822	0.299
	10	-0.442	0.062	0.183	-1.062	0.179
	11	-0.612*	0.036	0.006	-0.970	-0.253
	12	-0.773*	0.042	0.005	-1.194	-0.353
	13	-0.812*	0.078	0.045	-1.600	-0.024
	14	-1.403*	0.058	0.002	-1.985	-0.821
7	1	0.018	0.026	1.000	-0.245	0.282
	2	0.077	0.044	1.000	-0.367	0.520
	3	-0.002	0.028	1.000	-0.287	0.284
	4	0.137	0.021	0.247	-0.071	0.345
	5	0.115	0.046	1.000	-0.344	0.574
	6	0.087	0.048	1.000	-0.392	0.565
	8	-0.095	0.036	1.000	-0.453	0.263
	9	-0.175	0.022	0.123	-0.396	0.046
	10	-0.355	0.053	0.239	-0.890	0.180
	11	-0.525*	0.040	0.018	-0.932	-0.118
	12	-0.687*	0.040	0.006	-1.090	-0.284
	13	-0.725	0.083	0.084	-1.555	0.105
	14	-1.317*	0.090	0.012	-2.221	-0.412
8	1	0.113	0.043	1.000	-0.320	0.547
	2	0.172	0.047	1.000	-0.302	0.646
	3	0.093	0.039	1.000	-0.294	0.481
	4	0.232*	0.019	0.023	0.042	0.421
	5	0.210	0.032	0.261	-0.114	0.534
	6	0.182	0.051	1.000	-0.328	0.692
	7	0.095	0.036	1.000	-0.263	0.453
	9	-0.080	0.021	1.000	-0.287	0.127
	10	-0.260	0.083	1.000	-1.092	0.572
	11	-0.430	0.060	0.188	-1.038	0.178
	12	-0.592*	0.054	0.035	-1.131	-0.052
	13	-0.630*	0.058	0.037	-1.213	-0.047
	14	-1.222*	0.096	0.020	-2.191	-0.252
9	1	0.193	0.036	0.527	-0.169	0.555
	2	0.252	0.047	0.535	-0.221	0.725
	3	0.173	0.032	0.526	-0.151	0.498
	4	0.312*	0.014	0.002	0.168	0.455
	5	0.290	0.045	0.282	-0.167	0.747
	6	0.262	0.056	0.850	-0.299	0.822
	7	0.175	0.022	0.123	-0.046	0.396
	8	0.080	0.021	1.000	-0.127	0.287
	10	-0.180	0.074	1.000	-0.929	0.569

	11	-0.350	0.058	0.353	-0.937	0.237
	12	-0.512	0.053	0.057	-1.041	0.018
	13	-0.550	0.068	0.113	-1.230	0.130
	14	-1.142*	0.097	0.027	-2.114	-0.170
10	1	0.373	0.050	0.162	-0.134	0.881
	2	0.432	0.066	0.263	-0.236	1.100
	3	0.353	0.058	0.339	-0.232	0.939
	4	0.492	0.067	0.168	-0.183	1.167
	5	0.470	0.073	0.267	-0.260	1.200
	6	0.442	0.062	0.183	-0.179	1.062
	7	0.355	0.053	0.239	-0.180	0.890
	8	0.260	0.083	1.000	-0.572	1.092
	9	0.180	0.074	1.000	-0.569	0.929
	11	-0.170	0.029	0.392	-0.463	0.123
	12	-0.332	0.041	0.121	-0.749	0.086
	13	-0.370	0.129	1.000	-1.665	0.925
	14	-0.962	0.097	0.053	-1.936	0.013
11	1	0.543*	0.040	0.015	0.146	0.941
	2	0.602*	0.048	0.021	0.120	1.083
	3	0.523*	0.044	0.027	0.077	0.970
	4	0.662*	0.049	0.015	0.174	1.150
	5	0.640*	0.044	0.012	0.194	1.086
	6	0.612*	0.036	0.006	0.253	0.970
	7	0.525*	0.040	0.018	0.118	0.932
	8	0.430	0.060	0.188	-0.178	1.038
	9	0.350	0.058	0.353	-0.237	0.937
	10	0.170	0.029	0.392	-0.123	0.463
	12	-0.162	0.024	0.215	-0.399	0.075
	13	-0.200	0.105	1.000	-1.258	0.858
	14	-0.792	0.083	0.062	-1.629	0.045
12	1	0.705*	0.030	0.002	0.404	1.006
	2	0.763*	0.036	0.003	0.404	1.123
	3	0.685*	0.028	0.002	0.403	0.967
	4	0.823*	0.040	0.003	0.422	1.225
	5	0.802*	0.040	0.003	0.397	1.207
	6	0.773*	0.042	0.005	0.353	1.194
	7	0.687*	0.040	0.006	0.284	1.090
	8	0.592*	0.054	0.035	0.052	1.131
	9	0.512	0.053	0.057	-0.018	1.041
	10	0.332	0.041	0.121	-0.086	0.749
	11	0.162	0.024	0.215	-0.075	0.399
	13	-0.038	0.104	1.000	-1.084	1.008
	14	-0.630	0.090	0.203	-1.540	0.280
13	1	0.743	0.099	0.155	-0.256	1.743
	2	0.802	0.083	0.059	-0.034	1.637

	3	0.723	0.091	0.125	-0.195	1.642
	4	0.862*	0.074	0.028	0.122	1.602
	5	0.840*	0.071	0.026	0.130	1.550
	6	0.812*	0.078	0.045	0.024	1.600
	7	0.725	0.083	0.084	-0.105	1.555
	8	0.630*	0.058	0.037	0.047	1.213
	9	0.550	0.068	0.113	-0.130	1.230
	10	0.370	0.129	1.000	-0.925	1.665
	11	0.200	0.105	1.000	-0.858	1.258
	12	0.038	0.104	1.000	-1.008	1.084
	14	0-.592	0.095	0.309	-1.548	0.365
14	1	1.335*	0.107	0.022	0.254	2.416
	2	1.393*	0.070	0.003	0.691	2.096
	3	1.315*	0.096	0.015	0.346	2.284
	4	1.453*	0.095	0.010	0.500	2.406
	5	1.432*	0.082	0.006	0.608	2.255
	6	1.403*	0.058	0.002	0.821	1.985
	7	1.317*	0.090	0.012	0.412	2.221
	8	1.222*	0.096	0.020	0.252	2.191
	9	1.142*	0.097	0.027	0.170	2.114
	10	0.962	0.097	0.053	-0.013	1.936
	11	0.792	0.083	0.062	-0.045	1.629
	12	0.630	0.090	0.203	-0.280	1.540
	13	0.592	0.095	0.309	-0.365	1.548

Based on estimated marginal means

*. The mean difference is significant at the 95% confidence interval

b. Adjustment for multiple comparisons: Bonferroni.

Appendix III. Control primers designed from onion EST sequence used to confirm the expression of genes of interest in onion. Forward is top in each section and Reverse is below.

Gene	GeneBank Accession	Forward (RT-FOR) and Reverse (RT-REV) primer sequences (5'…..3')	Annealing temperature (^{o}C)	Product size (bp)	Gene size (bp)
ALL	L48614.1	TTCAGAAACTAAGAGAGAGGT CTTCCCATTCACACTTCACCC	53	160	4014
FKF1	GQ232754	AGGTCGCAATTGCCGATTCTTA AACGTGACCCGTTCATGGAAGT	60	815	2054
GIa	GQ232756	TGGTTGCTGCACATGTTTCTGA GTGTGCACTTGGATGCGATAGG	60	956	3555
GIb	GQ232757	TGGTTGCTGCACATGTTTCTGA GTGTGCACTTGGATGCGATAGG	60	956	3555
EF1α	CF437531	ACTCCAAGGCCCGTTATGATGA TGTGAGATGTGTGGCAATCCAA	60	582	775
FST-1	GQ214178	GCATGCTCGAGTGTGTGGATCT CATGGAGAACTGGAACGGTGTG	60	813	1872
LFY	JX275963	ACAACCCTCTCCCACCTCTTCC TTCTTCATCCAGGCAATGCAAA	60	642	1119
GGTP	AY517548	ATCTGCCACCACCTGCTCCTAC GCTAGACAAACGCAGCCTCCTC	60	538	1077
GGTP	AY517547	CACTTGGGCTTCATCGCTTCAT GTAGGAGCAGGTGGTGGCAGAT	60	500	912
GGTP	AY517549	CGTGCCTGGTTACATACCCAAA TGTTCTGTTCATTTGCATGTGTAGGT	60	541	600
GGTF	AF401622	AGGCAGCGGCATTCTATCAGTC GTAGGAGCAGGTGGTGGCAGAT	60	936	1905

