Gene Expression and Daylength Regulation of 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.

ABSTRACT

Onion bulb initiation is photoperiod-dependent, which is crucial for adapting new varieties for growth at different latitudes as well as aiding germplasm screening for choice of current varieties. Although much is known about the physiological aspects of onion development, little work has been done on the genetics. This project primarily aims to gain further understanding of the molecular mechanisms involved in the onion bulbing process based on the parallels with well characterised functional clock genes in Arabidopsis flowering pathway, and additionally to identify some novel genes differentially expressed in bulb tissues. A comprehensive set of spatial, diurnal and developmental quantitative expression experiments were carried out to investigate the bulbing response with different onion varieties under varied day-lengths.

All onion homologues of *Arabidopsis thaliana* flowering time genes showed the spatial expression patterns consistent with their role in Arabidopsis, together with clear diurnal expression patterns peaking at different times of the day for both long/short-day onions, indicating that their role in a daylength dependent bulbing process at the molecular level. Particularly, under intermediate daylengths, *AcFT1* expression level increased with daylength while *AcFT4* was highly expressed in all daylengths. Both genes were expressed in longer days, indicating that *AcFT1* is the predominant partner in the negative co-regulation of bulbing by the two genes.

Developmental time-course experiments detailed the gene expression in onion leaf and bulb tissues during the development of a long-day onion cultivar under long-day and short-day conditions. Both *AcFT1* and *AcFT4* showed significant increasing expression level during plant development, whereas *AcFT1* was expressed in long-days with *AcFT4* in short-days. The data further support the role of *AcFT1* as a promoter and *AcFT4* as inhibitor in onion bulb formation. In addition, a number of genes associated with onion carbohydrate metabolism, sulphur metabolism and bulb development were identified as having different tissue-specific expression and provide targets for future studies.

LIST OF ABBREVIATIONS

AcCOL1	*Allium cepa CONSTANS LIKE 1*
AcCOL2	*Allium cepa CONSTANS LIKE 2*
AcCOL3	*Allium cepa CONSTANS LIKE 3*
AcFKF1	*Allium cepa FLAVIN-BINDING, KELCH REPEAT, F-BOX 1*
AcFT1	*Allium cepa FLOWERING LOCUS T 1*
AcFT2	*Allium cepa FLOWERING LOCUS T 2*
AcFT4	*Allium cepa FLOWERING LOCUS T 4*
AcFT5	*Allium cepa FLOWERING LOCUS T 5*
AcFT6	*Allium cepa FLOWERING LOCUS T 6*
AcGI	*Allium cepa GIGANTEA*
AHCYL1	adenosylhomocysteinase-Like Isoform 1
AcLFY	*Allium cepa LEAFY*
AcTUA	*Allium cepa ALPHA TUBULIN*
AcTUB	*Allium cepa TUBULIN*
ALL	*ALLINASE*
AP1	*APETALA*
B locus	bolting gene locus
bp	base pairs
CDF1	*CYCLING DOF FACTOR 1*
CDL	critical day length
CE	controlled environment
cDNA	copy DNA
CO	*CONSTANS*
COL	*CONSTANS LIKE*
COP1	*CONSTITUTIVE PHOTOMORPHOGENIC 1*

CTAB	cetyltrimethylammonium bromide
d	day
DFS	days from sowing
DFS	defensin
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ECT	endochitinase
EDTA	ethylenediaminetetraacetic acid
EST	Expressed Sequence Tag
et al.	and others
FKF1	*FLAVIN-BINDING, KELCH REPEAT F-BOX*
FT	*FLOWERING LOCUS T*
FFS	beta-fructofuranosidase
Fru	fructose
GCL	glutamate--cysteine ligase
GI	*GIGANTEA*
Glu	glucose
GST	glutathione S-transferase
Hd1	*Heading date 1*
Hd3a	*Heading date 3a*
h	hours
H2A	histone H2A
ID	intermediate day
kb	kilobase pairs
LB	long-day bulb
LD	long day
LFS	*LACHRYMATORY FACTOR SYNTHASE*

LFY	*LEAFY*
LL	long-day leaf
MADS	MADs-Box transcription factor
Mbp	Megabase pairs
$MgSO_4$	magnesium sulphate
min	minutes
miR172	microRNA172
MLP	major latex protein
MRF1	MORN-motif repeat protein
mRNA	messenger RNA
NaOAc	sodium acetate
NC	natural Condition
NCBI	National Centre for Bioinformatic Information
PBF	Phytobiology Facility
PCR	polymerase chain reaction
PER12	peroxidase 12
PHYA	*PHYTOCHROME A*
PHYB	*PHYTOCHROME*
POLYCOMB	Polycomb Group Protein Embryonic Flower
PPFD	photosynthetic photon flux density
PP2A1	*PROTEIN PHOSPHATASE TYPE 2A*
PP2AA3	*PROTEIN PHOSPHATASE 2A REGULATORY SUBUNIT 3*
qRT-PCR	quantitative real-time PCR
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
RPL34	60S Ribosomal Protein L34

RT-PCR	reverse transcription- polymerase chain reaction
SAUR	small auxin up RNAs family
SB	short-day bulb
SD	short day
SDW	sterile distilled water
sec	seconds
SL	short-day leaf
SST1	sucrose 1-fructosyltransferase
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*
SPA1	*SUPRESSOR OF PHYA-105*
TIC	*TIME FOR COFFEE*
TIP-41	*TONOPLASTIC INTRINSIC PROTEIN-41*
TPS	trehalose-6-phosphate synthase
TSF	*TWIN SISTER OF FT*
TUBA2	tubulin alpha-2 chain
UBC9	*UBIQUITIN CONJUGATING ENZYME 9*
UBQ1	*UBIQUITIN EXTENSION PROTEIN*
VeGIN	Vegetable Genetic Improvement Network
v/v	volume by volume
w/v	weight by volume
ZT	zeitgeber time
ZTL	*ZEITLUPE*
1-FFT	fructan 1-fructosyltransferase
6-SFT	fructan 6-fructosyltransferase

1 INTRODUCTION

1.1 Introduction to onion

1.1.1 Classification and history

The onion (*Allium cepa* L.) is a subspecies and primary member of the genus *Allium*, and is a monocotyledonous flowering plant belonging to the order Asparagales (Stevens, 2008; Kuhl et al., 2004). Other important plants in the order include asparagus, garlic, leek, Aloe, orchids and Narcissus. Onion belongs to the family *Liliaceace* and the genus *Allium* (Table 1-1) (USDA Plant Database, 2019). The genus *Allium* was first described in 1753 by Linnaeus, refer to Greek αλεω (aleo, to avoid) by reason of their smell (Boswell, 1883). It comprises over 700 species including plants such as garlic (*A. sativum* and *A. scordoprasum*), shallot (*A. ascalonicum*), leek (*A. porrum*), or chive (*A. schoenoprasum*). Onions belong to the section *cepa* (Brewster, 2008). The plant's name of "onion" comes from the Latin *unio*, or *annianus*, and is associated with the Welsh einion, meaning "anvil" (Jones, 1963). There are other plants in the genus *Allium* that also have the common name of onion, such as the Welsh onion (*A. fistulosum*). But when used without qualifiers, onion usually refers to *Allium cepa*, also known as the "garden onion" or the "bulb onion", which is characterized by an edible, rounded bulb composed of concentric, fleshy, tightly packed and enlarged leaf bases.

Onion is believed to have originated in the Near East, which has the ancient history of tillage as of man themselves. It is frequently recorded in the old scriptures of both the Bible and Coran. In ancient times, alliums were not only grown for food but also known as ornamental onions, which were grown for their showy flower heads. Even when the plants die back, the dried flower heads look attractive in the garden. Onions grown for food made their way to Egypt via trade, where they became a crucial food plant in the ancient world. Egyptian slave laborers, those who constructed the pyramids, consumed them on a daily basis (Platt, 2003). Ancient Sumerians widely grew and cooked onions 4000 years ago, and the plant has been discovered at the royal palace at Knossos in Crete (Estes, 2000). The ancient Greek physician Hippocrates

wrote in the fifth and fourth centuries B.C. that a broad variety of onions were eaten regularly in Greece (Estes, 2000). The onion in Britain was introduced by Romans, then it may have been carried to the Americas (Burnie et al., 1999). However, there have also been those who have rejected and detested onions. In ancient India, there was a fair amount of revulsion to onions. Orthodox Brahmins, Hindu widows, Buddhists and Jains regarded onions as forbidden vegetables for their strong odour and stimulating action (Brewster, 2008).

Table 1-1 Botanical classification of Allium cepa (USDA Plant Database)

Kingdom Plantae – Plants
Subkingdom Tracheobionta – Vascular plants
Superdivision Spermatophyta – Seed plants
Division Magnoliophyta – Flowering plants
Class Liliopsida – Monocotyledons
Order Asparagales
Family Liliaceae
Genus *Allium* L. – onion

1.1.2 Worldwide cultivation and economic importance

Today cultivated *Allium cepa* is placed into one of two horticultural groups: The Common Onion group and the Aggregatum group. The former contains the typical bulb onion as well as the majority of existing cultivars. Onions in this group show dramatic variation in colour and shape, photoperiodic response, storage quality and pungency, as well as many other characteristics. Their bulbs are usually large and single. Species assigned to the Aggregatum group have active lateral bulbs, and thus form clusters of smaller bulbs. One subdivision of this group, multiplier onions, consists of varieties that can contain as many as twenty small, short and wide bulbs (Brewster, 2008).

In terms of the Common Onion varieties grown for their bulbs, they are now cultivated under a worldwide geographic range from temperate to tropical regions, and are important vegetable crops in most parts of the world. According to the latest statistics from FAO (Food and Agriculture Organization of the United Nations), present world production of dry onion is about 93.2 million tons of bulbs per annum from 4.9 million ha, and has been generally increasing for the past few decades (Figure 1-1). In the light of global weight of vegetables produced, only tomatoes exceed bulb onions in importance (Table 1-2). Biggest producers are China, India, Egypt and United States, accounting for about half of the world's dry onions production. Other countries with top annual production of 2016 are also shown in the figures (Figure 1-2, Figure 1-3).

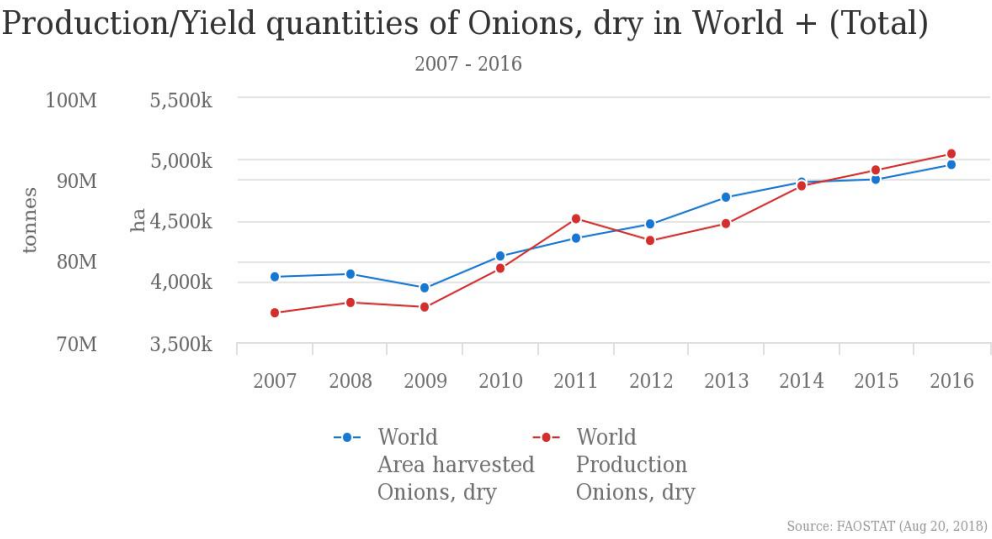


Figure 1-1 Increasing world production of onions, dry: 2007-2016 (FAOSTAT, 2018)

Table 1-2 Vegetable crops with top world production 2016 (FAOSTAT, 2018)

Area	Element	Item	Year	Production (tonnes)
World	Production	Tomatoes	2016	177042359
World	Production	Onions, dry	2016	93168548
World	Production	Cucumbers and gherkins	2016	80616692
World	Production	Cabbages and other brassicas	2016	71259199
World	Production	Eggplants (aubergines)	2016	51288169

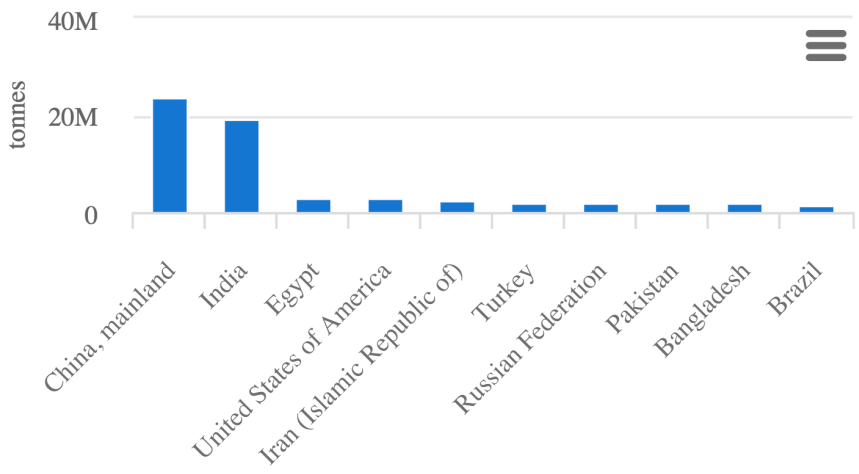


Figure 1-2 Production of onions, dry: top 10 producers of 2016 (FAOSTAT, 2018)

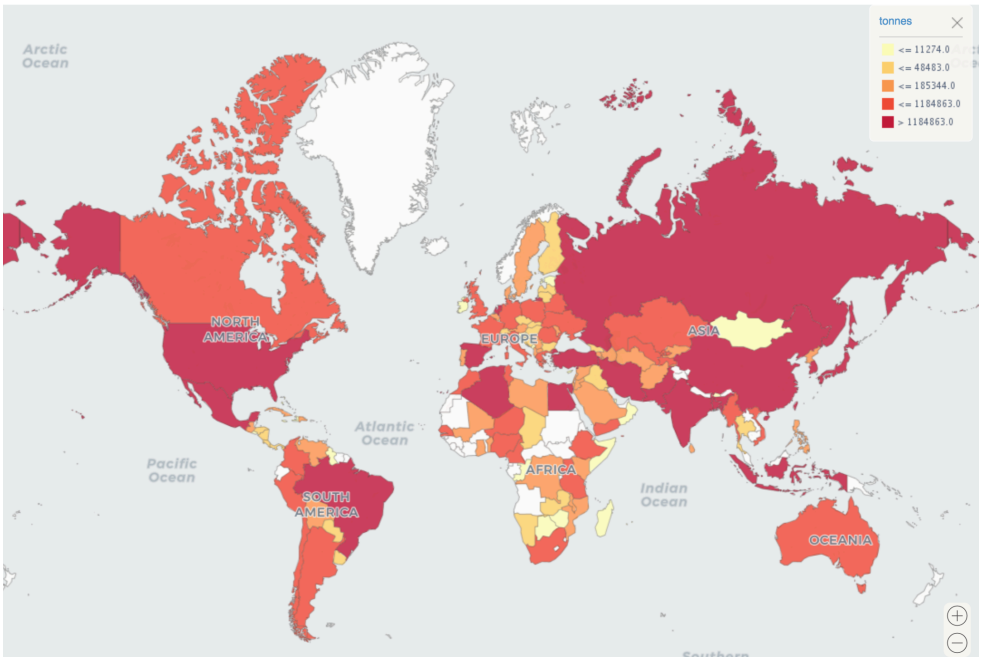


Figure 1-3 Production quantities of onions, dry by country, 2016 (FAOSTAT, 2018)

Onion trade is of great importance in the world-wide market. Unlike many other vegetables, onion bulbs can be transported for considerable distances without deteriorating if dried and packed properly. The post-production sector of onions accounts for about 60% of the economic activities in most developing countries, including the growing, handling, storage and marketing. In many growing regions, it

is a major source of income for rural families who sell their produce in local, regional and international markets (Table 1-3).