GSTF	AF401623	CCCAACGTCGTGAGAGTGATTG ATCTCCCACCAGGCCTTCAAGT	60	560	678
ATPS	AF403295	GCTGAAGCGTGTTGATCTGGAA TTGCGTGCCATTGTACCTCAGT	60	761	1585
GGCS	AF401621	GGTGGTGATGACAGAGCCATTG ACCTTCTCCAGGGACCTCCATC	60	969	1912
SATF	AF212156	ACCAGCTTTGGCCAGCTACTTG GCAGTAGTCCTAGGCGGCACAT	60	636	1094
ACAAV44 *F-box*	GQ232752	ACATGCACAACCATCGACGACT TTGCGAATCCTCTCCACCGTAT	60	576	1445
COL1	GQ232751	GAATCCGACGGTGCAGGTTAAG GCCTTTCTGGAAGCGTAGCGTA	60	522	1109
GA3ox1	AB303422	AACACTTCGACCTCGGATCAGC TATGGTCAGCAGGCTTGAGTCG	60	644	1208
SPS	EU164758	CCTGAACATTGTCGCACGTACC CGATAGCGCAAACCCTGAAATC	60	504	3267
AcGSTF1	AB300334	CCCAACGTCGTGAGAGTGATTG ATCTACTTGGCAGCAGCGGTCT	60	608	820
FT-LIKE PROTEIN 1	JX145040	TTGGAGATGTTGTGGATCCGTTT CAATGGATCAAGATGCGGTCAG	60	515	807
FT-LIKE PROTEIN 2	JX145039	GAAACGTTGTGGGCGATGTTCT GGAACGGAATGGTAGGATGCAG	60	579	834

Legends: *ALL: ALLINASE, ATPS: ATP-SULPHURYLASE, FKF1: FLAVIN-BINDING KELCH REPEAT PROTEIN; F-BOX 1 PROTEIN, GI: GIGANTEA, EF1α: ELONGATION FACTOR 1 ALPHA, FST-1: SUCROSE 1-FRUCTOSYLTRANSFERASE-LIKE, LFY: LEAFY, GGTP: GAMMA-GLUTAMYL TRANSPEPTIDASE, GGTF: GAMMA-GLUTAMYL TRANSFERASE, GSTF: GLUTATHIONE S-TRANSFERASE, GGCS: GAMMA-GLUTAMYLCYSTEINE SYNTHETASE, SATF: SERINE ACETYLTRANSFERASE, COL1: CONSTANS LIKE 1, GA3ox1: GIBBERELLIN 3-OXIDASE, SPS: SUCROSE-PHOSPHATE SYNTHASE, AcGSTF1: Allium cepa GLUTATHIONE S-TRANSFERASE 1, FT-LIKE PROTEIN: FLOWERING LOCUS T-LIKE PROTEIN.*

Appendix IV. Control primers designed from onion EST sequence used to confirm the expression of *FT* genes in *Renate F1* (Lee et al., 2013). Forward is top in each section and Reverse is below.

Gene	GeneBank Accession	Forward (RT-FOR) and Reverse (RT-REV) primer sequences (5'…..3')	Annealing temperature (^{o}C)	Product size (bp)	Gene size (bp)
AcFT1	KC485348	TTACATGGCAAGAGAAAGTGACCCAT CCTATTAGTAATCCGTGTATATTCT	50	537+8=545	726
AcFT2	KC485349	AAGGATGATGGATTCGGATCCGTTA CCATTCATCTATAAGTTCTCCTCCCA	61	528+8=534	572
AcFT3	KC485350	GGGAATGTTGTAGGCGATGTTT AGCCGTTTCCTGGTCGATACCT	50	508	745
AcFT4	KC485351	CACGATGTCTTTTGATCCTTTAGTT TGTGCTAATTCTCTGATCGAAACCTT	50	528+8=534	539
AcFT5	KC485352	TTCCATGTCAAGAGATCCTCTTGTT AGTGTCAGAGCCAGCCACTTCCT	58	540+8=548	604
AcFT6	KC485353	ATACATGCAAGTAAAAATGTTGCGA GCAGTCAGCAAAGCCCCGAGAACCT	58	558+8=566	841

Legends: *AcFT: Allium cepa FLOWERING LOCUS T*. Green colour means start codon and red colour means stop codon.

Appendix V. Onion gene sequences

AcFKF1

ATTCCAAATCCCAAACCAATTACAGCGAATTTGAGAATGGGTTTTATAGAAGAT
GATGTGCAGAGGAAAGGGAAGCGATTGAAATACTACACCGATGGTTCTATAGAT
TGGGAGGATGAAGAAGAAGAGAATGAAAATGAATACGAATCGGATGACGAGGA
GGAGGAAATAAGTATTGATTATGCTGGAGATTATTATAATCATCAGTATACGAG
CATCGATCGATTTGATTTTGAATTGAGATCTTCTGCTTCCTTTGTTGTGTCTGATG
CGATGGAACCCGATTTCCCGATTATTTATGTTAATTCCGTGTTTGAGGATTCTAC
TGGATACAGAGCTGATGAAGTTATAGGTCGCAATTGCCGATTCTTACAATTCCG
GGACCCACAAGCGCAAAGGCGGCACCCACTAGTGGACCCTACAGTGGTATCCGA
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AcGIa

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AcGIb

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AcCOL/AcCOL1

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AcCOL2

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AcCOL3

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FT-LIKE PROTEIN 1

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FT-LIKE PROTEIN 2

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AcFT1

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AcFT2

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AcFT3

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TCTATCATATTTGATGTGCCTAAAAGCATGCATATTCTYTTAACAAAGTTTTCAG
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AcFT4

GTACGATGTCTTTTGATCCTTTAGTTTTAAGTCGTGTTGTAGGTGATGTAATTGAT
GACTTTGATAGGCGTATACCTCTAAGGGTTATGTATAATGGAAGATCGGTTCTCA
ATGGAGTTGGTATGAAACCATCTATTATCAATGACAAACCTAGAGTTGAAATAG
GAGGTGTACCAAGAATGTTTTACACTCTTGTAATGGTCGATCCAGATGCTCCAAA
CCCGAGCAATCCTTCTTTAAAGGAATACTTGCACTGGATGCTTGTGGATATACCA
GCAAATATGGATGGTAGTTTCGGAACTGAACTAATGGAGTATGTGAGCCCACAG
CCAACTTCAGGGATACACCGCATTGTATTTGTTCTATATCAGCAACTGTGCAAGG
AAAGTGTGTTTGCTCCAGATGTTCGTCATAACTTCGACACCAGAAAGTTTGCCAG
AGAACATTATCTGGGACCGCCTGTAGCTGCATTATATTTCAACTGCCAACGTGAG
AATGGGTCTGGTGGCCGAAGGTTTCGATCAGAGAATTAGCCGTGC

AcFT5

CTAATATTAAAACATGTCAAGAGATCCTCTTGTTGTAGGAAACGTTGTGGGCGA
TGTTCTAGACCCATTTCAAAAATCGGCTTCACTAAGAATTACATACAATAATAGG
GAAGTTACTAACGGATCAGAGCTTAAACCATCAATGGTGATGAATGAGCCAAGG
ATTGAAATTGGAGGACGCGACTCTAGGACACTATACACCGTTGTTATGATAGAC
CCAGATTCACCTAGCCCAAGCAATCCAACAAAGAGGGAGTACTTGCACTGGATG
GTGACTGATATACCAGAAGCAAAAGATGCAAGTTTAGGAAATGAAATAGTGCC
ATATGAAAGTCCACAACCGACAGCTGGGATTCATCGATTTGTGTTTGTATTATTC
AAGCAGACAGTGAAACAAACTATATATGCACCAGGGTGGCGCCAAAATTTTAAC
TCTAGAGATTTTGCAGCGTACTATAGCTTTGGGCCGCCTGTTGCTGCTGTTTACTT
TAATTGCCAGAGAGAAAATGGATGTGGTGGAAGAAGGTACCGACCAGGAAGTG
GCTGGCTCTGACACTAGTCTTGATTTCAAATTGTCTACCTAGAATATTAGGGCCT
GCATCCT

AcFT6

TATACATGCAAGTAAAAATGTTGCGAGAGAGAGTAGCAAGGGATCCTCTAGTCT
TGGGACAGATAATTGGAGATGTTGTGGATCCGTTTACCAAATCCGTGAATCTCA
AAGTAGTTTATGGAGATAAGGAAGTGAGTAATGGCACAAGACTTCGTCAATCGA
TGGTTATAAATCAACCACGTGTTACCATTGAAGGACGTGACTCAAGGACTCTTTA
TAGCCTTGTTATGATAAACCCTGATGCACCAAGCCCAACTAATCCAACTCATAG
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AATTGTATTTGTACTATTCAAGCAGCAGATTCAACAAACGGTGTATGCACCTGGT
TGGCGGCAAAATTTCTATACTAGAGACTTTGCAGCTTATTATAACCTTGGTTCAC
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GGTTCTCGGGGCTTTGCTGACTGCATCTTGATCCATTGATCAATTTCAACATGGT
TAGCTTTCATAGGCAAGTTCGAAGAACTAGATTTATTATGTTTCTGAGCTTTCAC
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ATTTTTTTGCCAACTTAGTAATAAACTTCCTGTGTAACTGATGCTGTTTTTATACC
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TTCTTCAAAAAAAAAAAAAAAA

AcLFY

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CGACGTCTTCAACGGTTACGGAATACGTTACCTCACAGTTATCCACATAGGCGA
GCTCGGTATCACCCCCAGCACTCTCTTAGGCATGCGCGACGAAGAACTCGACGA
CATGATGACAACCCTCTCCCACCTCTTCCGTTGGGAGCTGCTCGTCGGAGAACGC
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GCGGCAAAGGGAGCACCCATTTATAGTTACGGAGCCGGGTGAAGTGGCGAGGG
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TTCTTGTTCTGGTTCAGGCGATGGCGAAAGAGCAAGGGGATAAGTGTCCAACAA
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TGTTGTTGGTAGCAGTAACTCTGTGTCCTCTATGTTCTAA

GA3ox1

AACTCATCTCCAATCACACAATGCCTTCCATTTCAATGGAACAACCAATCCACTT
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CCGGACTCCCACACTTGGCCCACCATCCAACCCAACCCTCCCAATGGGCCCAAC
GAATCAATACCCGTTATCGACCTTTCCTCGCCCGATGTAATCTCACTCATAGGTC
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CTTGCTTCACAATTTAGAGTCTCAAGCCAGAAGGCTGTTCTCTTTACCCACACAA
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CTGCTGACCATACTGTACCAAAGCAACACAAGTGGGCTGCAAGTGCTACGGCCC
AACAAGGAGTCGAGCCCGACCCAGTGGGTCACCGTGCCGCCCATCCCTGGTGCT
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CATATGCGGGCCTCCGGCCCATGTCAAGGTGTCACCTATTGTGAAGCCTGTGGG
GCCGGCATATAGGCCCATTACATGGAGAGAATACCTGGGTATTAAGGCTAGGCT
ATTTGACAAAGCCCTAGCATCTGTAATAATATCAGAAGAGGACCATAACGATAA
CAGCACATGCTTGCTTAGTTGCGTATGATATGTTACTGTACATGAGTATATTTTT
GGAGTAAATGTCATATAATAGTGTAGCATCCAATGAATGAATGGGAAGTGAAAT
TTTGCATGC

Appendix VI: Differential expressions of 22 genes of interest in the individual tissues of onion cv. *Renate F1* using gel red stained agarose gel electrophoresis.

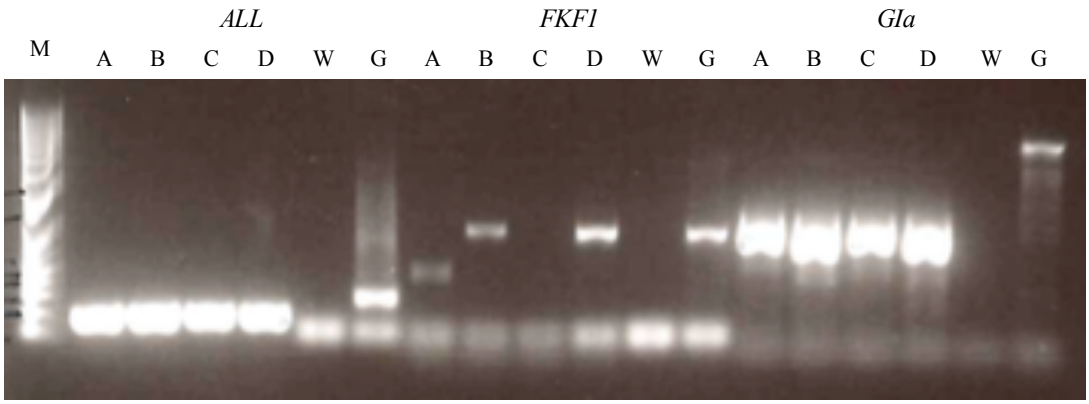

Figure A1. Gel red stained agarose gel showing *ALL*, *FKF1* & *GIa* differentially expressed in leaf (L) and bulb (B) materials grown under SD and LD conditions. M=marker, A=SD-L cDNA, B= SD-B cDNA, C= LD-L cDNA, D= LD-B cDNA, W=water control G=genomic DNA. The same marker was used as shown in Figure 4.2 of section 4.3.1.

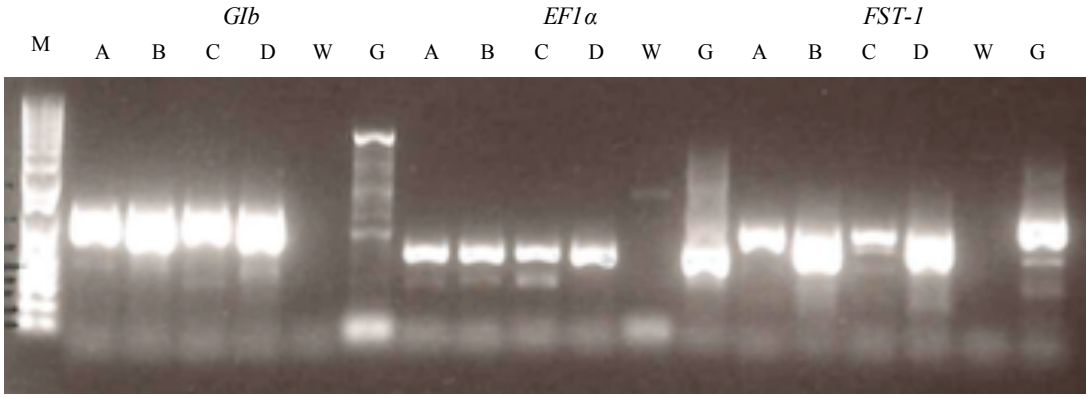

Figure A2. Gel red stained agarose gel showing *GIb*, *EF1α* & FS*T-1* differentially expressed in leaf and bulb materials grown under SD and LD conditions. M=marker, A=SD-L cDNA, B=SD-B cDNA, C= LD-L cDNA, D= LD-B cDNA, W=water control G=genomic DNA. The same marker was used as shown in Figure 4.2 of section 4.3.1.

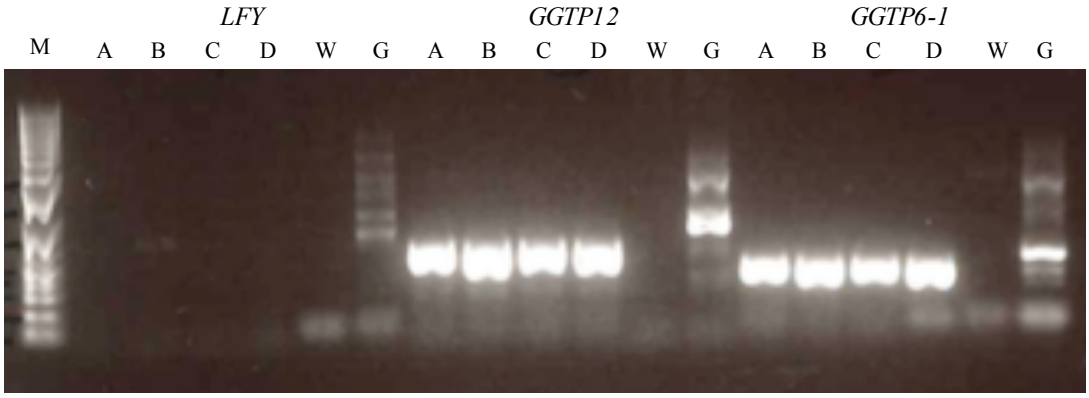

Figure A3. Gel red stained agarose gel showing *LFY*, *GGTP12* & *GGTP6-1* differentially expressed in leaf and bulb materials grown under SD and LD conditions. M=marker, A=SD-L cDNA, B=SD-B cDNA, C= LD-L cDNA, D= LD-B cDNA, W=water control G=genomic DNA. The same marker was used as shown in Figure 4.2 of section 4.3.1.

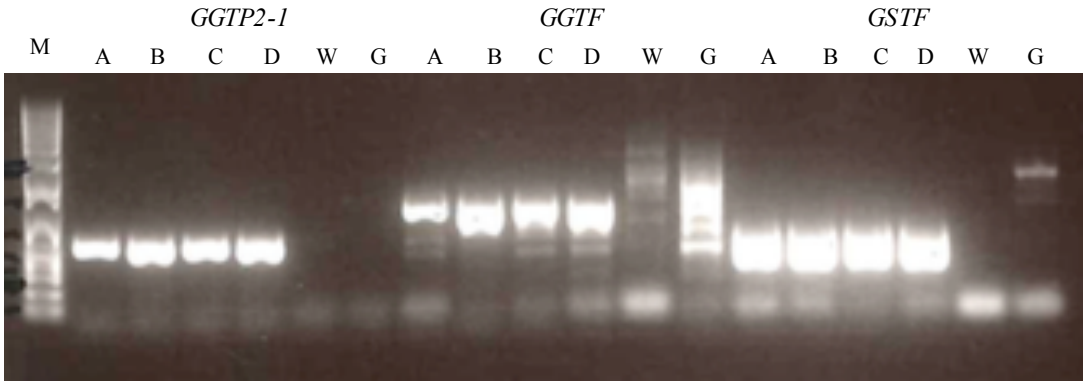

Figure A4. Gel red stained agarose gel showing *GGTP2-1, GGTF* & *GSTF* differentially expressed in leaf and bulb materials grown under SD and LD conditions. M=marker, A=SD-L cDNA, B=SD-B cDNA, C= LD-L cDNA, D= LD-B cDNA, W=water control G=genomic DNA. The same marker was used as shown in Figure 4.2 of section 4.3.1.