Table 1-3 Economic importance of onion trade (FAOSTAT, 2018)

	2012	2013	2014	2015	2016
Production (tonnes)	82492053	84577917	89216889	91170161	93168548
Area harvested (ha)	4468685	4688182	4811459	4834452	4955432
Export Quantity (tonnes)	6534224	7007820	6935588	6890034	7299372
Export Value (1000 US$)	2416987	3265754	2781683	3068336	2951710

1.1.3 Flavour and potential health benefits

For centuries, onions have been used to reduce inflammation and heal infections. As early as 1932 the *British Medical Journal* was noted by Stobie (1932) "Rub site of sting gently with a raw cut for ten minutes or so. The effect is dramatic, and the swelling goes down under observation. An onion should be in every business basket" (Block, 2010). Nowadays the major value of this crop product is in the kitchen for culinary use, for its characteristic flavour and odour. Besides their unique flavour, onions are highly valued for their nutritional value in supplying minor constituents such as minerals and trace elements of several health-related phytochemicals.

The nutritional value of onions can vary depending on the variety, growing conditions and climate. In general, onion has very low contents of lipids and sodium, and is cholesterol free, while still being a good source of vitamins and mineral content (vitamin C, B6, folic acid, calcium, magnesium, phosphorus, potassium and mineral chromium) (Estes, 2000). With regards to amino acid content, only arginine and glutamic acid are extraordinarily high. Onion has three diverse and highly valuable families of phytochemicals in its unique combination: organosulfur, fructans and flavonoids. These three families of compounds working independently or in perfect proportion in onions, contribute to several salutary effects on human health.

Organosulfur compounds

Onion plant smells when crushed (WHO, 1999). Organosulfur compounds (OSCs) is the reason for the pungent smell and flavour. In onions, over 70% of the total OSCs in a whole bulb is comprised of (+)-S-alk(en)yl-L-cysteine sulfoxide and γ-glutamyl peptide (Lawson, 1996). Allium plants (onions, shallots, leeks) contain high concentrations of alk(en)yl cysteine sulfoxides (ACSOs), and when the plant tissue is damaged (cut or crushed), the ACSOs are enzymatically degraded by the enzyme alliinase. This leads to the formation of new organosulfur compounds which cause the odour and flavour of onions, such as cepaenes and thiosulfinates (Dorsch and Wagner, 1991; Goldman et al., 1996). Simultaneously, γ-glutamyl cysteine is also converted to different organosulfur compounds, including *S*-allyl cysteine and *S*-allyl mercaptocysteine (Block, 1985). Evidence from several investigations has proved the medical functions of organosulfur compounds found in allium crops (Augusti and Mathew, 1974). Because of the presence of sulfur–oxygen or sulfur–sulfur linkages, the OSCs can work as anti-inflammatories, antiallergenics, and antimicrobials (Augusti, 1996; Block et al., 1997).

Flavonoids

Onion is also the richest source of dietary flavonoids, which are part of the polyphenol family and could also function as an antioxidant in human body. Onion flavonoids contribute to a large extent to the overall intake of flavonoids, and possess crucial biological roles. There are two types of flavonoid subgroups found in onion: the anthocyanins in red and purple varieties, and the flavonols such as quercetin in yellow and brown varieties. The high levels of quercetin provide the most abundant flavonoid in the human diet, deactivating molecules that are injurious to cells and delaying oxidative damage in bodily tissues (National Onion Association, 2011). The layers of onion (especially the dry outer layer) contain large amounts of quercetin (Gülşen et al., 2007), which are effective antioxidants against non-enzymatic lipid peroxidation and oxidation of low-density lipoproteins (LDL). The flavonoids in onions also showed strong benefits in other health maintenance, like antiviral, antimicrobial, anti-inflammatory, and anticancer activity, as well as protection of the heart and brain (Sagar et al., 2018).

Fructans

Fructans (polysaccharides) are small carbohydrate molecules and the principal type of carbohydrates storage in onions. In a study of 60 common vegetables, onions were reported to have the highest quantity of fructans (Roberfroid, 2004). They are nonreducing carbohydrates composed of fructosyl units and terminated by a single glucose molecule. Fructans can contribute to human health through multiple mechanisms despite their resistance to hydrolysis by human digestive enzymes. They can help maintain gastrointestinal health by sustaining beneficial bacteria. Studies show that fructans can be fermented by the colonic microbiota to produce short chain fatty acids (SCFAs), metabolic by-products that possess immunomodulatory activity (Franco-Robles and López, 2015). Besides, fructans are reported to have a role in several biological and pharmacological activities. The established beneficial properties include antifungal, antibacterial, antitumor, anti-inflammatory, antithrombotic, and hypocholesterolaemia (Lanzotti, 2006; Kumari and Augusti, 2007), as well as a part in treating diabetes for the researches show that fructans in onions can lower insulin levels and improve tolerance for glucose (Dey et al., 2002; Liu, 2007).

There are many other perceived health benefits associated with onion consumption. The healthy phytochemicals in onions work individually or combined together and have been used in many medicinal practices. The combination of sulfuric compounds and vitamin B6 work together to drop blood pressure, reduce the risk of stroke, prevent atherosclerosis and heart disease, by helping to lower bad cholesterol and raise good HDL-cholesterol (high-density lipoproteins cholesterol). Clinical trials also found that consuming onions increases oxylipins that help regulate blood fat levels and levels of cholesterol (González-Peña et al., 2017). Furthermore, evidence has been shown that regular consumption of onions helps greatly lower the risk of several common cancers, fight inflamed joints and osteoporosis and give a boost to bones due to the powerful compounds (Winston and Beck, 1999). Overall, the numerous potential health benefits together with the widespread consumption of onions further highlights the importance of this crop.

1.2 Physiology of the Onion Plant

1.2.1 Growth and lifecycle

Onion is a biennial crop. It only flowers and sets seeds in the second year after a long period of cold over the winter months to satisfy a vernalisation requirement. In the first-year spring, onion seed germinates one or two weeks after planting, and sends up leaves that photosynthesize to produce energy as the plant grows. Each leaf is composed of a photosynthetic leaf blade and a non-photosynthetic storage leaf base. During late spring and summer, leaves arise alternatively from a small flattened stem (base plate) from out to in. So that old leaves are on the outside and younger ones are on the inside of the stem. During its growth, the base of the leaves begins to swell, the leaf scales thicken and form the character of bulb (Figure 1-4). The energy by photosynthesis maintains plant growth during spring, summer and autumn. Meanwhile, leaf sheaths continue to swell, bladeless bulb is developed as central storage tissue. The base continues to develop to its full expansion, and the green leaves top down and die back. Onions grown for food are usually harvested in the late autumn when the bulbs reach a particular size. Mature onion bulbs can range in size from 5 mm to over 100 mm bulb diameter (Lancaster et al., 1996).

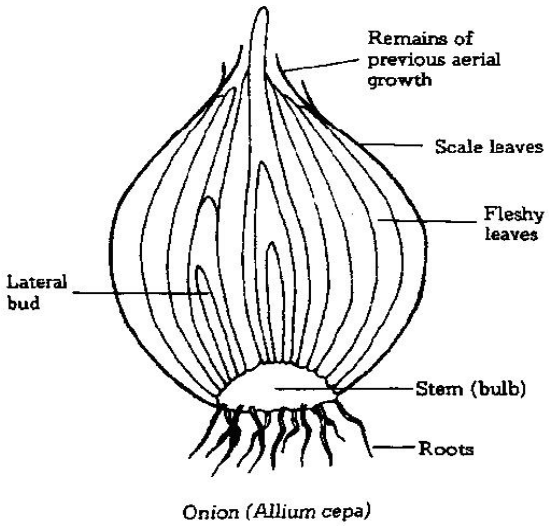


Figure 1-4 Bulb structure of a temperate onion.

However, if a plant is left in the ground without being harvested, the bulb functions as a vegetative overwintering stage in the life cycle. The onion uses the energy stored in its basal part to stay overwinter. When the temperature warms up during the second year, dormancy is broken in the following spring. Then onion sends up a flower stalk in response to the temperature and daylength signal. After the flower blooms and being pollinated during summer, the seeds are developed for future generations (Figure 1-5).

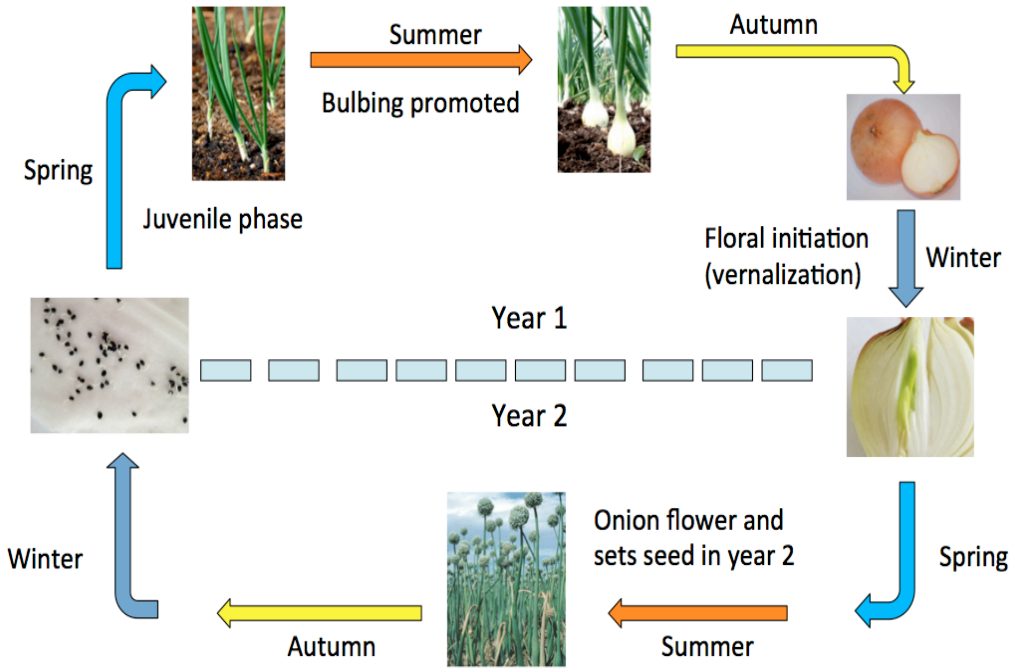


Figure 1-5 Life cycle of temperate onion (Brewster, 2008)

Bulb initiation can be quantified using the "bulbing ratio" (maximum bulb diameter/minimum sheath diameter, Figure 1-6). When this value increases to a value greater than two, bulbing is considered to have been initiated (Clark and Heath, 1962). The bulb diameter is usually measured in two different planes to allow for the fact that not all bulbs are round. Different methods of quantifying bulb initiation have been described, but the bulbing ratio is the preferred method as it can be measured non-destructively.

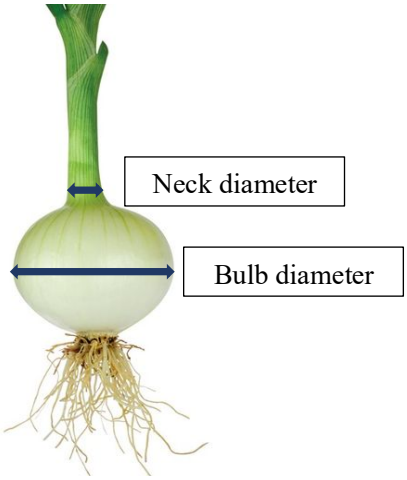


Figure 1-6 Measurements for the calculation of bulbing ratios

1.2.2 The physiology of bulb initiation and crop yield in onion

The target of onion production is a high yield of high-quality produce, which is the end result of many processes of plant growth and development (Brewster, 2008). The well-established principals that determine crop yield can also be applied to onions. Several factors are involved in affecting different phases of the growth of a bulb onion crop (Table 1-4).

The quality of light absorbed has a major effect on bulb initiation (Austin, 1972), which depends on the quantity of light irradiating the field, the duration of the growth phase in order to transfer photosynthate to harvestable material, and the percentage absorbed by leaves during the season of high solar irradiation (Brewster, 2008). It is known that phytochrome has a role in the photoperiodic control of bulbing (Lercari, 1984; Sobeih and Wright, 1986), and high levels of far-red light accelerate bulbing (Mondal et al., 1986). Bulbing will not be initiated under high red: far-red ratios.

The efficiency of photosynthate converted into sucrose is also important. The conversion from absorbed light to primary photosynthetic products can be affected by the temperature and water status of the leaves (Brewster, 2008). If leaves are under water stress, stomata would be closed causing increasing diffusive resistance to CO_2 entry, which also reduces photosynthetic efficiency (Brewster, 2008). The highest

photosynthetic efficiency only occurs with suitable temperature. Slightly elevated day or night temperatures have been shown to accelerate bulbing (Steer, 1980). A minimum thermal time has been suggested as the major factor controlling bulbing especially in short-day onions (Lancaster et al., 1996).

The optimum harvesting time which affects the proportion of photosynthetic output transferred to the harvested fraction is also crucial for growers. It has been reported (Brewster et al., 1986) that the harvesting would be taking place when 80% of plants have 'soft necks' (about 80% of dry shoot weight). However, if harvested two weeks later, an average of 89% of the shoot weight in the bulb can be reached.

For onions, the edible parts contain primarily simple sugars and fructan storage in bulbs, and the structural carbohydrates and protoplasmic constituents typical of most plant cells. These components will vary under different growth environments. The conversion coefficient between photosynthetic sucrose and the biochemical constituents is important, as we expect carbohydrate-rich tissues with high dry biomass weight rather than photosynthate utilization. It has been shown that this conversation efficiency is low in conditions of high light levels and high temperature, and high in lower irradiance and lower temperatures (Brewster et al., 1986). Likewise, the same study has shown a lack of irritation in dry weather could decrease this conversion efficiency, causing the weight losses due to respiration.

Table 1-4 Summery of the main phases of growth of a bulb onion crop (Brewster, 2008)

Growth Phase	← Emergence →	←Leaf Growth→	←Bulbing →
Usual Rate Controlling Factors	Temperature	Temperature	Photoperiod and temperature
Constraints	Soil water Potential above 1.1MPa	Water and nutrients Plentiful; no disease, pests or damage	Modified by leaf area index (red: far-red)

1.2.3 The bulbing process and the factors affecting it

Despite their broad geographical distribution, onions are very sensitive to climate. In Europe, onions had to be selectively adapted to local growing conditions. Onions require a number of specific conditions in order to achieve optimal growth, including bountiful sunshine, stone-free, loamy, well irrigated soil, excellent drainage, and necessarily, sufficient plant age. All these elements will affect flavour and bulb development. Early in 1986, Sobeih and Wright suggested that onions would not initiate bulbing until they have at least four leaves, implying the crop will not form bulbs until after the presence of a juvenile phase even under inductive conditions. Other factors most intensively studied are temperature and photoperiod (Brewster et al., 1977). It has been well known for decades that the bulbing process is promoted by long days and high temperatures (Magruder and Allard, 1937; Heath, 1945; Kato, 1964). Steer (1980) showed that elevated day or night temperatures could accelerate bulbing in phytotron-grown plants. Austin (1972) and Lercari (1984) showed that far-red light was necessary during onion bulb initiation. The multi-factor experiment (Lancaster et al., 1996) with three onion cultivars showed clear result that bulb initiation required a combination of a minimum thermal time and a minimum daylength. This suggests a major role in bulb initiation was the dual thresholds of temperature and photoperiod, while none of either weed competition, density, fertilizer or water were limiting.

One of the two factors that has been widely studied is the photoperiodic nature of onion bulbing process. The strong linkage between onion bulb formation and day length means the formation and quality of onion bulbs are highly influenced by the duration of days and nights. The concept of critical daylength (CDL) is defined as the point at which the photoperiod switches from being noninductive to inductive, which means that bulb initiation can only be triggered once the CDL is reached for the plant. The value of the CDL of onions varies among different varieties. The varieties of onion that require a shorter period (10 to 12 hours per day) of daylength to form bulbs are defined as ‘short day’ (SD) variety. Those that require the longest period of daylight (longer then 14 hours per day) to bulb are defined as ‘long day’(LD) variety. Those with the intermediate daylight requirements (12 to 14 hours per day) are defined as ‘intermediate’ (ID) variety. The formation of bulbs can only be triggered once the

onion plant is exposed to their CDL over a period of days. Studies (Mallor and Thomas, 2008) showed that the LD onion variety formed bulbs in long-day condition, whilst continued to grow leaves in short daylength condition. Under intermediate daylength conditions, the short-day onion variety successfully formed bulbs as the CDL is sufficient, whilst a long-day onion variety failed undergo bulbing because the CDL provided is not enough to trigger the formation of bulbs.