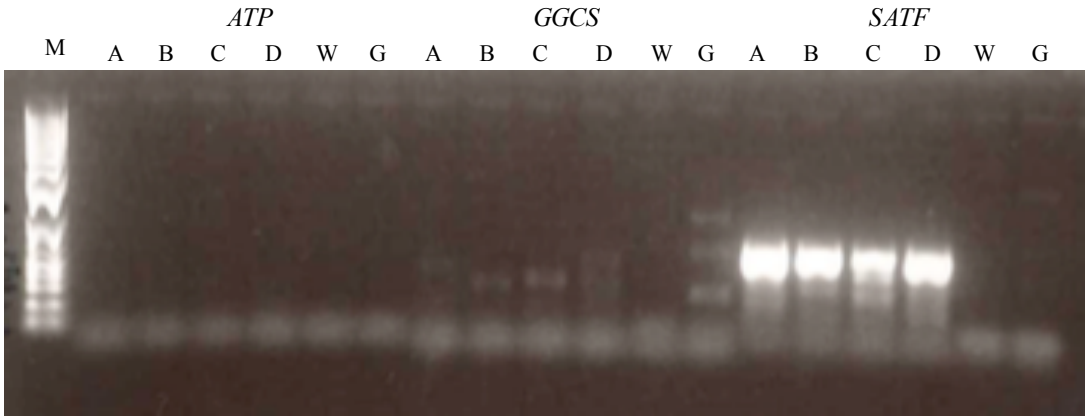

Figure A5. Gel red stained agarose gel showing *ATP*, *GGCS* & *SATF* differentially expressed in leaf and bulb materials grown under SD and LD conditions. M=marker, A=SD-L cDNA, B=SD-B cDNA, C= LD-L cDNA, D= LD-B cDNA, W=water control G=genomic DNA. The same marker was used as shown in Figure 4.2 of section 4.3.1.

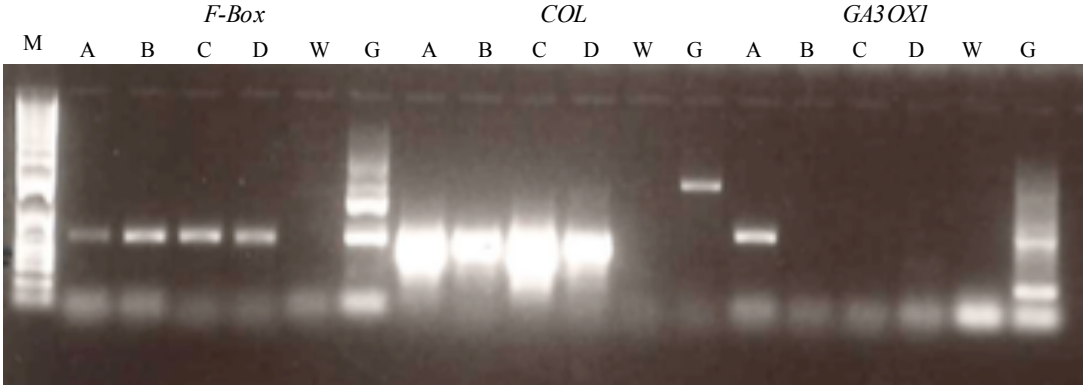

Figure A6. Gel red stained agarose gel showing *F-Box*, *COL* & *GA3OX1* differentially expressed in leaf and bulb materials grown under SD and LD conditions. M=marker, A=SD-L cDNA, B=SD-B cDNA, C= LD-L cDNA, D= LD-B cDNA, W=water control G=genomic DNA. The same marker was used as shown in Figure 4.2 of section 4.3.1.

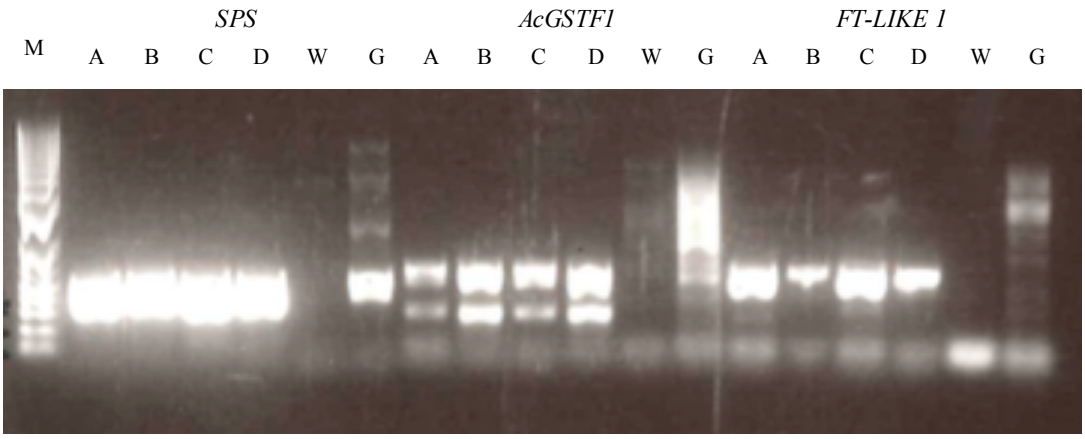

Figure A7. Gel red stained agarose gel showing *SPS*, *AcGSTF1* & *FT-LIKE PROTEIN 1* differentially expressed in leaf and bulb materials grown under SD and LD conditions. M=marker, A=SD-L cDNA, B=SD-B cDNA, C= LD-L cDNA, D= LD-B cDNA, W=water control G=genomic DNA. The same marker was used as shown in Figure 4.2 of section 4.3.1.

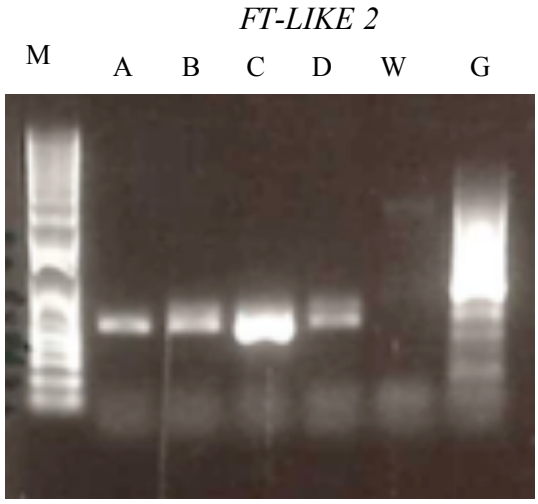

Figure A8. Gel red stained agarose gel showing *FT-LIKE PROTEIN 2* differentially expressed in leaf and bulb materials grown under SD and LD conditions. M=marker, A=SD-L cDNA, B=SD-B cDNA, C= LD-L cDNA, D= LD-B cDNA, W=water control G=genomic DNA. The same marker was used as shown in Figure 4.2 of section 4.3.1.

Appendix VII. qRT-PCR primers used to detect reference genes and their similarities with *Renate F1* onion transcriptome sequence**.** Forward is top in each section and Reverse is below.