1.2.4 The distribution of onion varieties

The concept of CDL explains why latitude makes a difference with the distribution of onion varieties (Figure 1-7) (Thomas et al., 2006). It is common sense that farmers and gardeners choose their onion types depending on latitudes. In the higher latitudes, due to the long distance from the equator, daylight length varies greatly throughout the year. Although winter days are very short, summer days are long. So long-day onion varieties will have a chance to produce abundance vegetative top growth before the day length triggers bulbing. These long days before bulb initiation contribute to larger bulb size. If a short-day onion variety was grown in these areas, the plant would start bulbing too early before having a chance to grow very big, also a few small leaves could not produce a good size bulb. However, in the lower latitudes, there is much less variation in day length between seasons. If long-day onion cultivars were planted near the equator, they would not experience enough day length to trigger the bulbing process, plants continue to grow leaves without swollen at bases and the crop would not bulb at all. In the United Kingdom, LD cultivars are mostly planted. The reason for this being that the longest summer daylength in the UK is between 16 and 18 hours, depending on location.

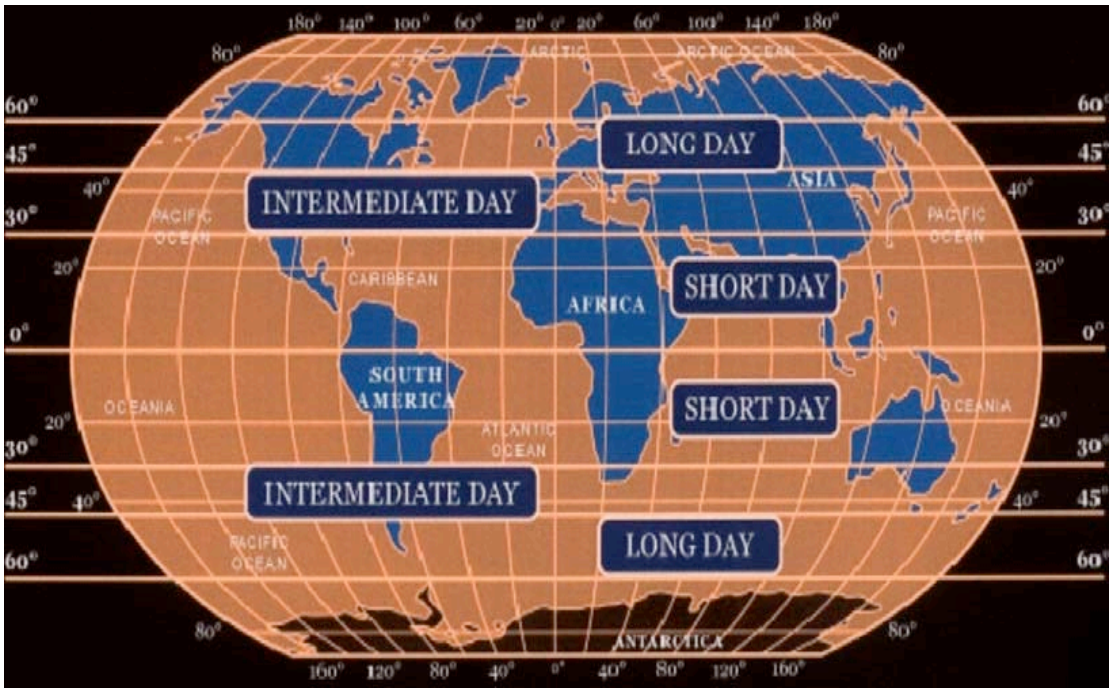


Figure 1-7 World distribution of onion varieties

1.2.5 The onion genome

In terms of genetics and genomics, relatively little is known about the onion genome compared to many other crops. This is partly due to the huge size of the onion genome (32pg/2n). Onion in a diploid plant (2n=2x=16) with a nuclear genome of 16,415 Mbp per 1C, approximately equal to hexaploid wheat and 34 and 6 times larger than rice and maize, respectively (Arumuganathan and Earle, 1991), whilst with a low base chromosome number. It is reported that the level of duplication in the onion genome is greater than many other diploids such as Arabidopsis, barley, tomato and rice (King et al., 1998). The relatively high level of duplication may suggest that the onion is of polyploid origin. Sequencing data from SEQUON (Sequencing the Onion Genome) project (Finkers et al., 2015) presented a great number of unexpected contigs suggesting that the onion genome contains a large number of ancient repeats which are distributed all over the genome. Significant initial efforts were made by King et al. (1998) to bring about the first publicly available RFLP (restriction fragment length polymorphisms) based genetic map of onion. The data indicates that tandem, transpositional, or retropositional duplication of specific chromosomal regions and possibly subsequent rearrangements have occurred in onion's large diploid genomes.

There are several studies suggesting tandem duplication of chromosomal segments being an important factor in the evolution of large-genome *Allium* species for showing high percentage of repetitive DNA (McCallum et al., 2001; Kuhl et al., 2004).

Additionally, an onion EST database has been created where sequencing was carried out in order to assess the genomic differences between the Asparagales (of which onion is a member) and the Poales (e.g. rice, barley), the two most economically important monocot orders (Kuhl et al., 2004). Subsequently, several other genetic maps of onion using AFLP (van Heusden et al., 2000a, b) and RAPD (Shigyo et al., 1997; Friesen and Klaas, 1998) markers have also been reported. These are now available online and is known as the *A.cepa* Gene Index (DFCI, 2008). The material used for the construction of a normalised cDNA library originated from RNA isolated from leaf, bulb, root and callus tissue.

In recent years, studies have started selectively targeting some of the genes affecting prominent trait loci, like for bulb colour (Kim et al., 2004; Khar et al., 2008), carbohydrate assimilation (Masuzaki et al., 2006; McCallum et al., 2006; Yaguchi et al., 2008), flavonoid biosynthesis (Masuzaki et al., 2006), pungency (McCallum et al., 2007, 2011; Thomas et al., 2011; McManus et al., 2012) lachrymatory factor synthesis (Masamura et al., 2012), and bulb initiation and flowering (Lee et al., 2013). However, sequencing of onion whole genome still remains challenging and yet to be done for its largest size and poor density of single genes (Shukla et al., 2016).

1.2.6 Genetic transformation in onion varieties

As the given importance of onion as a crop, varieties are commonly grown worldwide both open-pollinated and hybrid cultivars. Hybrids are predominating in most regions of the world and seeds are produced using the system of cytoplasmic male sterility (CMS). Most onion breeders focus on some important bulb traits such as pungency and flavour, health related phytochemicals (flavonoids, anthocyanins, fructo-oligosaccharides and organosulphur compounds), as well as some plant characteristics like pests and disease resistance or bolting resistance. However, due to the biennial generation time, as well as onion's large genome size, cross-pollinated nature and

high inbreeding depression, the development of expected hybrids for the crop have been time consuming and expensive process (McCallum 2007). The current utilization of the biotechnological approaches is now focusing on marker-aided selection, production of double haploids, gene editing and cytoplasmic conversions (Harvey, 2018).

Marked-aided selection (MAS) with desirable traits has been widely utilized among breeders. The markers would allow for the precise identification of plants with the target genetic constitution at an early stage of development enabling the crossing or selfing of the plants only with the desired genotype (Harvey, 2018). MAS is an indirect selection where the selection of interest in based on a maker rather than the trait itself.

Doubled haploids (DHs) performed similarly to sexually derived inbred in hybrid combinations (Hyde et al., 2012), whilst DH plants are derived from a single pollen grain and doubled artificially to form homozygous diploids. The study of hybrids using DHs as parents found the hybrids to be found uniform and promising compared to conventionally developed hybrids (Alan et al., 2004). In onion breeding programme, a heterotic superiority of DH derived hybrids has been reported for mean row weight and mean bulb weight (Hyde et al., 2012). DHs have been successfully utilized in onion genomic research for identification of gene and genomic regions affecting quality traits like colour, restorer of male sterility (Ms) locus, bolting, fructan content etc (Khar et al., 2019). In terms of commercial production, despite DH lines are successfully used in numerous crops, onion breeding programs have not yet exploited their potential.

In 2008, RNA interference (RNAi) was applied to onions in order to produce a tearless onion (Eady et al., 2008). This was achieved by silencing the lachrymatory factor synthase (LFS) gene which generate the enzyme that catalyses the production of lachrymatory factors, the compounds which irritate the eyes when onions are chopped. This transgenic onion variety has been reported to have increased accumulation of secondary organosulphur compounds with known health benefits (Aoyagi et al., 2011; Thomson et al., 2013). However, due to the high regulatory cost and uncertainty of consumer acceptance, the cultivar was never commercialised (Harvey, 2018).

1.3 Photoperiodic nature of the onion bulbing process

1.3.1 The Photoperiodic Control of Flowering in Arabidopsis

Photoperiod is defined as the proportion of light and dark hours in a daily cycle of 24 hours (Jackson, 2009). Accordingly, photoperiodism is the events (flowering, tuberization, bud set and many other responses) triggered by the duration of illumination or pattern of light/dark cycles. Often the wavelength of the illuminating light is important to both plants and animals. Circadian rhythm refers to the regular cycle of behaviour with a period of approximately 24 hours. The constant variation of day length is a reliable indicator for plants to determine the annual transition from developmental growth to other progression, such as flowering, tuberisation and bulb formation. For agronomically relevant plant species, the correct timing to modulate developmental transition and organogenesis switch are crucial for both crop production and plant survival. Day length perception through complex photoperiod sensors offers an adaptive advantage to plants because photoperiod is the most reliable indicator for seasonal progression. By coupling photoperiodic information with the internal clock that functions as a mode of invariant time keeper, plants are able to anticipate seasonal changes and initiate e.g. flower development according to the coming season (Nagel, 2012).

The molecular genetics of photoperiodic floral induction has been well characterized at the molecular and genetic levels in *Arabidopsis thaliana*. It is a facultative long-day plant and flowers only after being exposed to light periods longer than a certain critical length. For this model plant, leaf-to-apex communication initiates flowering in response to photoperiod (Navarro et al., 2011; Abelenda et al., 2014). The flowering time genes in Arabidopsis mainly function in six different pathways: autonomous; vernalisation; gibberellin; temperature; light quality; and photoperiod (Jack, 2004). It appears that genes that act primarily in the photoperiod pathway can sometimes act as a component of an interacting network of pathways in regulating Arabidopsis flowering. The isolation of Arabidopsis mutants that had a compromised flowering response led to the identification of the *GIGANTEA* (*GI*), *FLAVIN KELCH F BOX 1*

(*FKF1*), *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) genes, which have a role in the same genetic pathway. Plants are able to measure time by means of the circadian clock, which is an endogenous timekeeping mechanism, controlled by various feedback loops (Jackson, 2009). In the leaf, light is perceived by different photoreceptors, and inputs into the clock. The clock derives the rhythmic expression of some of the key elements (*FKF1* and *GI*) to integrate the input signals from the photoperiodic pathway, including the zinc-finger protein *CO* (Putterill et al., 1995) and the RAF kinase inhibitor–like protein *FT*. The *FT* protein is translocated to the apical meristem through the phloem and forms a *FT/FD* complex (Pnueli et al., 2001; Abe et al., 2005; Wigge et al., 2005; Purwestri et al., 2009; Taoka et al., 2011), which then activates several genes (namely *AP1*, *SOC1*) and subsequently trigger *LEAFY* (*LFY*) expression leading to floral initiation in Arabidopsis (Kardailsky et al., 1999; Kobayashi et al., 1999; Nakamichi, 2011; Golembeski and Imaizumi, 2015).

Specifically, light interacts with the circadian clock and entrains it to 24-hour cycles (Michael et al., 2003). This entrainment of the clock is achieved through both cryptochromes in blue light and phytochromes in red light (Phytochrome B is the major photoreceptor for circadian control in high intensity red light and Phytochrome A is important in low intensity red light) (Somers et al., 1998). Then the photoreceptors transmit the light signal into the clock, promoting a complex of *GI* and *FKF1* (Sawa et al., 2007). Both of the genes are circadian regulated (Fowler et al. 1999, Park et al., 1999, Nelson et al. 2000), and control flowering by regulating *CO* transcription through the degradation of *CYCLING DOF FACTOR 1* (*CDF1*), a repressor of *CO* (Imaizumi et al. 2005, Sawa et al. 2007). Under long-day conditions, the activation of *CO* transcription by sufficient *FKF1-GI* complex will trigger the expression of floral integrating genes *FT*, *SOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*) and *TSF* (*TWIN SISTER OF FT*). The *FT* protein is then translocated to the apical meristem and activates other genes which finally trigger *LFY* gene expression in apical meristem leading to floral initiation (Jackson, 2009; Massiah, 2007). It is worth noting that, the induced *FT* expression by *CO* is only activated when the existence of *CO* protein overlaps a period of illuminated time. If the plant is grown in the dark, *COP1* (*CONSTITUTIVE PHOTOMORPHOGENIC1*) and *SPA1* (*SUPPRESSOR OF PHYA-105*) proteins

promote the degradation of the *CO* protein (Laubinger et al., 2006; Jang et al., 2008), leading to a delay or failure in flowering under short daylengths (Figure 1-8).

However, there are increasing studies continuing adding complications to this Arabidopsis flowering pathway. It is reported that MicroRNA's may also be involved, with miR172 regulating photoperiodic flowering through *FT* independently of *CO* (Jung et al., 2007). The recent study showed the phloem companion cell protein MRF1 (MORN-motif repeat protein, MORN-MOTIF REPEAT PROTEIN REGULATING FLOWERING1) could also be a positive floral regulator (You et al., 2019), underlying the important epigenetic modifications regulating this photoperiodically driven process. It appears that as more elements involved are discovered, the genetic pathway becomes more complex. There may be other genes, which are yet to be characterised, that have a significant role in the photoperiod response.

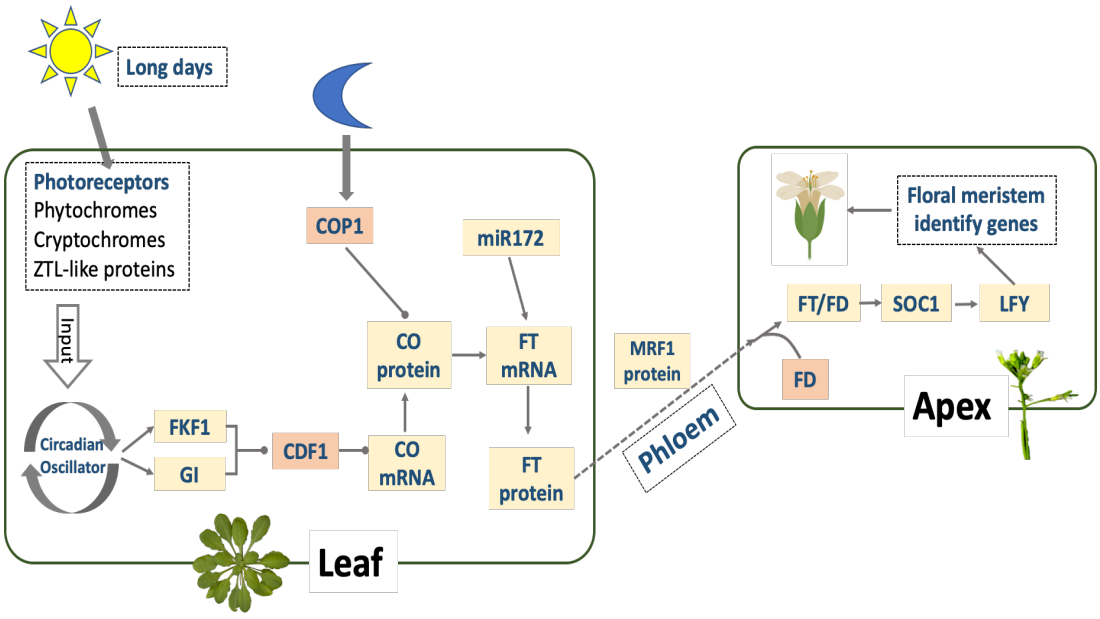


Figure 1-8 Daylength perception in Arabidopsis. Yellow boxes indicate floral promoters, red boxes indicate floral inhibitors. Pointed arrows indicate positive regulations, round arrows indicate negative regulations.

1.3.2 Comparing Photoperiodic Bulb Initiation with Photoperiodic Floral Initiation

However, flowering is not the only response that plants show to photoperiod. There are intense studies in molecular mechanism of responses such as tuberisation, bud break and the onset of cold hardiness or dormancy that also rely on the basic photoperiodic detection mechanism and common features with the control of flowering. In terms of onion, a similar mechanism can be related to bulb formation, which is also a photoperiodically driven process (Lancaster et al. 1996). At the physiological level, bulb initiation in LD onion is regulated in a similar way to the photoperiodic regulation of flowering in Arabidopsis: induced by long day, signal perception is in the leaf and response is at the apex. In Arabidopsis, the site of flowering is the apex presents at the highest point of the shoot. However, the apical meristem in onion is the most inner leaf down inside the basal tissue, where the bulb forms (Summerfield, 1991).

Similarly, photoperiodically driven bulb formation can be explained as follows. Initially, sunlight is sensed by the photoreceptors phytochrome (PHY) which can detect and absorb far-red to red light (600-750 nm) with the help of a linear tetrapyrrole chromophore contained in phytochrome (Park, 2003). Afterwards light is allowed to entrain the circadian clock, which controls the timing of the physiological and biochemical processes that occur in onions (Devlin, 2000). Then the circadian clock induces the translation of the mRNA of circadian regulated flowering-time genes such as *GI and FKF1*. Under LD conditions, sufficient *GI-FKF1* complexes are formed to co-activate *CO* transcription during the day. *CO* initiates the transcription of *Flowering Locus T* genes, leading to bulb initiation in onion varieties. Onion plants will also flower after a period of vernalisation (Brewster, 1994). However, during first year under inductive daylength conditions, onions will initiate bulbing, and flowering will be inhibited (Brewster, 2008).

1.3.3 Daylength perception in other plants

There is conservation of the genes of photoperiod response in many plant species, whereas different regulation of their homologues has also been reported in various plants. The photoperiodic control of flowering in Arabidopsis is proposed to be the model for conservation across plant species. Homologues of the major flowering time

genes *FKF1*, *GI*, *CO* and *FT* have been characterised in many species (Table 1-5). Different plants have different daylength requirements, leading to the differences in genetic regulation of these genes. Studies on SD plant *Pharbitis nil* (Liu et al., 2001) and rice (Kojima et al., 2002), as well as LD plant sugar beet (Chia et al., 2008) and barley (Kikuchi and Handa, 2009), all have shown both conservation and variation of the functional genes in different photoperiod responses.

There is a strong conservation of the photoperiod pathway genes in the SD plant rice, whilst the flowering response is only induced by SDs instead of LDs. The rice *FT* orthologue *Hd3a* is inhibited by its *CO* orthologue *Hd1* under long days, so the flowering is induced under SD conditions (Kojima et al., 2002). From this case we can see that although Arabidopsis and rice share the same set of *CO*-*FT* genes, and both function in promoting flowering under inductive conditions, while the role of *Hd1* acts as a repressor under non-inductive conditions is not found in Arabidopsis *CO*. In barley (a LD plant like Arabidopsis), study provided there was still floral promoting pathway by *FTs* under non-inductive SD conditions (Kikuchi and Handa, 2009), raising the question of the differences in the genetic control of photoperiod response. In potatoes, in which photoperiod-driven tuberisation is only induced by SDs, evidence suggests that floral and tuberisation transitions are mediated by two different *FT*-like paralogues that respond to independent environmental cues (Navarro et al., 2011).

Table 1-5 Conservation of the Arabidopsis flowering time genes CO, FT and GI across plant species (Rashid, 2016).

Species	Genes	Key References
Rice	*CO, FT, GI*	(Yano *et al.*, 2000; Kojima *et al.*, 2002; Hayama *et al.*, 2003)
Wheat	*CO, FT, GI*	(Nemoto *et al.*, 2003; Xiang *et al.*, 2005; Li and Dubcovsky, 2008)
Barley	*FT, GI*	(Dunford *et al.*, 2005; Faure *et al.*, 2007)
Pea	*CO, FT, GI*	(Hecht *et al.*, 2005; Hecht *et al.*, 2007)
Maize	*CO, FT*	(Danilevskaya *et al.*, 2008; Miller *et al.*, 2008)

Species	Genes	Key References
Perrenial ryegrass	*CO*	(Martin *et al.*, 2004)
Darnel ryegrass	*FT*	(King *et al.*, 2006)
Potato	*CO*	(Martínez-García *et al.*, 2002)
Oilseed rape (and other Brassicas)	*CO*	(Robert *et al.*, 1998)
Japanese morning glory	*CO, FT*	(Liu *et al.*, 2001; Hayama *et al.*, 2007)
Sugar beet	*CO*	(Chia *et al.*, 2008)
Radish	*GI*	(Curtis *et al.*, 2002)
Barrel medic	*CO, FT, GI*	(Hecht *et al.*, 2005; Paltiel *et al.*, 2006)
Tomato	*FT*	(Lifschitz *et al.*, 2006)
Norway spruce	*FT*	(Gyllenstrand *et al.*, 2007)
Squash	*FT*	(Lin *et al.*, 2007)
Moss	*CO*	(Zobell *et al.*, 2005)
Lombardy poplar	*FT*	(Igasaki *et al.*, 2008)
Red goosefoot	*FT*	(Cháb *et al.*, 2008)

1.4 Genes of interests

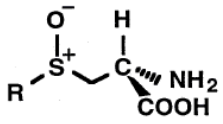
1.4.1 Genes of circadian regulation

The mechanism of Arabidopsis flowering and onion bulb initiation in response to daylength were characterised. In this thesis, we chose some of the key genes involved in this regulation. The candidates are selected as ***GI***, ***FKF1***, ***CO***, ***FT****s* and ***LFY***, not only for their roles as Arabidopsis flowering time genes, but most importantly, they have been isolated with their homologs in onion varieties. Besides of those, we also

have two novel sequences ***PHYA*** (Phytochrome A) and ***TIC*** (Time for Coffee) of interest, adding more comprehension and understanding to the circadian regulation in the onion bulbing process.

1.4.2 Genes of onion quality traits and plant defense

Alliums are well characterized by their remarkable sulphur-containing compounds for generating distinctive smell and pungency. In these allium crops most of the sulphur is in the form of non-protein amino acids which include the precursors of the volatile flavour compounds (Brewster, 1994). These precursors are odourless amino acids with a general name of S-alk(en)yl-L-cysteine sulfoxides, which the structure instructed as followed:



S-Alk(en)yl-L-cysteine sulfoxide

The R moiety represents the -L- cysteine sulfoxide group varies from different allium crops as follows:

Table 1-6 Flavour precursors detected in some of the edible alliums and their relative amounts (Brewster, 1994)

Allium species	Cysteine Sulfoxides (R moiety)			
	1) CH_3- S-melthyl	2) CH_3-CH_2-CH_2- S-propyl	3) CH_3-CH=CH- S-(1-propenyl)	4) CH_2=CH-CH- S-allyl
Onion	+	++	+++	0
Shallot	++	++	+	0
Chives	+	+	++	0
Leek	++	++	+	0

Garlic	++	+	0	+++
Chinese chives	++	+	+	+++

Among these precursors, compound 4 is characterized specially in garlic with the name of alliin (S-allyl--cysteine sulfoxide), which is confined to the cytoplasm. Its corresponding enzyme is called alliinase (S-alk(en)yl-L-cysteine sulfoxide lyase), which exists only in the cell vacuole. A similar compartmentalisation is found for the precursors and alliinase, in the other allium species. Only when the fresh tissue is damaged, does the enzyme have access to the precursors, explaining the reason for the plants' eye-irritating smell when cutting them. After disrupting the cell, the highly reactive sulfenic acid is released and spontaneously produces a wide range of volatile products. In garlic, it catalyses the formation of allicin, while in onion, it causes the formation of lachrymatory factor (LF) with the involvement of Lachrymatory Factor Synthase (LFS) (Silvaroli et al., 2017). In this thesis, we chose some of the related genes as our candidates, including ***ALL*** (alliinase) and ***LFS***, hoping to add some insights in developing tear-less or mild onion varieties.

The flavour precursors give rise to many other compounds as well. For instance, the γ-glutamyl peptides and its related chemicals occur in quite large quantities in onion bulbs. Additionally, these compounds are reported to have strong effects to other organisms and very likely to play an important role in plant chemical defence (Brewster, 1994). There have been various studies showing the health benefits of some mineral nutrients that can work as natural defense of different crops in response to the action of diverse types of pathogens (Garcia-Mina, 2012). Therefore, we chose some of the genes related to plant defense system in this thesis as well, including ***GCL*** (Glutamate-cysteine ligase), ***GST*** (glutathione S-transferase), ***DFS*** (defensins), ***ECT*** (endochitinase-like protein) and ***AHCYL1*** (adenosylhomocysteinase-like). More details of these genes will be described in the individual chapters.

1.4.3 Genes of carbohydrate and sugar metabolism

The non-structural or storage carbohydrates of alliums make up 80% of the dry matter (Brewster, 1994). Allium crops are almost totally lacking in starch but store carbohydrates as fructans, which are long chain polymers of fructose that occur widely in plant kingdom (Hendry and Wallace, 1993). Fructans can be hydrolysed to fructose and glucose, and are stored in plant vacuoles. In onion, the predominant bulb fructan type differs with cultivars, whilst all fructan biosynthesis is initiated by the enzyme ***SST1*** (sucrose 1-fructosyltransferase), which catalyses fructose to bind to another sucrose molecule and form 1-kestose for additional fructose chains (Vijn et al., 1998).

Besides *SST1* for one of the target sugar metabolism genes, we also selected invertases (beta-fructofuranosidase, ***FFS***) and ***TPS*** (trehalose-6-phosphate synthase). *FFS* (invertases) catalyse sucrose hydrolyzation to produce hexoses (fructose and glucose) available for cellular activity such as cell division and expansion, differentiation and maintenance, respiration and cell wall synthesis (Benkeblia et al., 2004). The gene is also essential in sugar source to sink transportation system in the phloem of leaf blades. ***TPS*** is responsible for the biosynthesis of trehalose, which is not only a valuable nutrient in onion, but also reported to play a role in successful transition from vegetative growth to flowering in higher plants (Dijken et al., 2004).

1.4.4 Genes of leaf morphology and plant development

Plant development is a complex process that involves coordination of specific developmental events. It is already described that bulbs are formed because of the swelling of leaf bases and the accumulation of carbohydrates during photosynthesis. The transition from vegetative growth to swelling storage for leaf is crucial for onion bulb formation, seeing that the production of consumable bulbs relies on this developmental switch. In this thesis, we also choose some of the genes potentially involved in leaf morphology and plant development. The selected genes including ***MADS*** *box* which has been described as "molecular architects" in flower morphogenesis(Kumar et al.，2016); ***POLYCOMB*** which is putatively having roles in differentiation and silencing target genes (Di Croce and Helin, 2013); ***PER12*** (peroxidase) which is believed to reduce rates of cell wall expansion and leaf

elongation (Bacon et al., 1997); ***SAURs*** (Small auxin up RNAs) which causes rice's pleiotropic developmental defects and reduced auxin levels (Xu et al., 2017); ***RPL34*** (ribosomal protein) which triggers a promoted growth activity in floral organs and wound-inducible in tobacco leaves (Dai et al., 1996; Yu and An, 2000); ***TUBA2*** (α-tubulin) which has been found with pollen specific expression in plant species and has a role in cell wall thickening in plant development (Oakley et al., 2007); and ***H2A*** (one type of protein histone) which is suggested can promote flowering time in Arabidopsis (Su et al., 2017). However, despite their potential role in onion bulb formation, we did not have an opportunity for detailed study, but will discuss them further in the individual chapters.

1.5 Aims and objectives

New research and development is aiming at some major changes in the onion industry. One opportunity is the development of day-length insensitive varieties. Based on the genetic control in Arabidopsis day-length dependent flowering process provided, the similar mechanism has been reported in Allium crops (Taylor et al., 2010; Lee et al., 2013; Manoharan et al., 2016; Yang et al., 2016), indicating that day-length insensitivity may be selected and utilised in these allium crops. Former group member Rashid (2020) has identified several genes involved in daylength adaptation in onion, and conducted a comprehensive set of quantitative expression experiments under 8 hours and 16 hours of daylengths growth conditions. However, due to the limit number of genes and the large gap between daylengths settings, the results did not explain the different photoperiod response in onion varieties. Thereupon, we aimed to address these knowledge gaps in this project.

1.5.1 Project aims

- Gain further understanding of the mechanisms involved in the daylength-dependent bulbing response of onions.
- Aid breeders by providing them with information on the onion orthologues of important genes in order to speed up breeding and enable us to meet the challenge

to produce food for an increasing world population and make a contribute to global food security.

1.5.2 Specific objectives:

- Understand how *FT* genes interact in different daylength types and different photoperiods to control the bulbing response.
- Determine the spatial distribution of bulbing-related genes in leaf and bulb tissues.
- Identify genes differentially expressed in bulb tissues during bulb formation.

2 GENERAL MATERIALS AND METHODS

This chapter details the plant materials and general methods that were used in this study. 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 2015 to the July 2018. Unless otherwise stated, the methods and protocols set out in this chapter were used throughout the project.

2.1 Standard materials

2.1.1 Allium cepa L.

Three onion (*Allium cepa* L.) varieties with different daylength responses were used in this study. These included long day (LD) onion variety "*Renate*" F1 (Elsoms Seeds Ltd., Spalding, UK), the short day (SD) variety "*Hojem*" from VeGIN, UK (Vegetable Genetic Improvement Network Project Diversity Set), and another LD variety "*Marco*" (S.E. Marshall & Co Ltd, Cambridgeshire, UK). The seeds of variety *Hojem* were obtained from the Warwick HRI Genetic Resources Unit and provided by Dr. Andrew Taylor, Warwick Crop Centre. The seeds of varieties *Renate* and *Marco* were purchased directly from the seed companies.

2.1.2 Arabidopsis thaliana

Arabidopsis thaliana co-2 (Landsberg background) mutant as well as Landsberg erecta (Ler) wild-type seed was provided by 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).

2.2 Growth conditions

All plants used in this project were grown under the operation of the central monitoring system, which monitors and logs temperature and humidity in rooms and cabinets. Carbon dioxide content and light were also monitored during plant growth.

The plant materials used for this project were grown either in the Phytobiology Facility (PBF) or Gibbet Hill (GH) Chambers and the growth conditions monitored and logged by central monitoring systems.

The PBF combines Controlled Environment and Grodome facilities for the growth of a range of plants under defined environmental conditions (university website: https://warwick.ac.uk/fac/sci/lifesci/intranet/staffpg/support/phytobiology). In this project, PBF Grodome and PBF Controlled Sanyo 2279 Cabinets were used (Tale 2-1). **Phytobiology Facility (PBF) Grodome** is specifically designed with sustainable energy technology providing a single span polycarbonate clad structure offering fully contained independent compartments to accommodate different research programmes. The **Shared Compartment** is a large chamber (48m^2) set with natural daylengths combined with 16-hour supplementary light (400 W SONT lamps) from 03:00 to 19:00. Plants were grown here for LD treatment. The **Specific Compartment** is a smaller chamber (24m^2) with the same environmental control but also has daylength control blackout blinds that can be individually programmed. Plants were grown here for a range of daylengths (natural daylight, short-day or intermediate-day). All compartments used in this project were set at 22°C day and 18°C night with 60% relative humidity and ambient CO_2 concentration. The **PBF Sanyo 2279 Cabinets** (SANYO Electric Co., Ltd.) are controlled environment cabinets that were set with different day-lengths treatment for the diurnal experiments. The photoperiods were provided by fluorescent bulbs (General Electric 60W, HU), and tungsten bulbs (Philips 32W, NL) for light extension. **Gibbet Hill Panasonic Chambers MLR-351** are PANASONIC controlled Environmental Cabinets, that provide a Photosynthetic Photon Flux Density (PPFD) of 100 Wm^{-2} in the main fluorescent light photoperiod.