Gene	GeneBank ID (*Renate F1* sequence)	GeneBank ID (NCBI database sequence)	Forward (qRT-FOR) and Reverse (qRT-REV) primer sequences (5'…..3')	Product size (bp)	Nucleotide sequence length (bp)		Similarity between NCBI database sequence and onion transcriptome sequence	
					NCBI Reference sequence	*Renate F1* transcriptome	% Nucleotide	% Amino acid
UBQ1/ L40	KY072872	AY059080.1	AATCAGCTAAGGCCGAAGAAGAAGAT TCAATTCAAGATGCAAAGTACACCAA	149	418	751	82	97.7
UBL	KY072874	NM_129118.4	CTGTCCTTCATCTTGTGCTTGCTCT CAAGACCGAAACAAACCATCAAATTA	148	712	596	84	94.8
ACT 2	KY072870	AK317453.1	CTTCTCTCAGCACTTTCCAACAGATG CCAAATCAAACTTGCATGATAACCAG	149	1632	1761	80	92.8
TUA	KY072876	BT000718.1	GATTACGAAGAAGTTGGAGCTGAAGG GCACTTCACCAGAACCCACATATACT	106	1646	1665	78	95.1
G6PDH	KY072878	AK317337.1	AACTTTGTTAAGCTCGTCGCATGGTA AAGACGCAAACACCACCTACTAATAA	145	1405	1506	82	87.2
UBC 9	KY072873	AF325019.2	GAAGCTGGACCCAGAAGTATGCTATG TATAGGGAAGTAGCGAAGCAATGCAC	95	896	972	82	96.6
TIP-41	KY072882	NM_119592.4	GTCAAACACAGAACTGAAAGGCTGAA AATAAATGATTGTTGGCTCGCATCTA	146	1254	1456	71	66.5
PP2AA3	KY072881	BT002601.1	ATCGATAAAGCCGTGTCTAGTTGAGC GCGACAAATCATGATACTTTCTGACA	134	1794	2065	77	84
PP2A-1	KY072880	AY096543.1	CAGATACAACACGCAAGACTCCTGAT CCCACTCCCTTTGTAGCACAAATCT	127	952	1518	79	90.2

ACT	KY072869	GU570135.2	AGGAGTTATGGTTGGAATGGGACAAA TGTATGATGCCAAATCTTCTCCATGT	145	387	238	99	100
ACT-like	KY072871	JN797640.1	CAAAGGGAGAGTACGATGAATCTGGT CAACCAAATCAAAGACACGATAACGA	122	699	1619	85	97.9
TUB-7	KY072877	DQ897861.1	CACTCCACGAAGTACGACGAGTTCT CTATTTAACAGCCTCCGCCCTCTT	105	635	265	97	100
AcTUB	KY072875	AA451549.2	GTCTTCAGAGGCAAGATGAGCAC TCAGTCCAGTAGGAGGAATGTCG	138	1004	1728	98	100
EF1-α	KY072879	CF437531.1	TGGCATCCAACTCTAAGGACGAT AATGTGAGATGTGTGGCAATCCA	131	775	1924	86	96.9

Legends: *UBQ1/L40: UBIQUITIN EXTENSION PROTEIN, UBL: UBIQUITIN-LIKE PROTEIN RUB2, ACT2: ACTIN 2, TUA 5: ALPHA TUBULIN-5, G6PDH: GLUCOSE-6-PHOSPHATE DEHYDROGENASE, UBC 9: UBIQUITIN CONJUGATING ENZYME 9, TIP-41: TONOPLASTIC INTRINSIC PROTEIN-41, PP2AA3: PROTEIN PHOSPHATASE 2A REGULATORY SUBUNIT 3, PP2A-1: PROTEIN PHOSPHATASE TYPE 2A, ACT: ACTIN, ACT-LIKE: ACTIN LIKE, TUB-7: TUBULIN-7, AcTUB: Allium cepa TUBULIN, EF1-α: ELONGATION FACTOR 1-ALPHA.*

Appendix VIII. Primers for qRT-PCR used to estimate the expression of genes of interest in onion. Forward is top in each section and Reverse is below.

Gene	GeneBank Accession	Forward (qRT-FOR) and Reverse (qRT-REV) primer sequences (5'…..3')	Annealing temperature (^{o}C)	Product size (bp)	Primer concentration for qPCR (μM)
AcFKF1 (2054 bp)	GQ232754	TTTGGCATATTGGGTACTGTAGGTA ACGAGCACAATCAGATTTATACAACAGC	60	130	0.3
AcGI (3555 bp)	GQ232756	CACAGATGGATTGCTTGTTGATG ATTGGCTACGAGATGAACTGCTC	61	94	0.3
AcCOL1 (1109 bp)	GQ232751	AGAGAAGCGAAAGAATAGAAAGTT ATCCGCATAAGAATCGTTGTC	55	127	0.2
AcCOL2 (1323 bp)	KY012331	ATTGATCAGGTGCTGAAGGGATTG AATCGTCACCATTAAACTACACTGAAA	65	143	0.2
AcCOL3 (1623 bp)	KY012332	CGAAGTTGAGTTTCACCGTGTACTTG TGCTCTGGTACTGAGCATACAACAAA	60	135	0.2
AcFT1 (726bp)	KC485348	AAACCATCACAAATAACTCAGCA GTTTCTCGCCCAAAGTTCG	56	185	0.2
AcFT2 (572bp)	KC485349	AAGTTGCTAATGGACGCGAGTTTAAG CACCAACACAAGTGCATAAGAGTTCC	61	104	0.2
AcFT3 (745bp)	KC485350	AGGAAGTTACTAACGGGTGTGAA CAAAGCTTGCATCTTTTGACC	60	201	0.2
AcFT4 (539bp)	KC485351	TGAAATAGGAGGTGTACCAAGAAT TTCCGAAACTACCATCCATATTTG	60	143	0.3
AcFT5 (604bp)	KC485352	GAAATTGGAGGACGCGAC CTTGCATCTTTTGCTTCTGGTA	60	137	0.2
AcFT6 (841bp)	KC485353	TCGTCAATCGATGGTTATAAATCA TTTCCATAACTTGCATCGACTGT	60	180	0.2

AcLFY (1119 bp)	JX275963	AGCGTGCTATCAACCGATAGTAGTGA AGCTTAGTCGGAACATACCAAATGGA	60	108	0.3
GA3ox1 (1208 bp)	AB303422	GCTATTTGACAAAGCCCTAGCATCTG ATCATACGCAACTAAGCAAGCATGTG	63	86	0.3

Legends: *AcFT: A. cepa FLOWERING LOCUS T, AcFKF1: A. cepa FLAVIN-BINDING KELCH REPEAT; F-BOX 1 PROTEIN, AcGI: A. cepa GIGANTEA, AcCOL: A. cepa CONSTANS LIKE, GA3ox1: GIBBERELLIN 3-OXIDASE 1, AcLFY: A. ceap LEAFY.*

Appendix IX. Significance of the differences in gene expression in *Renate F1* during development under different daylengths. Differences are significant at 95% confidence interval when $P < 0.05$ and non significant when $P > 0.05$.

Table A1. ANOVA table showing the significance of the differences in *AcCOL1* expression in *Renate F1* between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Daylength	3.389	2	1.694	131	<0.05
Days from sowing	0.346	5	0.069	5.348	<0.05
Daylength x Days from sowing	0.362	10	0.036	2.796	<0.05
Error	0.466	36	0.013		

Table A2. ANOVA table showing the significance of the differences in *AcCOL2* expression in *Renate F1* between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Daylength	0.962	2	0.481	21.61	<0.05
Days from sowing	6.346	5	1.269	57.04	<0.05
Daylength x Days from sowing	6.783	10	0.678	30.48	<0.05
Error	0.801	36	0.022		

Table A3. ANOVA table showing the significance of the differences in *AcCOL3* expression in *Renate F1* between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Daylength	5.691	2	2.845	129.1	<0.05
Days from sowing	0.978	5	0.196	8.88	<0.05
Daylength x Days from sowing	4.382	10	0.438	19.89	<0.05
Error	0.793	36	0.022		

Table A4. ANOVA table showing the significance of the differences in *AcFT1* expression in *Renate F1* between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Daylength	395.6	2	197.8	485.1	<0.05
Days from sowing	3.91	5	0.782	1.918	>0.05
Daylength x Days from sowing	16.22	10	1.622	3.978	<0.05
Error	14.68	36	0.408		

Table A5. ANOVA table showing the significance of the differences in *AcFT4* expression in *Renate F1* between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Daylength	439331	2	219665	188.7	<0.05
Days from sowing	174085	5	34817	29.92	<0.05
Daylength x Days from sowing	331990	10	33199	28.53	<0.05
Error	41899	36	1164		

Table A6. ANOVA table showing the significance of the differences in *AcFT5* expression in *Renate F1* between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Daylength	388.2	2	194.1	212.3	<0.05
Days from sowing	117.9	5	23.58	25.79	<0.05
Daylength x Days from sowing	224.7	10	22.47	24.58	<0.05
Error	32.91	36	0.914		

Table A7. ANOVA table showing the significance of the differences in *AcFT6* expression in *Renate F1* between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Daylength	20823	2	10411	25.34	<0.05
Days from sowing	7843	5	1569	3.817	<0.05
Daylength x Days from sowing	12201	10	1220	2.969	<0.05
Error	14793	36	410.9		

Table A8. ANOVA table showing the significance of the differences in *AcLFY* expression in *Renate F1* between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Daylength	24.58	2	12.29	6.355	<0.05
Days from sowing	171.8	5	34.35	17.76	<0.05
Daylength x Days from sowing	92.98	10	9.298	4.808	<0.05
Error	69.63	36	1.934		

Appendix X. Significance of the differences in gene expression in *Hojem* and *Renate F1* during development under 12 h daylength. Differences are significant at 95% confidence interval when P < 0.05 and non significant when P >0.05.