Table 2-1 General conditions for growth compartment

Conditions	PBF Grodome Compartment		PBF Sanyo 2279 Cabinets	GH Panasonic Chambers
	Shared Compartment	Specific Compartment		
Humidity Range	70%	70%	60%	60%
Day/Night temperature (°C)	20/18	22/18	22/18	22/18

Conditions	PBF Grodome Compartment		PBF Sanyo 2279 Cabinets	GH Panasonic Chambers
	Shared Compartment	Specific Compartment		
Supplementary Lamp Type	400 W SONT lamps	400 W SONT lamps	Fluorescent + Tungsten lamps	Fluorescent + Tungsten lamps
CO_2 Injection	ambient	ambient	ambient	ambient
Irrigation	Manual	Manual	Manual	Manual

Initially, onion seeds were sown into modular Plantpak P40 trays (HSP, Essex, UK) containing Levington F2 compost. After sowing, the trays with seeds were watered heavily and the trays covered with a 634x413 mm clear autoclave bag (CPS Flexible Ltd.) to maintain the humidity. The trays were put into the appropriate growth environment for each experiment. After 50% of germination, autoclave bags were removed, and after approximately 4 weeks seedlings were potted up into 9 cm pots containing Levington M2 compost. Thenceforth the plants could be translocated to different growth environment and used for specific experiments.

2.3 Standard Methods

2.3.1 RNA extraction, DNase treatment and first strand cDNA synthesis

Total RNA was extracted from leaf and bulb material from onion grown under specific growth conditions using the Z6 buffer method, following the manufacturer's (Roche manufacturing Ltd., Republic of Ireland) guidelines. Approximately 100 mg of frozen plant tissue was homogenised using a pestle and mortar. Followed by further grinding using a Dremel drill in liquid nitrogen. In this step, Z6 buffer reagent and β-Mercaptoethanol were added in order to remove RNase. Two extra reagents, 3M sodium acetate (NaOAC) and 7.5M lithium chloride, which removes carbohydrates and polysaccharides, respectively, were included to obtain high quality RNA. After isolation, the quality and quantity of total RNA was measured with the Thermo Scientific NanoDropTM 1000 Spectrophotometer (NanoDrop Technologies, Inc., USA).

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

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

2.3.2 Genomic DNA extraction and purification from gel

Genomic DNA extractions from onion leaf were carried out using a modified CTAB method (Doyle and Doyle, 1987; Porebski et al., 1997). Leaf tissue was ground using a pestle and mortar and then placed in a microfuge tube followed by homogenising with a microfuge Dremel drill. Pre-warmed CTAB buffer was added (500 µl) and the material homogenised further. The reaction mixer was then incubated at 65 ºC for 10 minutes. Dichloromethane: isoamyl alcohol (24:1 v/v) was then added (500 µl) and samples were centrifuged at 16,500 x g for 2 min (in a microfuge) at room temperature. After centrifugation, the top phase was then transferred to a fresh tube and 300 µl of isopropanol was added. The reactions were then centrifuged at 16,500 x g for 3 min and the supernatant removed. Wash buffer was added (500 µl) and samples were left at room temperature for 2 min. After incubation, the samples were centrifuged at 16,500 x g for 3 min and the supernatant removed. A pulse spin was given using table-top centrifuge and the pellet was air dried for approximately 15 min. The pellet was re-suspended in 200 µl of sterile distilled water. This protocol was provided by Dr Andrew Taylor (Warwick Crop Centre) and Linda Brown (Warwick HRI).

2.3.3 Quantification of samples

DNA and RNA samples for all experiments were quantified using a NanoDrop™ ND-1000 spectrophotometer (Thermo Scientific). A volume of 1 µl was loaded onto the spectrophotometer.

2.3.4 Target gene selection

The onion ESTs and transcriptome sequences database were obtained from Rashid (PhD Thesis, 2016), and was used to select target genes and design primers. The database was originally obtained by using the BLAST algorithm blasting the sequence of each gene in Arabidopsis homologs against the onion EST database (DFCI, 2008). The genes were selected for their key features either in putative functions or differential expressions between onion tissues. The chosen sequences were BLASTed with other plant species obtained from the publicly available NCBI database. A contig was constructed for each gene using the SeqMan package of DNAStar (DNAStar Inc.). Alignments were also carried out with DNAStar software and phylogenetic trees constructed. 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.

2.3.5 Primer design

Primers for PCR and Real-time PCR quantification was designed using Primer3Plus (http://www.bioinformatics.nl/cgi bin/primer3plus/primer3plus.cgi). For Real-time PCR, the primers were checked for binding in the amplicon in a region free of secondary structure using the mfold (http://mfold.rna.albany.edu/?q=mfold). All primers in this project were synthesised by Invitrogen Ltd and Sigma-Aldrich® (UK).

2.3.6 Standard PCR (polymerase chain reaction)

PCR reactions were set up in 20 µl volumes containing 0.4 units KOD Hot Start DNA Polymerase (Merck Bioscience, Cat No. 71086-3), 1 µl of template, 1 x KOD Hot Start DNA Polymerase reaction buffer, 0.2 mM each dNTP, 2 mM $MgSO_4$, 0.3 µM of each forward and reverse primer and SDW (sterile distilled water) to make the total

volume of 20 µl. Reactions were 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 deemed appropriate for the primer pair for 10 seconds and extension at 70 °C for 1 min per kb of product.

2.3.7 Agarose gel electrophoresis for DNA and RNA

Agarose gel electrophoresis was carried out to check the quality of RNA and DNA after extraction, or to check the product after PCR reactions. Agarose gels were composed of 1-2% (w/v) ultra-pure agarose (Invitrogen Ltd., Cat. No. 15510) with 1x Tris-acetate-EDTA buffer (TAE buffer) (VWR International, Cat. No. 44125D), and 0.05 µl/ml Gel Red Nucleic Acid Gel Stain (10000X in Water). One volume of Orange G ($C_{16}H_{10}N_2O_7S_2Na_2$, Sigma-Aldrich®, Cat. No. O3756, UK) loading dye was added to five volumes of 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 size of products. Gels were run in a tank containing 1x TAE running buffer for the electrode distance at room temperature (25 °C), and were carried out at 100-120 mA for 30-90 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 photographs were recorded using a G: BOX gel documentation system (Syngene, UK).

2.3.8 qRT-PCR

After the first strand cDNA was obtained, the expression of reference and candidate genes was analysed by Real-time PCR quantification using the CFX384 TouchTM Real-time PCR machine from BioRad (Bio-Rad Laboratories Ltd., UK). All primer pairs used were initially tested to ascertain the optimum primer and cDNA concentrations. Each reaction contained 5 µl GoTaq® qPCR Master Mix, 0.5 µl of cDNA, either 0.2 or 0.3 µM of each primer (primer details see Appendix), 0.2 µL fluorescein dye and SDW to make up 10 µl reaction volume. Each sample was run in triplicate and the average CT value calculated, which represents the number of PCR cycles when a product is first detected. The optimisation and selection of dilution series for making standard curves were conducted. For each experiment, eight

reference genes were examined (*PP2A1*, *PP2AA3*, *TIP41*, *AcTUA*, *AcTUB*, *UBQ1*, *UBC9* and *UBL*). The top three with the best stability and performance were selected and further analysed together with the target genes. The general PCR condition is presented in Table 2-2. After each run, the quality and other running information of PCR was checked on the software screen. The acceptable PCR efficiency (standard curve, slopes on log amplification curves) is between 90-110%, R^2 higher than 0.985 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).

Table 2-2 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 (Annealing temperature) for 1:00 min Plate Read 4. GOTO step 2 (39 cycles) 5. 95.0°C for 10 sec 6. Melt Curve 60.0°C to 95.0°C: Increment 0.5°C 5 sec

The Real-time data obtained were then analysed using qbase plus software (Biogazelle, Belgium) after completion of each PCR run. Three replications were used and data indicated as means and normalized against expression levels of the reference genes for each sample. For each individual experiment, the best three reference genes were selected out of seven based on their stability for further analysis (listed in the Appendix). The expression of target genes was normalized against reference gene expression levels.

3 GENE IDENTIFICATION AND SPATIAL EXPRESSION

3.1 Introduction

The mechanism of Arabidopsis flowering as well as onion bulb initiation in response to daylength were characterised in Chapter 1. Both are photoperiodically driven processes, daylength perception occurs in the leaves, and response in the meristem (Thomas et al., 2006). In Arabidopsis, the circadian clock derives the rhythmic expression of some key genes including *FKF1* and *GI* (Somers et al., 1998), which then form a complex to degrade a transcriptional repressor of *CO* (Sawa et al., 2007). *CO* positively regulates *FT* (Jung et al., 2007). After that the *FT* protein is translocated to the apical meristem through the phloem and forms a *FT*/*FD* complex (Pnueli et al., 2001; Abe et al., 2005; Wigge et al., 2005; Purwestri et al., 2009; Taoka et al., 2011). This complex activates several genes (namely *AP1*, *SOC1*) which subsequently trigger *LFY* expression and cause flowering in Arabidopsis (Nakamichi, 2011; Yoo et al., 2005; Golembeski et al., 2014).

Considering the hypothesis of this thesis is that the homologues of some major genes involved in Arabidopsis photoperiodic flowering pathway are also proposed to be functionally conserved in onion, it is necessary and important to firstly examine the expression sites of these genes. Before considering their consistency in functions, we firstly aimed to see the spatial patterns of these genes and relate them with Arabidopsis. In the field of daylength perception site, as with Arabidopsis, the leaf blade of onion is the receptor for the photoperiodic control of bulb initiation (Brewster, 1990; Kato, 1964). Studies with onion (Rabinowitch, 2018) showed that after removing parts of leaf blades, a number of new leaves developed before bulb initiation. In terms of the site of response, these two species are separated. For Arabidopsis, the inflorescence meristem, which is a floral structure, is produced from the shoot apical meristem when environmental and internal signals cause a change of fate (Komeda, 2004). For onion, leaf sheaths swell and produce a storage structure from bladeless bulb scales at the basal meristem (Lancaster et al., 1996; Summerfield, 1991). In this chapter, we expected to see the homeotic conversion of photosynthetic leaves to floral structures (such as sepals, petals, stamens and carpels) which occurs in flower development, also occurs in onion through the conversion of photosynthetic leaves to bulb scales.

Three sets of experiments were conducted to further identify the photoperiodic nature of the onion bulbing process. Sequences with differential daylength responses or involved in onion development and nutrition traits were investigated.

The first: **Daylength and Renate development experiment** was set up to measure the bulbing response of *Renate* F1 onions grown in constant NC, LD and SD conditions, together with plant transfer from LD to SD for further understanding of the bulbing response.

The second: **Tissue-specific gene expression experiment** discusses a more widespread identification of some novel genes that are potentially linked to the circadian regulation, bulb initiation, plant development, or nutrition level in onion. Separate onion materials (SD-Leaf, SD-Bulb, LD-Leaf and LD-Bulb) were used and expression of the candidates was compared with that found in the onion transcriptome RNA-seq database. Predicted functions as well as distribution and diversity of these novel sequences were combined to develop a wider system for future onion genetic studies.

The third: **Spatial gene expression in *Renate* leaves experiment** was performed to determine spatial expression of the genes of interest in *Renate* F1 onion. The first batch of candidates were chosen from the genes related to the circadian clock, aiming to find the expression point of these candidates and examine their consistency in character with Arabidopsis: whether in the site of perception (green leaf), or in the site of response (basal tissue). The second batch of candidates were selected based on their function to provide more bulb-specific genes, especially those for quality traits such as carbohydrates and organosulphur content. The spatial expression pattern of these novel genes can provide a clearer understanding of their functions in respect of quality traits.

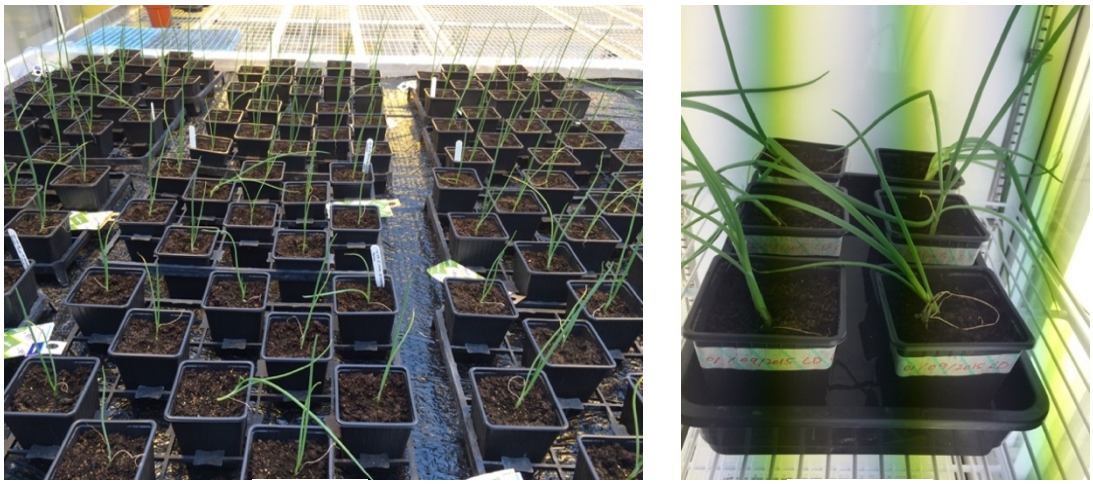
3.2 Material and methods

3.2.1 *Daylength and Renate development*

Onion *Renate* F1 seeds were planted on 29/06/2015. Initially, seeds were sown in modular trays and kept under natural conditions (NC) in the glasshouse as described in Chapter 2. After 4 weeks, plants were potted up into 9 cm pots containing Levington M2 compost and left in PBF NC until 15/09/2015. Then plants were separated into three groups: Group 1 plants were kept in PBF glasshouse with mild summer natural long-day (14.5h initially to 13.3h at the end of the experiment), combined with high pressure sodium discharge lamps supplemented from 06:00 to 18:00 GMT. Group 2 and 3 were transferred into two growth Panasonic chambers with artificial lights on Gibbet Hill campus. One was set with LD condition (10h of fluorescent light + 8h of low-level incandescent light), another was set with SD condition (10h of fluorescent light) (Table 3-1). Otherwise plants were kept in dark within the photoperiod chambers until the start of the next cycle (Figure 3-1).

Table 3-1 Growth conditions for Daylength and Renate development

Growth condition	PBF NC		Cabinet SD		Cabinet LD	
Temperature (°C) (Day: 9:00-19:00 Night: 19:00-9:00)	Day: 20	Night:18	Day: 22	Night:18	Day: 22	Night: 18
Supplementary light	6:00-18:00		Fluorescent: 9:00-19:00		Fluorescent: 9:00-19:00 Incandescent: 17:00-1:00	



PBF Cabinet LD

Figure 3-1 Growth of Renate plants under NC in PBF and LD in cabinet

Onion leaf layer materials were taken weekly at ZT10 for seven times, twice before transfer date and five times after the transfer date. Five plants, selected using a random number generator, were harvested from each growth condition. Visible leaf number was counted for each plant. Bulb and neck diameter measurements were taken using calipers and "Bulbing ratios" calculated by dividing the maximum bulb diameter by the neck diameter (Clark and Heath, 1962). Bulbing is considered to have been initiated when the bulbing ratio reaches a value greater than two. Finally, onions were dissected from out to in for separated layer samples, representing sequential leaves from oldest to youngest (approximately 8 layers per plant). Tissues for five replicate plants were pooled and quick frozen with liquid nitrogen before being kept in -80 °C for longer storage.

Selected plants Harvested Dissected

Figure 3-2 Indication of sampling process with LD plants in 12/10/2015

3.2.2 *Tissue-specific gene expression*

3.2.2.1 Gene selection

All the genes selected had not been previously published or available from public *allium* databases. The candidates were selected from the onion transcriptome sequence database provided by the Thomas Group (Rashid, 2020) and chosen due to their differential expression either between SD and LD growth condition, or between bulb and leaf tissue in the RNA-seq data. Each sequence selected was BLASTed with other plant species obtained from the publicly available NCBI database. Genes reported to be involved in photoperiod regulation and other important pathways were selected. 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 their homologue genes in other plant species were also calculated. Sequences were aligned and phylogenetic trees were constructed using predicted amino acid sequences. Primers (Forward and Reverse) were then designed and synthesised for each candidate. The mfold web server was used to check primer characteristics in order to avoid unwanted secondary structures in the PCR product. Primers used for obtaining the full-length of target genes in onion are presented in the Appendix.

Eventually twenty-four novel onion transcription sequences were selected as genes of interest. Predicted function as well as homologs in other species were obtained from publicly available NCBI database BLAST result. Among the twenty-four selected sequences, seven were selected on the basis of their differential bulb expression between SD and LD growth conditions (Table 3-2). The sequences on this list were all primarily found in bulb, but were specifically expressed under short-daylength or long-daylength. The remaining seventeen genes were chosen based on their large differences in expression between leaf and bulb provided by the RNA-seq data. These candidates were only bulb-specific, which means they were previously found to have higher expression at the base of the leaf. Twelve of them were characterised with predicted function from their homologues in other plant species when BLASTed with NCBI (Table3-3). Five of them, however, showed very low percentage identities with

homologue in other plant species. These genes of unknown functions are not listed here (see Appendix).

Table 3-2 Target genes selected based on differential expression (in bulb) between SD and LD

Description in the transcriptome database	Target name	Predicted function
LD Bulb-specific	*DFS*	Defensin-Like Protein 6-Like
	GCL	Glutamate--Cysteine Ligase, Chloroplast Precursor
	MADS	Mads-Box Transcription Factor 14-Like
	PHYA	Phytochrome A, Putative [*Ricinus Communis*]
LD Bulb-specific	*TIC*	Protein Time for Coffee-Like [*Vitis Vinifera*]
SD Bulb-specific	*POLYCOMB*	Polycomb Group Protein Embryonic Flower 2-Like
	PER12	Peroxidase 12-Like

Table 3-3 Target genes selected based on differential expression between leaf and bulb (all bulb-specific)

Target name	Predicted function
ALL	Alliin Lyase Precursor, Putative [*Ricinus Communis*]
MLP	Major Latex Protein 423-Like [*Solanum Lycopersicum*]
SAUR	SAUR Family Protein [*Populus Trichocarpa*]
LOC	Protein LOC100795348 [*Glycine Max*]
ECT	Endochitinase-Like [*Solanum Lycopersicum*]
DFS	Defensin J1-2-Like [*Cucumis Sativus*]
GST	Glutathione S-Transferase T1-Like [*Cucumis Sativus*]
RPL34	60S Ribosomal Protein L34-Like [*Cucumis Sativus*]

Target name	Predicted function
AHCYL1	Adenosylhomocysteinase-Like Isoform 1 [*Solanum Lycopersicum*]
TUBA2	Tubulin Alpha-2 Chain, Partial [*Vitis Vinifera*]
LFS	Lachrymatory Factor Synthase [*Zea Mays*]
H2A	Histone H2A.1-Like [*Cicer Arietinum*]

3.2.2.2 Sample preparation

The LD onion variety *Renate* F1 was used throughout this preliminary RT-PCR experiment. Onion plants were grown at the Phytobiology Facility glasshouses providing two growth conditions as: natural LD (daylight was 16 h 38 min to 15 h 7 min) and controlled SD (8 h natural day light from ZT0). Sampling was carried out at 62d at ZT10. The middle part of the first and the second newly expanded leaf were cut into 1cm pieces as leaf samples, and the middle to the basal portion of bulbs were chopped into small pieces and harvested as bulb samples. Three plants were harvested and pooled as biological replicates (Figure 3-3).

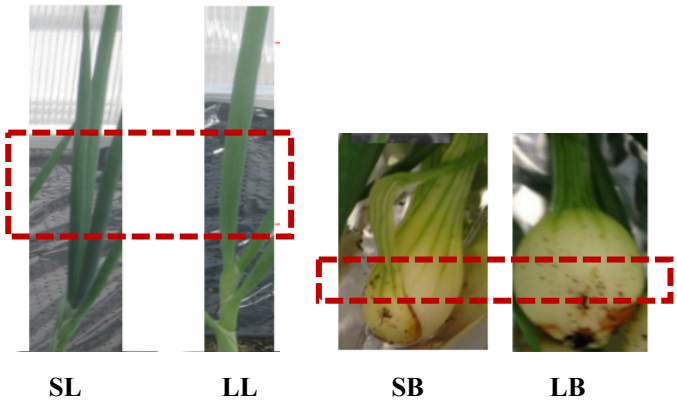


Figure 3-3 Middle parts of leaves and middle parts of bulbs were harvested (3 plants). SL=short-day leaf, LL=long-day leaf, SB=short-day bulb, LB=long-day bulb

Total RNA was extracted from leaf and bulb materials for onions grown under LD and SD, followed by cDNA synthesis from DNase treated RNA. To check if cDNA

samples used for analyses were of good quality and suitable sources for sequence amplification, PCR was carried out using Alliinase primers, and products were run on a gel. Optimum annealing temperature was chosen by conducting gradient PCR for each target with newly designed primers. The expressions were examined by standard PCR with primers and appropriate annealing temperatures for preliminary isolation of the genes. A cycle number of 35 was used for each specific gene for the first trial, if not specified, cycle number of 39, 29 and 22 were also used.

3.2.3 *Spatial gene expression in Renate leaves*

3.2.3.1 Plant material sampling and handling

Onion Renate plants were grown under NC in the glasshouse at Phytobiology Facility from middle June to August for approximately two months, the daylight was 15 h 42 min to 11 h 52 min accordingly. At the last day (63 days from sowing), plants were harvested at ZT10 and leaves were separated. The 5th leaf was taken and cut into 12 pieces of 1 cm starting from the base (Figure3-4), 6 plants were pooled together. The samples were immediately frozen in liquid nitrogen before storing at -80°C. Plants were selected for harvesting using a random number generator in a Completely Randomised Design.

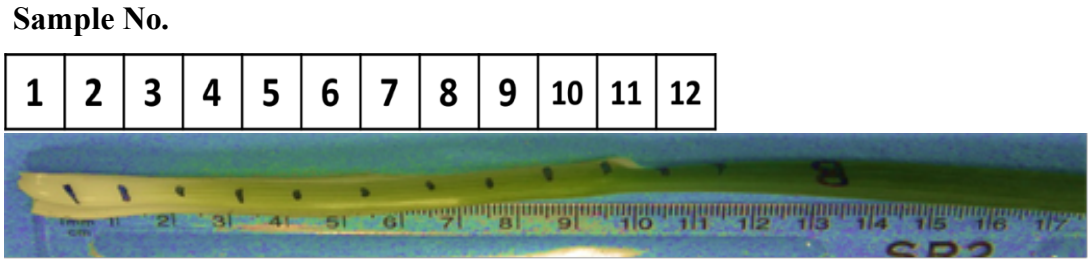


Figure 3-4 Leaf section samples for spatial experiment

From leaf dissection indication in the figure above, it can be seen that sample 1 and sample 2 were close to the basal meristem, indicating they were the youngest parts. Samples 3 and 4 are transition parts. From sample 5 to sample 12, tissues get older with greener colour. The section of a whole leaf can be considered as a time series of leaf development.

The identification of the genes of interest was obtained in two ways: either from past papers or from the onion transcriptome database. *GI*, *FKF1*, *LEAFY* and the *FT*s were chosen from published literatures. *COL2* was identified and firstly described by Rashid (PhD Thesis, 2016). It was characterised as one of three Arabidopsis *CO*-like homologs isolated in onion, while only *CO*-like 2 showed significant circadian pattern similar to Arabidopsis (described in Chapter 1). Other targets (namely *DFS*, *MADS*, *LFS*, *GCL* and *PER12*) were obtained from the onion transcriptome database as discussed in the tissue-specific gene expression experiment (3.2.2.1). The qPCR primer pairs for the targets from the literatures were directly obtained from their original sources, and the primer pairs for the targets from the onion transcriptome were designed following the procedure described in Chapter 2. Extraction of total RNA, DNase treatment and synthesis of cDNA using 2µg total RNA were followed by the protocols as described in Chapter 2. The primary expression of each candidate was initially examined with pooled cDNA samples (Renate leaf and bulb samples under LD). Optimal annealing temperatures for new obtained primers were tested by conducting gradient PCR.

3.2.3.2 qRT-PCR setup and analysis

The relative expression of the target genes was examined by qRT-PCR using the CFX384 TouchTM Real-time PCR machine from BioRad (Bio-Rad Laboratories Ltd., UK), as described in Chapter 2. The qRT-PCR conditions were followed as described in Chapter 2 with primer pairs' appropriate annealing temperatures. The optimal primer concentration was determined for each candidate. Three replicates were analysed for each sample and the average CT value was calculated. qRT-PCR data was analysed after completion of each PCR run and data indicated as means and normalized against expression levels of the reference genes for each sample. Normalisation was achieved by using Biogazelle qBase+ software. The average spatial expression was calculated and standard errors were included. Standard curves (using 10-fold serial dilutions) were plotted using cDNA synthesised from approximately 2 µg of total RNA extracted from pooled *Renate* leaf and bulb samples.

The optimal primer concentrations and annealing temperatures are listed in the Appendix. Eight housekeeping genes were tested (*UBQ1*, *TIP41*, *PP2A1*, *UBL*, *PP2AA3*, *AcTUA*, *AcTUB* and *UBC9*) and the best three were selected (*UBL*, *PP2A1* and *TIP41*) (Appendix).

3.3 Results

3.3.1 *Daylength and Renate development*

After five times of sampling after transfer (40 days), clear differences were observed in plants' bulbing ratios and the morphology of newly formed leaves (Figure 3-5). Plants grown in SD conditions showed no signs of bulb initiation as the average bulbing ratio was not significantly above 2 when finishing the experiment. Plants grown in NC had larger bulbing ratio than those grown in LDs, while both conditions showed increasing bulbing ratios above 2 after transfer. Under NC, bulbing process was already slowed down before the end of the experiment as the process neared completion. With regard to visible leaf number, the value showed the opposite pattern to bulb ratios. Plants grown in SD produced considerably more green leaves than all other conditions, then followed by LD condition. There showed a continuous reduction in visible leaf number for plants in NC after one week of transferring as older leaves died back and were lost but not replaced by younger leaves.

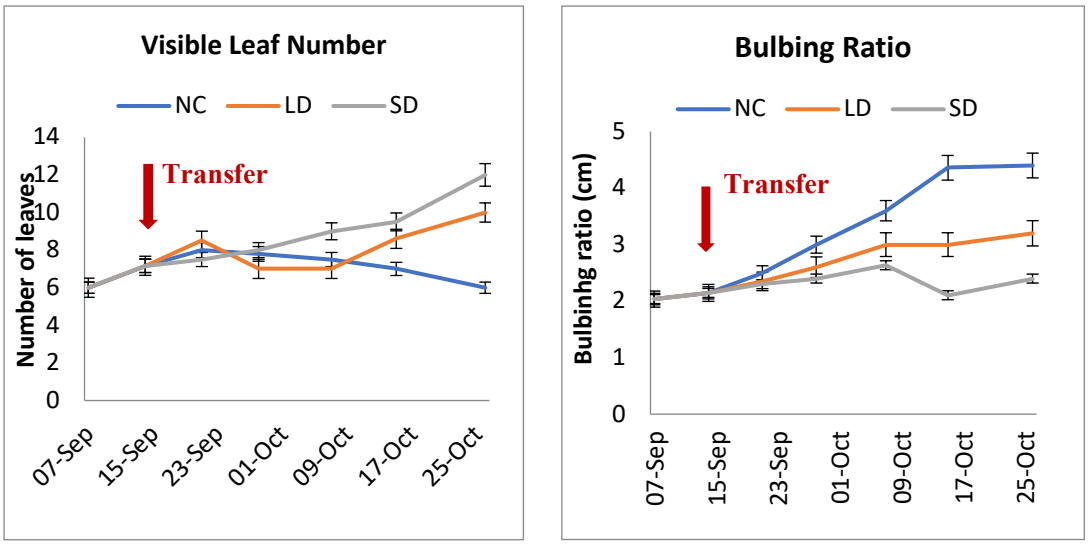


Figure 3-5 Average leaf number and bulbing ratio for NC, LD, SD conditions. Red arrows indicate the date plants were transferred into different growth conditions. Error bars represent the SEM. Five plants were used as technical replicates.

The table below shows morphological features of the plants and the harvested leaves one month after transfer (17/10/2015). From the size and the shape of the harvested onions, it can be seen bulb development performed the best under NC, presenting

largest bulb size and bulbing ratio. The LD treated plants showed enlarged bases similar to those in NC, only with smaller bulbing ratio and less bulb layers. There was no enlargement of the base for SD plants. From the morphology of the dissected leaves, it can be seen that the plants in SD cabinet showed new green leaves that continued to be produced until the experiments ended whilst plants in NC and LD stopped forming new green leaves in their inner layers.

Table 3-4 Morphology of dissected sequential leaves from Renate plants grown in SDs, LDs and NC (17/10/2015)

Growth condition	Harvested onions	Dissected layers
PBF NC		
Cabinet LD		
Cabinet SD		

Furthermore, in order to test for reversibility of the bulbing process, one onion plant was moved from the LD cabinet into the SD cabinet in the middle of the experiment, and was kept for three weeks before dissection. The effect of daylength transfer on bulbing ratio and visible leaf number were shown to be noticeable. From the figure it

can be seen the plant stopped making bulb scales and returned to leaf development. *Renate* F1 onions respond quickly to a transfer to non-inductive conditions. The return to vegetative growth following a transfer from LD to SD conditions was significant.

Figure 3-5 Bulbing plants turned back into leaf development after 3 weeks transferred to SD

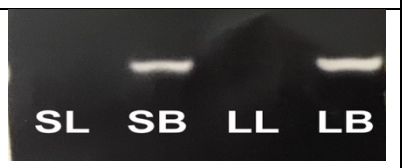
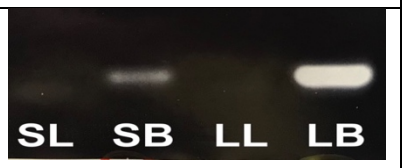
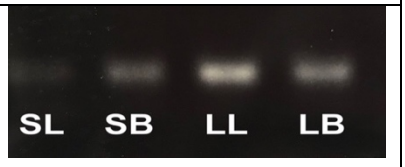
3.3.2 *Tissue-specific gene expression*

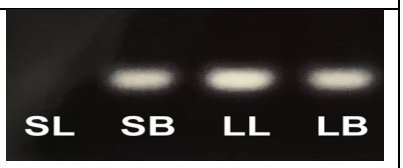
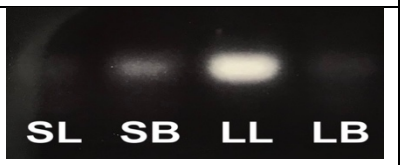
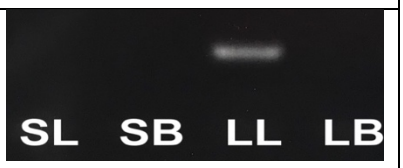
Nineteen novel sequences were identified and isolated from Renate. The expression of the genes in individual tissues of SD leaf, SD bulb, LD leaf and LD bulb was examined using standard PCR. Several genes showed evidence of differential expression in leaf vs bulb and LD vs SD or vice versa.

For the genes selected based on their differential expression in SD and LD bulb, gel images showed their expression in SL (SD-leaf), SB (SD-bulb), LL (LD-leaf) and LB (LD-bulb) samples (Table 3-5). *DFS* (Defensin-Like) and *GCL* (Glutamate--Cysteine Ligase) showed a clear bulb-specific expression pattern. For both genes, the bulbs grown under LD showed higher expression level compared with the bulbs grown in short-day. The results are consistent with the onion RNA-seq data which described *DFS* and *GCL* as LD bulb-specific. *MADS* (Mads-Box Transcription Factor) showed intensive expression in LD-leaf samples, while both bulb samples showed weaker bands. It can be seen that *MADS* was expressed slightly higher in LD-bulb then in SD-bulb, confirming what the transcriptome database suggested. For two circadian

regulation related genes *PHYA* (Phytochrome A) and *TIC* (Time for Coffee), both showed with highest expression level in LD-leaf samples. There was no amplification in SD-leaf samples for either gene. Some products were found in bulb tissues, but the expression levels were very low. Differential expression between SB and LB provided by RNA-seq data were not found in these two genes in this experiment. *PER12* (Peroxidase 12-Like) showed consistent result with the transcriptome database confirming its SD-bulb-specific expression. However, POLYCOMB was not expressed in any bulb samples. The only amplification of *POLYCOMB* mRNA was found in LD-leaf material. The reason for its absence in bulb material may be its generally low expression throughout the plant.