Table A9. ANOVA table showing the significance of the differences in *AcFT1* expression at 12 h between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Variety	403.1	1	403.1	4.026	<0.05
Days from sowing	2877	13	221.3	2.211	<0.05
Variety x Days from sowing	2761	13	212.4	2.122	<0.05
Error	7007	56	100.1		

Table A10. ANOVA table showing the significance of the differences in *AcFT2* expression at 12 h between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Variety	512	1	512	3.35	>0.05
Days from sowing	3446	13	265	1.734	>0.05
Variety x Days from sowing	3661	13	281.6	1.843	>0.05
Error	8558	56	152.8		

Table A11. ANOVA table showing the significance of the differences in *AcFT4* expression at 12 h between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Variety	53.61	1	53.61	35.36	<0.05
Days from sowing	205	13	15.77	10.4	<0.05
Variety x Days from sowing	260	13	20	13.19	<0.05
Error	84.91	56	1.516		

Table A12. ANOVA table showing the significance of the differences in *AcFT5* expression at 12 h between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Variety	0.415	1	0.415	0.432	>0.05
Days from sowing	33.51	13	2.577	2.679	<0.05
Variety x Days from sowing	42.58	13	3.276	3.405	<0.05
Error	53.87	56	0.962		

Table A13. ANOVA table showing the significance of the differences in *AcFT6* expression at 12 h between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Variety	0.806	1	0.806	0.214	>0.05
Days from sowing	509.4	13	39.18	10.44	<0.05
Variety x Days from sowing	814	13	62.62	16.68	<0.05
Error	210.2	56	3.754		

Table A14. ANOVA table showing the significance of the differences in *AcLFY* expression at 12 h between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Variety	29.77	1	1.317	166.2	<0.05
Days from sowing	17.07	13	1.313	7.334	<0.05
Variety x Days from sowing	17.12	13	29.77	7.357	<0.05
Error	10.03	56	0.179		

Appendix XI. Significance of the differences in spatial gene expression in *Renate F1* leaf under LD and SD conditions. Differences are significant at 95% confidence interval when $P < 0.05$ and non significant when $P > 0.05$.

Table A15. ANOVA table showing the significance of the differences in spatial expression of *AcCOL2* in *Renate F1* leaf between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Daylength	5.984	1	5.984	37.93	<0.05
Days from sowing	178	11	16.19	102.6	<0.05
Daylength x Days from sowing	19.86	11	1.806	11.44	<0.05
Error	7.573	48	0.158		

Table A16. ANOVA table showing the significance of the differences in spatial expression of *AcFT1* in *Renate F1* leaf between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Daylength	17.75	1	17.75	28.91	<0.05
Days from sowing	184.1	11	16.74	27.26	<0.05
Daylength x Days from sowing	232	11	21.09	34.35	<0.05
Error	29.47	48	0.614		

Table A17. ANOVA table showing the significance of the differences in spatial expression of *AcFT4* in *Renate F1* leaf between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Daylength	1890	1	1890	110.5	<0.05
Days from sowing	17729	11	1612	94.24	<0.05
Daylength x Days from sowing	7622	11	692.9	110.5	<0.05
Error	820.9	48	17.1		

Table A18. ANOVA table showing the significance of the differences in spatial expression of *AcFT5* in *Renate F1* leaf between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Daylength	0.048	1	0.048	1.046	>0.05
Days from sowing	25.21	11	2.292	50.44	<0.05
Daylength x Days from sowing	9.747	11	0.886	19.5	<0.05
Error	2.181	48	0.045		

Table A19. ANOVA table showing the significance of the differences in spatial expression of *AcFT6* in *Renate F1* leaf between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Daylength	1.163	1	1.163	2.946	>0.05
Days from sowing	30.47	11	2.77	7.019	<0.05
Daylength x Days from sowing	70.94	11	6.449	16.34	<0.05
Error	18.95	48	0.395		

Table A20. ANOVA table showing the significance of the differences in spatial expression of *AcLFY* in *Renate F1* leaf between treatments

Sources of Variation	Sum of squares	Degrees of Freedom (df)	Mean Square	F-Statistic (F) value	Probability (P) value
Daylength	129.3	1	129.3	15.32	<0.05
Days from sowing	2939	11	267.2	31.66	<0.05
Daylength x Days from sowing	2637	11	239.8	28.41	<0.05
Error	506.3	48	8.439		

Appendix XII. Housekeeping genes used for normalisation in the qRT-PCR experiments

Name of experiment	Housekeeping gene
Developmental time-course experiment in onion cv. *Renate F1* under different daylengths	*PP2A1, UBL* and *TIP41*
Diurnal time-course experiment in onion cv. *Renate F1* under LD and SD conditions	*PP2A1, TIP41* and *UBL*
Leaf spatial expression experiment in onion cv. *Renate F1* under LD and SD conditions	*PP2AA3, TIP41* and *UBL*
Developmental time-course experiment in onions cv. *Hojem* and *Renate F1* under 12 h daylength	*PP2A1, UBL* and *PP2AA3*
Diurnal time-course experiment in onion cv. *Hojem* under LD and SD conditions	*PP2A1, UBL* and *PP2AA3*

Legends: *PP2A1: PROTEIN PHOSPHATASE TYPE 2A, UBL: UBIQUITIN-LIKE PROTEIN RUB2, TIP-41: TONOPLASTIC INTRINSIC PROTEIN-41, PP2AA3: PROTEIN PHOSPHATASE 2A REGULATORY SUBUNIT.*

Appendix XIII. Media recipes

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 XIV. Gene specific, M13 and vector primers used for transformation studies

Gene	Forward Primer (RT-FOR) (5'…….3')	Reverse Primer (RT-REV) (5'…….3')	Product size (bp)	Gene size (bp)
AcCOL2	ATGCCTAGTTCGAGGACAATG	CTAAGACGAAGTTTGTAACAAAG	1077	1323
AcCOL2 adaptor	GGGGACAAGTTTGTACAAAAAAGCAGGCT ATGCCTAGTTCGAGGACAATG	GGGGACCACTTTGTACAAGAAAGCTGGGT CTAAGACGAAGTTTGTAACAAAG	1110	1323
M13	GTTGTAAAACGACGGCCAGT	CACAGGAAACAGCTATGACC		
pDONR 207	TCGCGTTAACGCTAGCATGGATCTC	GTAACATCAGAGATTTTGAGACAC		5585

Legends: *AcCOL2*: *Allium cepa CONSTANS LIKE 2*

Appendix XV: Published paper (Acta Hortic. 1143. ISHS 2016. DOI 10.17660/ActaHortic.2016.1143.2 Proc. VII Int. Sym. on Edible *Alliaceae*)

Genetic regulation of day length adaptation and bulb formation in onion (*Allium cepa* L.)

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Keywords: Onion, bulb, day length, gene, transcriptome sequence

Abstract

Genetic studies aimed at onion improvement have been limited because of outcrossing, high heterozygosity, very large genome size with high level of repetitive DNA and limited sequence data. Onion bulb initiation is photoperiod-dependent and day length regulation in onion is crucial for adapting new varieties for growth and development under different latitudes. In comparison to the photoperiodic regulation of flowering, relatively little is known about genetic regulation of the bulbing process. We are testing the hypothesis that the genetic regulation of bulb formation is analogous to the genetic regulation of flowering and aim to identify genes involved in day length adaptation in onion. A comprehensive set of developmental and diurnal experiments have been set up to investigate the bulbing response and to generate materials for molecular analyses. Bulbing ratios were used to measure the bulbing response of onion plants under long day (LD) and short day (SD) conditions, and the reversibility of the bulbing process. Twenty-two partial cDNAs representing genes potentially involved in onion bulbing have been identified and isolated. Eight of these were shown to be differentially expressed in bulb and leaf tissue and with respect to photoperiod. RNA-Seq analysis was performed to generate onion transcriptome reference sequence and for more widespread identification of genes differentially expressed in response to photoperiod. A total of 13665, 12604, 484 and 964 significantly differential expressed transcripts were detected in short day (SD) leaf vs bulb, long day (LD) leaf vs bulb, SD leaf vs LD leaf and SD bulb vs LD bulb, respectively.

INRTODUCTION

Onion (*Allium cepa* L.) belongs to the family Alliaceae and is one of the most important vegetable crops cultivated worldwide (Brewster, 1994). In 2012, the total production of onion was 82.85 million tonnes, which was only exceeded by tomato (FAO, 2014). It is used both in the green and mature stage for salad and spice. Onions form part of the diet of millions of people across the globe, providing flavour and valuable phytochemicals with health related properties, including alkenyl cysteine sulphoxides, flavanols and fibre. An onion plant is composed of leaves which arise alternately from a small flattened stem (bulb), or base plate, which is a vegetative overwintering stage in the life cycle of the plant, where the older leaves are on the outside and younger leaves on the inside of the stem (Lancaster et al., 1996). Being a biennial species it takes more time to improve onion crop by conventional methods such as hybridization, recombination and selection (Lawande et al., 2009).

Onions are categorized into long-day, intermediate-day and short-day varieties. Each onion variety will form a bulb only when it gets a certain hours of daylight each day for a certain number of days. Long-day onions start forming bulbs when the day length reaches 14-16 hours while intermediate and short day onions will start making bulbs much earlier in the year when there are only 12-14 hrs and 10-12 hrs of daylight, respectively (Mettananda and Fordham, 1997). Elevated day or night temperatures also influence bulb formation with varieties being highly adapted to their local conditions (Magruder and Allard, 1937; Heath, 1945; Kato, 1964; Brewster et al., 1977; Steer, 1980). Bulb initiation can be measured by 'bulbing ratio', the ratio of the maximum diameter at the base to the minimum at the neck/sheath, increases to greater than two (Brewster, 1997). The age of the plant also affects bulbing in onion and bulbs grow more rapidly in older plants than younger ones, which will not bulb unless they have at least 4 leaves. Bulbing involves homeotic conversion whereby photosynthetic leaves are transformed to swollen bulb scales at the responsive meristem. Bulbing is a reversible process and plants grown under inductive conditions promote bulbing but if transferred to non-inductive condition, they revert back to vegetative growth (Brewster, 1997). It also involves the accumulation of sugars such as glucose, fructose, sucrose and fructans, and water at the leaf base. In bulbing, photoperiodic signals are detected in the leaves and involve phytochrome A.

Day length sensitivity places a significant barrier to breeding programmes as elite characters found in onions from a particular day length group cannot be transferred to another day length group by cross breeding because the specific day length response of the progeny will be unknown. In addition, crossing onions with different day length requirements is difficult, as the progeny will be compromised. Identifying the genes responsible for day length requirement of bulb formation will help understand the basis of the difference, which is important for adapting new varieties for growth and development at different latitudes. Complete genomes have been sequenced for several species, which include *Arabidopsis thaliana* (Arabidopsis Genome Initiative, 2000) and the crops rice (*Oryza sativa*), maize (*Zea mays*), wheat (*Triticum aestivum*) and legumes such as soybean (*Glycine max*) and barril medic (*Medicago truncatula*) (Goff et al., 2002; Yu et al., 2002; Ware et al., 2002; Lunde et al., 2003; Shoemaker et al., 2002; Young et al., 2003). However, due to difficulty in developing, maintaining and exchanging genetic stocks, there are low numbers of genetic studies aimed at onion improvement (McCallum et al., 2012). Onion bulb formation is day-length dependent and is thus similar to the day length response of flowering (Taylor et al., 2010). In comparison to the knowledge gained regarding photoperiodic regulation of flowering, relatively little is known about genetic regulation of bulbing process (Taylor et al., 2010; Lee et al., 2013). This project addresses this and will involve the identification of genes and regulatory mechanisms involved in the bulbing process.

METARIALS AND METHODS

Time-course experiment

The experiment was conducted to characterize the response of bulb initiation in relation to day length. Onion plants cv. Renate F1 (LD) were grown in natural conditions (NC) of glasshouse. At 30 d, leaf and bulb materials of onion was harvested and sampled (pool material from 3 plants, replicated) at weekly intervals for 2 weeks and then twice in a week for rest of the harvesting period. At 49 d from sowing, rest of the plants were divided into 3 groups, one group was transferred to

LD (16 h photoperiod including 8 h fluorescent followed by 8 h incandescent light), one to SD (8 h fluorescent light) using controlled environmental conditions, and one group left in NC. Both environmental SANYO cabinets were set at 22°C day and 18°C night temperatures with 60% relative humidity and ambient CO_2 concentration, and provided with a Photosynthetic Photon Flux Density (PPFD) of 100 Wm^{-2}. Bulbing ratios were monitored using vernier caliper. Sampling was carried out at ZT10 (zeitgeber time) and involved removing the middle part of the first newly expanded leaf and the middle to the basal portion of bulb, and freezing in liquid nitrogen before storing at -80°C. The harvested materials were used for molecular analysis.

Gene identification and isolation

Key genes were selected which have known functions in *Arabidopsis* flowering and regulate other important pathways such as sucrose and gibberellins pathways. The sequences of each gene in *Arabidopsis* were obtained from NCBI database (www.ncbi.nlm.nih.gov). An onion EST sequence was obtained by blasting the sequence of each gene in *Arabidopsis* homologs against onion database (www.ncbi.nlm.nih.gov/nucest/?term=onion). After obtaining the EST sequences, they were aligned with *Arabidopsis* sequences using MegAlign™. ESTs were then used to design primers. From alignment information, the positions of sequence identity were obtained and primers (Forward and Reverse) for each gene amplification designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

RNA extraction and

Total RNA was extracted from leaf and bulb materials of onion grown under LD and SD from developmental time course Experiment 1 using TRIZOL (Invitrogen, USA), following the manufacturer's guidelines. After isolation, the quality and quantity of total RNA was measured with the Thermo Scientific NanoDropTM 1000 Spectrophotometer (NanoDrop Technologies, Inc., USA). The TURBO DNA-*free treatment* kit (Ambion, USA) was used to eliminate the genomic DNA contamination following the manufacturer's guidelines. A PCR was set up to check free of genomic DNA contamination using primers for *ALLINASE* gene and visualized on RNA gel electrophoresis.

Illumina sequencing

Leaf and bulb material was harvested from the two sets of plants (SD and LD) and used to prepare libraries for Illumina sequencing in the Life Sciences genome centre. Samples were multiplexed to obtain differentiation between long and short day samples and for biological replication.

Synthesis of cDNA synthesis and PCR amplification

cDNA was synthesised using 1 µg total RNA using iScript™ cDNA Synthesis Kit (BIO-RAD) for RT-PCR following the manufacturer's guidelines.

Agarose gel electrophoresis of RNA and DNA

Agarose gels composed of 1-2% (w/v) ultra pure agarose (Invitrogen, USA) with 1x Tris-acetate-EDTA buffer (TAE buffer) and 0.05 µl/ml GelRed™ Nucleic Acid Gel Stain, 10,000X in Water. Orange G loading buffer (Sigma-Aldrich, UK) was mixed with each sample prior to loading on a gel. 1Kb plus DNA ladder

(Invitrogen, USA) was also loaded on gel to estimate the product size. The gel was electrophoresed in 1x TAE buffer. After electrophoresis, the samples on the gel were visualised and recorded by a G: BOX gel documentation system (Syngene, UK).

DNA purification and sequencing of PCR products

DNA products were purified from PCR and agarose gel using QIAquick PCR Purification Kit (QIAGEN) and QIAquick Gel Extraction Kit (QIAGEN), respectively, following the manufacturer's guidelines. Total amount of 10 µl (Premix 5 µl template of 20-80 ng/µl conc. + 5 µl Primer of 5 pmol/µl conc.) PCR products were sent to GATC Biotech for sequencing and the data were interpreted using 4Peaks Chromatogram and edited using SeqMan™.

RESULTS AND DISCUSSION

Time-course experiment

In developmental time-course Experiment, to see the response of Renate F1 onion bulbing ratio to different day lengths, plants were transferred to constant LDs and SDs at 49 days from sowing as well as kept under natural conditions (NC). After 14 d of transfer, the bulbing ratio increased in plants transferred to LD or kept under NC but not in those transferred to SD (Figure 1a & 1b). This might be due to the day length dependent bulb formation in temperate onion, which requires at least 14 hours of daylight for stimulating bulbing (Mettananda and Fordham, 1997; Taylor *et al*., 2010). A clear difference was also observed between plants given at different day lengths. After 14 days of transfer, the NC and LD plants showed increased bulb diameter, which consequently increased bulbing ratio in LDs (Figure 8). In addition, even after 14 days of transfer, SD plants continued producing new leaves, which leads to an increase in neck diameter and therefore a decrease in the bulbing ratio.

Gene identification and isolation

Twenty-two key genes were identified in Renate F1 onion and eight were shown to be differentially expressed. All were shown to represent the expected gene through sequencing of PCR products.

Differentially expressed onion genes under different day lengths

PCR products were run on a gel to determine whether any of the genes were obviously expressed differentially in onion leaf and bulb grown under SD and LD photoperiodic conditions (Figure 2). The data indicate that the cDNA samples used for the analyses were of good quality and suitable sources for sequence amplification. Several genes showed evidence of differential expression in the samples as shown in Figure 2. This experiment gives an indication of gene expression patterns but these need to be verified by real time qPCR. It was unusual to find expression of *FKF1* in bulb materials but not leaf, which could be confirmed by real time qPCR. It was reported that *Arabidopsis FKF1* is expressed highest at ZT10 (Taylor et al., 2010). No LFY mRNA bands were found in leaf under both SD and LD conditions but found in bulb under both conditions at the right product size as expected (642 bp), which might be similar to the expression of *Arabidopsis LFY* gene at the shoot apical meristem which together with *AP1* triggers the expression of the floral homeotic genes at the floral apical meristem and cause flowering (Yoo et al., 2005). In onion, the meristem is in the basal plate, which would be in the bulb samples. *GA3ox1*, was only found for SD leaf at the right product size as expected (644 bp) but not found for SD bulb, LD leaf and LD bulb. *FST-1, GGTF, FT-LIKE*

PROTEIN 1 and 2 mRNA bands were found in all 4 samples under both SDs and LDs.