Table 3-5 Gel images of standard PCR expression results with comparison in the onion transcriptome database. target genes with differential expression (in bulb) between SD and LD. The RNASeq reference transcriptome generation and expression analysis was carried out by Siva Samavedam, School of Life Sciences, University of Warwick using Galaxy Biotinformatics Platform

Target Name	Predicted Function	Relative Expression in the transcriptome		Gel image results
		Bulb SD	Bulb LD	
DFS	Defensin-Like Protein 6-Like	19.5	**419.5**	 (35 PCR cycles)
GCL	Glutamate--Cysteine Ligase	4.2	**65.7**	 (29 PCR cycles)
MADS	Mads-Box Transcription Factor 14-Like	4.4	**39.7**	 (32 PCR cycles)

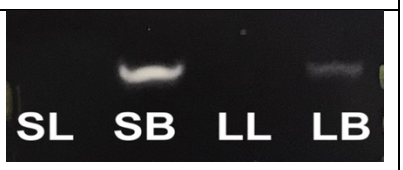
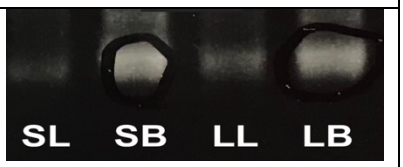
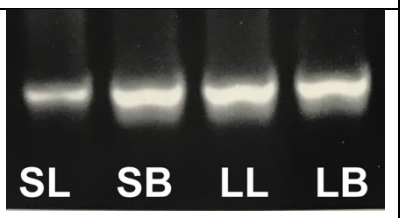
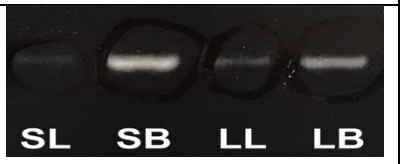
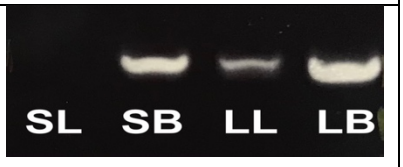
Target Name	Predicted Function	Relative Expression in the transcriptome		Gel image results
		Bulb SD	Bulb LD	
PHYA	Phytochrome A, Putative	1.3	**12.4**	 (29 PCR cycles)
TIC	Protein Time for Coffee-Like	0.0	**4.9**	 (29 PCR cycles)
POLYCOMB	Polycomb Group Protein Embryonic Flower 2-Like	**4.9**	1.0	 (35 PCR cycles)
PER12	Peroxidase 12-Like	**41.1**	5.3	 (29 PCR cycles)

For the genes selected for their bulb-specific expression pattern, targets were divided into two major groups based on their predicted function. One group is related with plant defence and onion's phytochemical content, another is linked with other aspects of plant development.

For the genes predicted to function in plant defence response, the results of their expression level were presented in Table 3-6. Markedly, all the targets showed higher expression in bulb material, consistent with their pattern in the RNA-seq data. Besides their significant bulb-specific pattern, *ALL* (Alliin Lyase Precursor) and *AHCYL1* (Alliin Lyase Precursor) appeared to have higher expression level under SD than LD. *ECT* (Endochitinase) was expressed equally in all bulb samples without being affected

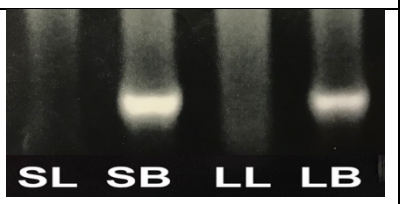
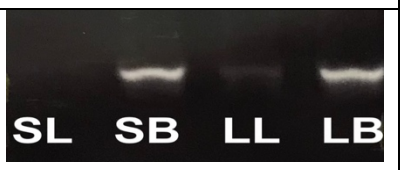
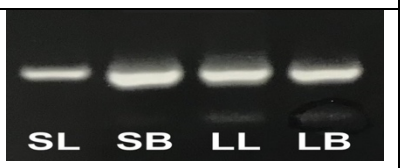
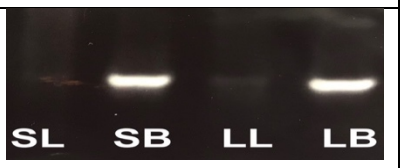
by day-length. *GST* (Glutathione S-Transferase) was also expressed generously and constantly throughout the plant, while slightly less in SD leaf material. *LFS* (Lachrymatory Factor Synthase) had the widest and brightest band under LD bulb sample as expected. There was no *LFS* band under SD leaf sample.

Table 3-6 Gel images of standard PCR expression results with genes of plant defence response. The RNASeq reference transcriptome generation and expression analysis was carried out by Siva Samavedam, School of Life Sciences, University of Warwick using Galaxy Bioinformatics Platform

Target Name	Predicted Function	Relative Expression in the transcriptome		Gel image results
		Leaf	Bulb	
ALL	Alliin Lyase Precursor	0.8	379.3	 (25 PCR cycles)
ECT	Endochitinase-Like	97.2	1261.3	 (29 PCR cycles)
GST	Glutathione S-Transferase T1-Like	142.1	939.9	 (29 PCR cycles)
AHCYL1	Adenosylhomocysteinase-Like Isoform 1	76.2	344.8	 (29 PCR cycles)
LFS	Lachrymatory Factor Synthase	110.6	1931.9	 (35 PCR cycles)

The remaining putative bulb-specific targets were predicted to have functions in other aspects in plant growth and development (Table 3-7). *SAUR* (Small auxin up RNAs), *RPL34* (60S Ribosomal Protein) and *H2A* (Histone H2A) showed considerable base expression pattern. *TUBA2* (Tubulin Alpha-2) was expressed generously in every sample, but with widest band under SD-bulb material.

Table 3-7 Gel images of standard PCR expression results with genes of plant growth and development. The RNASeq reference transcriptome generation and expression analysis was carried out by Siva Samavedam, School of Life Sciences, University of Warwick using Galaxy Biotinformatics Platform

Target Name	Predicted Function	Relative Expression in the transcriptome		Gel image results
		Leaf	Bulb	
SAUR	SAUR (Small auxin up RNAs) Family Protein	0.3	317.4	 (25 PCR cycles)
RPL34	60S Ribosomal Protein L34-Like	91.5	376.7	 (35 PCR cycles)
TUBA2	Tubulin Alpha-2 Chain, Partial	39.5	343.4	 (29 PCR cycles)
H2A	Histone H2A.1-Like	30	394	 (35 PCR cycles)

3.3.3 Spatial gene expression in Renate leaves

3.3.3.1 Spatial expression of *FT*s

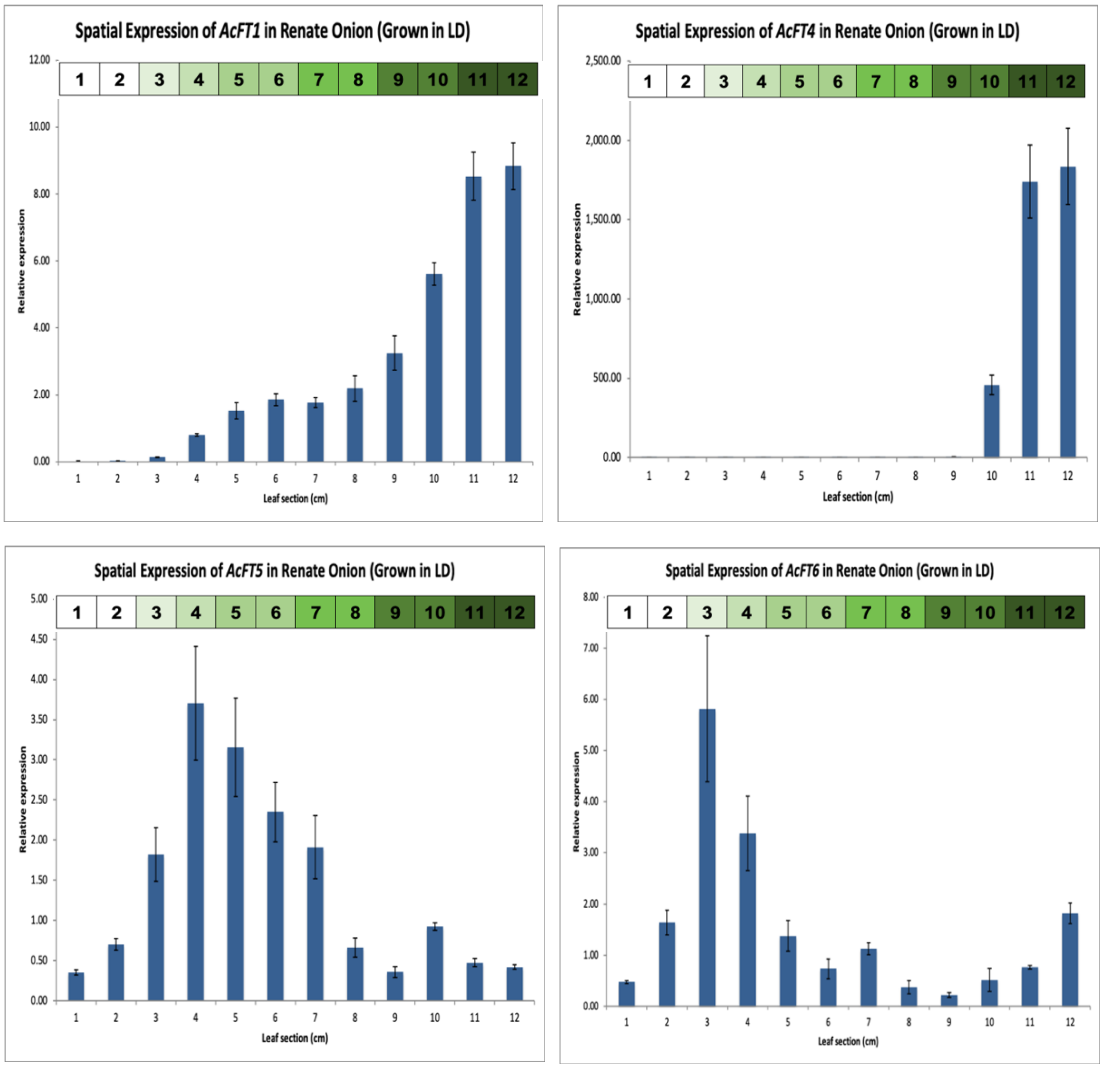


Figure 3-6 Spatial expression of AcFTs along Renate leaf section samples, relative to average of PP2A1, AcTUB and UBL. Error bars represent the SEM.

FT1 was expressed throughout the leaf starting from the transition parts, and the expression increased along with darker green colour. There was no *FT1* expressed at the basal tissue. *FT4* was expressed only in the greenest parts of leaf blade. There was no expression of *FT4* at basal materials or younger leaves (Figure 3-6).

In contrast, *FT5* was expressed throughout the leaf from base to top, and peaked in the transition part (Sample 4). The expression was also abundant in young leaf tissue (sample 5, 6,7). Generally, it showed higher expression in neck regions rather than basal part or leaf blade. *FT6* showed the similar pattern, with highest expression at the transition part of the leaf (sample 3). There was very low expression of *FT6* in leaf materials (sample 5-12).

3.3.3.2 Spatial expression of circadian rhythm-related genes

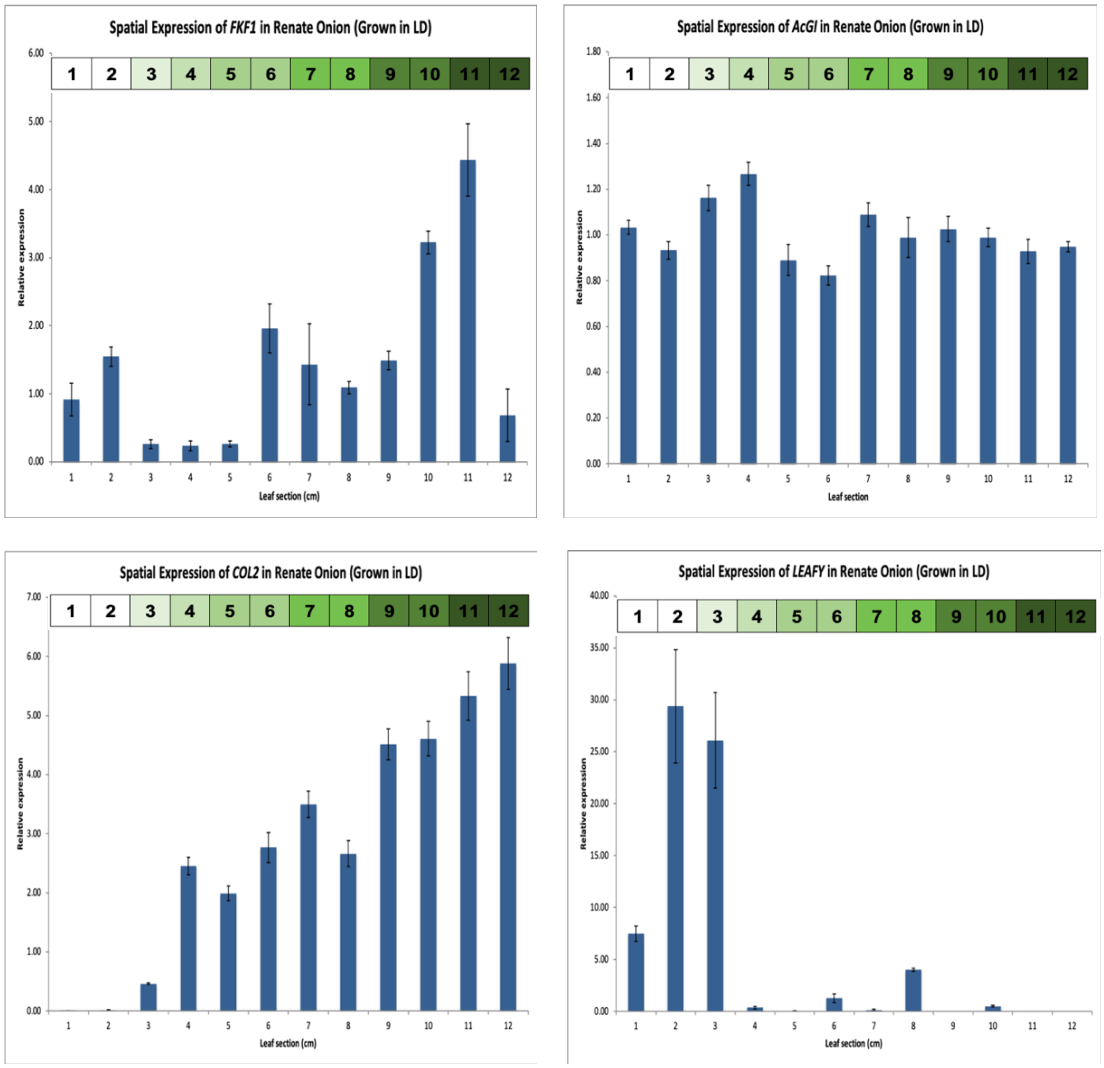


Figure 3-7 Spatial expression of FKF1, GI, COL2 and LEAFY along Renate leaf section samples, relative to average of PP2A1, AcTUB and UBL. Error bars represent the SEM.

FKF1 showed a more leaf-specific expression while low expression at the base and almost no expression in the intermediate transition parts. The expression of *GI* was abundant and generous throughout the plant, but no tissue specific expression pattern was showed. *COL2* was expressed throughout the leaf starting from transition part (sample 3). It showed high expression starting from 4cm and stayed at the top to the highest point. No expression was found at base. *LFY*, on the other hand, was mostly expressed at base, near apical meristem. The highest expression presented in the middle of the bulb. There was almost no expression of *LFY* in leaf blade starting from transition sample 4 (Figure 3-7).