Sequence comparison of differentially expressed genes

The onion partial cDNA sequences of differentially expressed genes obtained from gene isolation were blasted against an onion Expressed Sequence Tag (EST) database (www.ncbi.nlm.nih.gov/nucest/?term=onion) to determine similarity (Table 1).

Isolation of *FLOWRING LOCUS T* genes

This experiment was performed to identify different *FT* genes in Renate F1 onion grown under SD and LD photoperiodic conditions (Figure 3). Five out of 6 *FT* genes were isolated and detected in Renate F1 onion using the primers described in Lee *et al*., 2013 (Table 2). They were shown to represent the expected gene through sequencing of PCR products. *FT2* was not detected and *FT3* shared 83% identity with *FT5*. The reason might be due to the use of different materials (Lee et al. 2013). *FT-LIKE PROTEIN 1* and *FT-LIKE PROTEIN 2* genes were also detected in Renate F1 onion which is similar to *FT6* and *FT5*, respectively and which might have effect on bulb formation. All *FT* sequences were compared with Renate F1 onion transcriptome reference sequences using MegAlign and present their similarity in Table 2.

RNA-Seq analysis

RNA-Seq analysis was performed to generate onion transcriptome reference sequence and for more widespread identification of genes differentially expressed in response to photoperiod. All sequences obtained were used for onion gene assembly with the assistance of the Life Sciences Bioinformatics support officer. 12604 significantly differential expressed transcripts were detected in long day (LD) leaf vs bulb, 13665 in short day (SD) leaf vs bulb, 484 in SD leaf vs LD leaf and 964 in SD bulb vs LD bulb of onion. Red colours in heat maps show up regulation and green colours showing down regulation of the transcripts. Comparison of transcriptome sequences with sequence databases allowed to identify sequences for genes that have known function in the day length regulation of flowering, e.g. including *FT, CO, FLC, TEM, FD, SOC*. Three *COL* genes including two novel sequences (*COL2* & *COL3*) identified through RNA-Seq analysis. In addition, comparison of transcript abundance between the long-day and the short-day treatment would enables to identify genes involved in the bulbing process itself.

CONCLUSIONS

Day length dependent bulb initiation and development were characterised in Renate F1 (LD) onion and at least 14 h of light are required to initiate bulbing. Bulbing ratio increased in plants transferred to LD or kept in NC but not in those transferred to SD. Twenty-two key genes were identified in Renate F1 onion and eight were shown to be differentially expressed in leaf and bulb under LD and SD conditions. Five out of 6 *FT* genes were isolated and detected in Renate F1 onion transcriptome sequence. *FT2* was not detected and *FT3* shared 83% identity with *FT5*. *FKF1* gene was isolated in bulb materials. *LFY* was found in bulb under both LD and SD conditions. *GA3ox1* was only found in leaf of SD condition. *FST-1, GGTF, FT-LIKE PROTEIN 1 and 2* genes were found in leaf and bulb of both LD and SD conditions. Differentially expressed transcripts and two new *COL* genes were identified in onion by RNA-Seq analysis.

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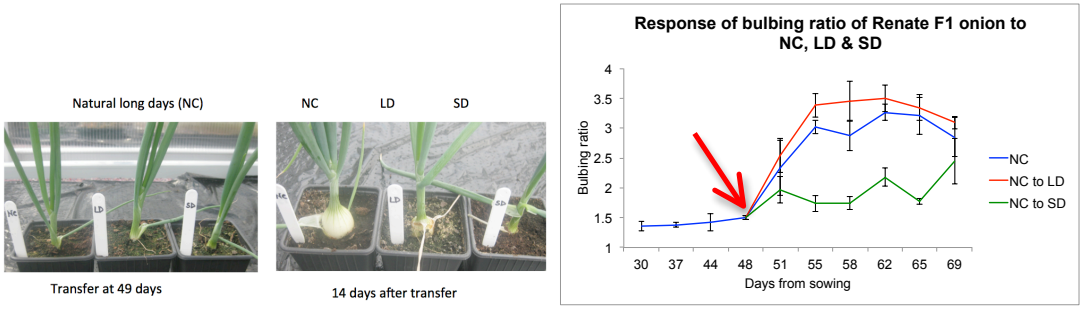
Figures:



a) b)

Figure 1. a) Bulbing response of Renate F1 onion and b) Comparison of Renate F1 onion grown under different day lengths at different stages of development. Error bars represent the SEM & red arrow indicates transfer point. Three plants were used in each data point.

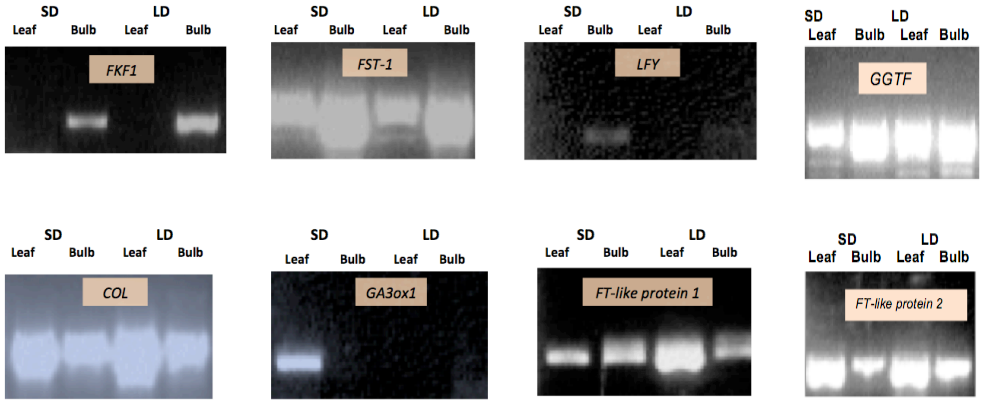


Figure 2. Gel red stained agarose gel showing onion genes differentially expressed in leaf and bulb materials grown under SD and LD conditions.

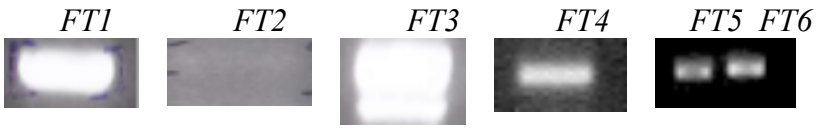


Figure 3. Gel red stained agarose gel showing onion genes expressed in pooled samples of leaf and bulb grown under SD and LD conditions. (*FT: FLOWERING LOCUS T*).

Tables:

Table 1. Similarity between onion EST and onion partial sequence obtained in Renate F1

Gene	Onion partial cDNA sequence obtained from gene isolation (bp)	Onion partial EST sequence (bp)	Similarity between onion EST and onion partial sequence obtained	
			% Nucleotide	% Amino acid
FKF1	814	2054	99	98
FST-1	813	1830	97	99
LFY	642	1081	99	98
GGTF	936	1905	99	97
COL	522	1109	99	100
GA3OX1	645	1208	97	98
FT-LIKE PROTEIN 1	515	807	99	99
FT-LIKE PROTEIN 2	579	834	100	100

FKF1: FLAVIN-BINDING KELCH REPEAT PROTEIN, FST-1: SUCROSE 1-FRUCTOSYLTRANSFERASE-LIKE, LFY: LEAFY, GGTF: GAMMA-GLUTAMYLTRANSFERASE, COL: ACABR20 CONSTANS-LIKE PROTEIN, GA3OX1: GIBBERELLIN 3-OXIDASE, FT-LIKE PROTEIN: FLOWERING LOCUS T-LIKE)

Table 2. Primers used to detect *FT* genes and their similarities with Renate F1 onion transcriptome sequence

Gene	GeneBank ID	Forward and Reverse primer sequences (5'…..3')	Product size (bp)	Nucleotide sequence length (bp)		Similarity between *FT* sequence in NCBI and Renate F1 onion transcriptome sequence	
				Genes (NCBI web)	Transcriptome seq.	% Nucleotide	% Amino acid
AcFT1	KC485348	TTACATGGCAAGAGAAAGTGACCCAT CCTATTAGTAATCCGTGTATATTCT	545	726	1821	80	-
AcFT2	KC485349	AAGGATGATGGATTCGGATCCGTTA CCATTCATCTATAAGTTCTCCTCCCA	534	572	459	100	94.8
AcFT3	KC485350	GGGAATGTTGTAGGCGATGTTT AGCCGTTTCCTGGTCGATACCT	513	745	921	84	85.3
AcFT4	KC485351	CACGATGTCTTTTGATCCTTTAGTT TGTGCTAATTCTCTGATCGAAACCTT	534	539	773	100	100
AcFT5	KC485352	TTCCATGTCAAGAGATCCTCTTGTT AGTGTCAGAGCCAGCCACTTCCT	548	604	921	100	100
AcFT6	KC485353	ATACATGCAAGTAAAAATGTTGCGA GCAGTCAGCAAAGCCCCGAGAACCT	566	841	360	99	97.8