3.3.3.3 Spatial expression of bulb-specific genes

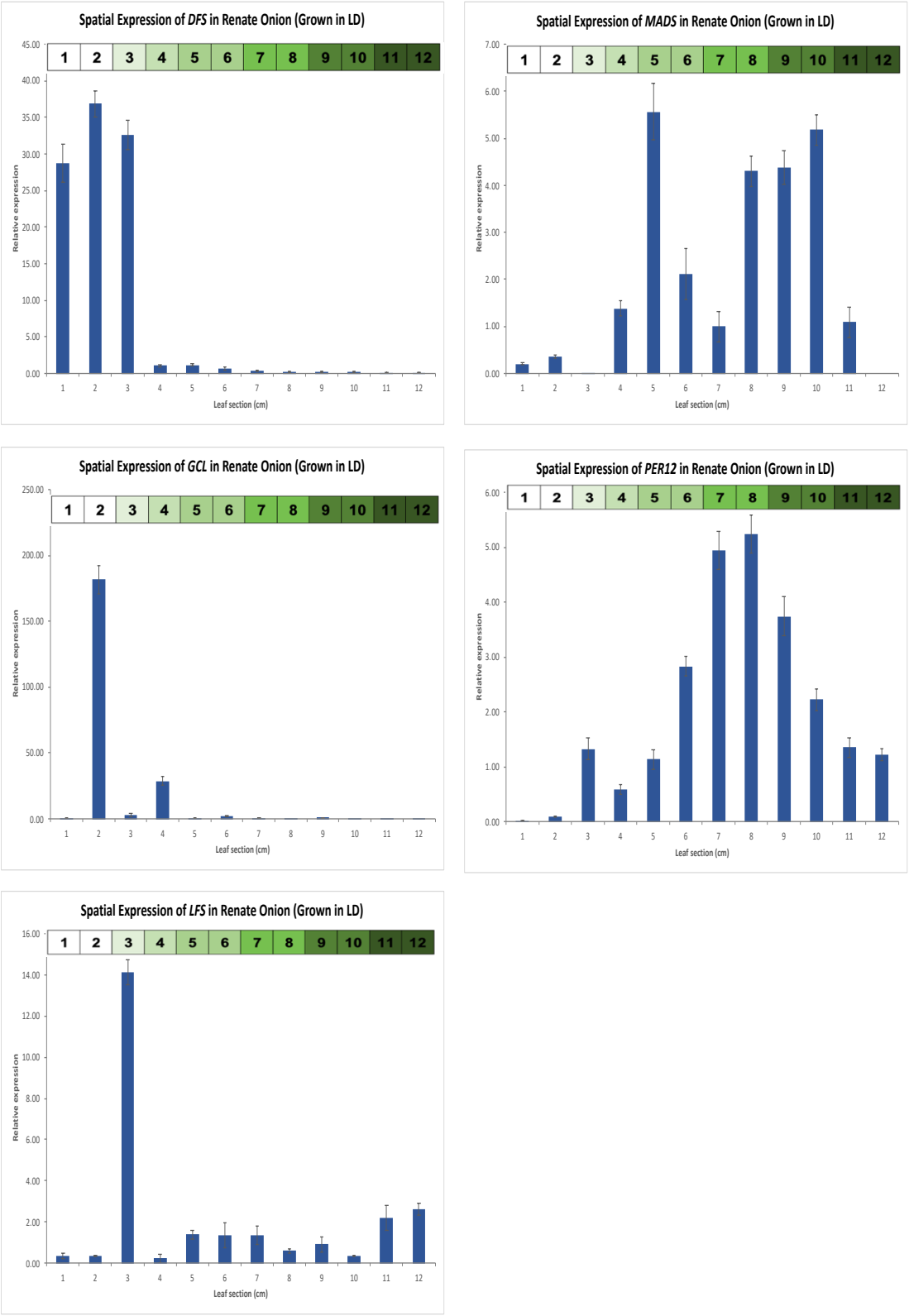


Figure 3-8 Spatial expression of DFS, MADS, GCL, PER12 and LFS along Renate leaf section samples, relative to average of PP2A1, AcTUB and UBL. Error bars represent the SEM.

Three of these putative bulb-specific candidates in the onion transcriptome database were confirmed in this experiment, while two of them showed different patterns (Figure 3-8). *DFS* is LD bulb-specific in the onion RNA-seq data, and this was confirmed by the RT-PCR result showing that the major expression of *DFS* was in first three basal samples. There was very low expression in young leaf material (sample3-7) and no expression further in leaf (sample 8-12). *MADS* is LD bulb-specific in the transcriptome database. In preliminary gene expression experiment (section 3.3.2) *MADS* showed highest expression in long-day leaf material. This spatial experiment showed consistent result with the preliminary experiment, confirming *MADS* is mainly expressed in leaf blade. *LFS* was expressed throughout the plant but significantly highest at 3cm point of the upper base. The result supports its pattern in the onion transcriptome database as well as the preliminary experiment, and more precisely compared with the predicted bulb pattern. *PER12* showed its peak in younger leaf material, and the expression declined at both ends of the plant (basal part or older leave). However, the onion transcriptome as well as the tissue-specific gene expression experiment all support its bulb-specific pattern.

3.4 Discussion

3.4.1 *Daylength and Renate development*

The experiment figures showed the differences in bulbing ratio among three growth conditions with onion development, it can be confirmed that the pattern of bulbing ratio over time is affected by daylength. Bulb initiation in *Renate* F_1 onions was stimulated by longer photoperiod, which is consistent with its classification as a LD variety.

The experiments also showed LDs of about 14h are sufficient to initiate bulbing, 10h of artificial light or less will not enable bulb formation. Plants grown in NC showed the best bulb formation among the three conditions. This is probably due to higher light levels, which have been shown to accelerate bulbing (Mondal, 1986).

The experiment provided further evidence that bulbing is also a reversible process previously discussed Taylor (PhD Thesis, 2009). When onions are transferred to non-inductive conditions after initiation of bulbing, the plants revert to vegetative leaf growth. It is also a supportive result for the theory that individual leaves become committed to a particular daylength response (Cookson et al, 2007).

This section establishes the basis to study the expression of putative photoperiod response genes at a molecular level. The photoperiodic nature of bulb initiation not only leads to the hypothesis that the genes controlling photoperiodic flowering also control photoperiodic bulbing, but also the location of the genes is important. The characterization of several onion putative photoperiod response genes is discussed in the spatial experiment of this chapter, focusing on whether they play a role in light perception in leaves or to the response at the base.

3.4.2 *Tissue-specific gene expression*

3.4.2.1 Genes of plant defence response and onion nutrition

Plants respond to pathogens by activating a variety of defence responses, including the induction of a large number of defence- related genes (Dixon and Harrison, 1990). The nutritional level of plants and their defence mechanisms are highly interrelated. A large number of studies showed the beneficial effects of some mineral nutrients on not only natural defence of in response to the pathogens but also human health (Thomma et al, 2002; Sharma et al, 2011; Li et al, 2015). In onion, plant defence is also linked with onion's nutrition level and its pungent flavour. In this experiment, several plant defence related genes were chosen. We expected them to be expressed in onion bulbs. Study of these candidates may help for breeding "tear-less" or on the contrary "high nutrition" onion varieties.

DFS is predicted to translate a defensin-like protein. Defensins are small basic peptides that have a characteristic three-dimensional folding pattern that is stabilized by eight disulfide-linked cysteines. Lacerda's (2014) experiments show transgenic plants overexpressing defensins are strongly resistant to fungal pathogens. Some plant defensins are not merely toxic to microbes but also have roles in regulating plant growth and development. Defensin is also linked with flavonoid, one of the heathiest polyphenolic natural products in onion, and plays important roles as defence compounds and as signalling molecules in reproduction (Koes et al., 1994, Gurjar et al. 2012). This experiment showed the expression of *DFS* is in the bulb, confirming these healthy defence sulfur-related compounds accumulate in that part of the plant.

GCL was chosen for its link with glutamate--cysteine ligase and chloroplast precursor. The major function we expect is a role in onion sulphur metabolism and bulb phytochemical content level. *GCL* can be used to provide conditions in promoting growth and stimulating glutathione biosynthesis during onion bulb formation. Cameron and Pakrasi (2010) suggested that cellular glutamate-cysteine ligase and glutamylcysteine content declined in exposure to dark and blue light during photoheterotrophic growth. In this experiment, *GCL* showed its significant abundance

in long-day bulb tissue, which not only implies a higher phytochemical level in bulb, but also supports Cameron's study that these chemical content declines in dark.

ALL is a putative Alliin Lyase precursor. The action of the enzyme Alliinase operates in the biochemical pathway that produces the compounds responsible for the onion's characteristic flavour (Shen, 2000). Amplification of *ALL* in *Renate* F1 samples showed its base specific expression as expected, which explains the pungent flavour of onion bulb. However, its considerably higher expression under SD base rather than LD bulb was not expected. This is probably because this experiment used the same sample size for all daylength conditions, while actually the size of a LD bulb is much larger than a SD bulb, so there is no proof of a higher overall biochemical level in a short-day grown plant.

ECT is predicted to code for an endochitinase-like protein in the onion transcriptome database. Chitin is the main structural component of the cell walls of fungal and insect pathogens which cause plant diseases. The enzymes responsible for hydrolysing chitin, namely chitinase, are receiving attention in regard to their development as biopesticides or chemical defence proteins (Schlumbaum et al. 1986). Chitinase attacks chitin molecules in fungal cell wall and insect skeletons. Chitinases have been reported to be involved in response to an array of stimuli including mechanical injury, phyto-hormones, temperature alteration, salinity, metal stress, and pathogen invasion (Mohapatra and Nanda, 2018). In addition, chitinase has been observed to accumulate during normal development in healthy tissues (Lotan et al. 1989; Neale et al. 1990). A recent study in onion chitinase by Mohapatra (2018) found nine highly homologous onion contigs with other plant chitinases having conserved motifs, and proposed four of them have a role in defence response. This experiment showed the bulb-specific expression pattern of *ECT*. The result is expected and may benefit in characterizing the gene in future onion genetic studies.

GST is related to glutathione S-transferase T1-Like protein. Glutathione S-transferase (*GST*s) is comprised of a large superfamily of multifunctional proteins. *GST*s play roles in both normal cellular metabolism as well as in the detoxification of a wide variety of xenobiotic compounds. They protect plant cell membrane structure and protein activity when plants are subjected to environmental stresses (salt, heavy metal,

drought, low temperature herbicide etc.). In addition, plant *GST*s play a role in the cellular response to auxins and during the normal metabolism of plant secondary products like anthocyanins and cinnamic acid (Marrs, 1996). Type I *GST*s also appear to function as defence genes or cellular protectant genes, producing proteins in response to pathogen attack, wounding, senescence, and the resulting lipid peroxidation that accompanies these processes (Dudler et al., 1991; Bartling et al., 1993; Zhou and Goldsbrough, 1993). In this experiment, *GST* was generously expressed throughout the plant in bulbs and LD leaf tissues. The abundance of *GST* indicates its key function in plant defence as well as many other aspects in plant development.

AHCYL1 (adenosylhomocysteinase-like isoform 1) is an enzyme that converts S-adenosylhomocysteine to homocysteine and adenosine, and further promote transmethylation reactions. It is mostly studied in human for its putative key role in the control of methylations via regulation of the intracellular concentration of adenosylhomocysteine (Yang et al., 2003). Many diseases were found to be associated with changes in adenosylhomocysteinase function in animals (Matthews et al., 2009). In higher plants, adenosylhomocysteinase was reported to play a part in plant growth and development in tobacco and Lupins (Brzezinski et al., 2008). Reduced *AHCL* expression in tobacco causes lack of apical dominance and floral abnormalities (Fulneček et al., 2011). In tomato, some evidence indicated *AHCYL* genes play a role in plant response to biotic and abiotic stress response (Li, etc., 2015). Co-silencing of three tomato *AHCL* genes confers increased immunity to pathogen infection, and also enhanced drought stress tolerance. The experiment showed *AHCYL1* was expressed at a higher level in SD base materials compared to LD. The lower expression in LD may indicate a silencing in expression of this gene, and thus leading to stronger defence response and higher phytochemical content.

LFS (Lachrymatory Factor Synthase) is well known for its producing lachrymatory factor (propanthial S-oxide), the one causes onions' pungency when chopped up. *LFS* not only gives onions their savour but also has a role in plant's defence mechanism against microbes and animals. There are studies focussed on the developing "*LFS*-suppressed onion", which means a non-lachrymal onion that still retains its characteristic flavour and high nutritional value by down-regulating the activity of

this enzyme, and hence leading to tear-free cutting onion variety (Eady et al, 2008; Thomson et al, 2013). In this experiment, *LFS* showed most expression in LD bulb tissue, suggested there was more of *LFS* for this LD variety Renate.

3.4.2.2 Genes linked to circadian regulation

As onion bulb formation is highly daylength regulated, the genes related to circadian clock are of great importance to this project. Studies of these genes can help to find their potential role in onion daylength-dependent bulbing process. In this experiment, two sequences were found and selected from the onion transcriptome database for their putative relation with circadian rhythmic.

PHYA (Phytochrome type A) is one of the basic photoreceptors in plants and has a key function in photoperiodical regulation. Lercari (1982) suggested phytochrome promoted carbohydrate accumulation by far-red light. Research indicated the lower the R:FR, the higher the bulbing ratio and the earlier the bulb swelling (Mondal, 1986). The influence of the spectral ratio R:FR indicated the involvement of the phytochrome in a high irradiance reaction. The results for this experiment showed *PHY* was expressed intensively in LD leaf sample. The higher expression in leaf may be linked with its role as photoceptors to perceive daylight. There was also expression in both bulb materials, implying *PHYA* has a wider role in other aspects in plant growth and development.

TIC (TIME FOR COFFEE) was first identified to be required for proper clock activity, such as maintaining circadian period and amplitude, and regulating clock-driven output responses (Hall et al., 2003; Ding et al., 2007). We chose *TIC* as one of the targets for it is not only involved in circadian clock but also related with leaf morphology (Duc et al., 2009). It is known to be involved in clock resetting at dawn. In Arabidopsis, *TIC* mutants (*TIC* overexpressing plants) displayed a shortened circadian period compared to the wild type (Ding et al., 2007). *TIC* is also known to be required for proper growth, development, and metabolic homeostasis (Duc et al., 2009). *TIC* overexpression plants display developmental defects including altered leaf morphology and pigment biosynthesis, and other developmental defects. In this

experiment, the LD leaf-specific expression pattern of *TIC* can be potentially linked with altered leaf morphology for long-day grown onions. The gene indicates its role in leaf to bulb transition in onion development.

3.4.2.3 Genes of plant growth and development

The ***MADS*** box is a highly-conserved sequence motif found in a family of transcription factors. The family of *MADS* domain proteins has been subdivided into several distinct subfamilies. Most *MADS* domain factors play important roles in developmental processes. Most prominently, the *MADS* box genes in flowering plants are the "molecular architects" of flower morphogenesis. In plants, they are associated with numerous development processes and most notably those related to reproductive development: flowering induction, specification of inflorescence and flower meristems, establishment of flower organ identity, as well as regulation of fruit, seed and embryo development (Kumar et al.，2016). We choose *MADS* as candidate to see if it functions in determine whether the younger leaves develop into photosynthetic tissue or bulb scales. The result of this experiment showed the major expression for *MADS* appeared in LD leaf samples. This was unexpected as it is inconsistent with the onion transcriptome database. The LD leaf-specific pattern for *MADS* indicate it has multiple functions in specific growth stage during onion development.

The ***POLYCOMB*** genes found in other plant species refers to conserved amino acid sequences present in chromosomal proteins that contribute to the epigenetic control of gene expression by altering regional organization of the chromatin structure. Homologs of all poly-comb classes have been widely characterized in yeast, mammals and plants. We suggest that the poly-comb gene has a role in regulating various aspects of onion development. The experiment showed an unexpected result that *POLYCOMB* is only expressed in LD leaf material, which may imply its unknown function in epigenetic control of gene expression in onion bulb formation.

PER12 is related to peroxidase. There are wide physiological functions of peroxidases in plants, covering active oxygen metabolism, formation of lignin and suberin, degradation of auxin, oxidation of other compounds and responses for various environmental stresses especially pathogen attack. The experiment showed consistent result with the onion transcriptome where the highest levels of *PER12* mRNA were found in SD base materials. This may be linked with its role of degradation of auxin in short-day grown *Renate* onion bulbs.

Small auxin up RNAs (***SAURs***) are the largest family of early auxin response genes. *SAUR* functions have remained elusive, while recent genomic studies have implicated *SAUR*s in the regulation of a wide range of cellular, physiological, and developmental processes. Crucial mechanistic insight into *SAUR* function was provided by Ren (2015) that *SAUR*s inhibit PP2C.D phosphatases to activate plasma membrane ATPases and promote cell expansion. In this experiment, it is clear that *SAUR* was expressed in bulb material but not in the leaf. This bulb-specific *SAUR* may due to bulb's character of apical meristem and indicate the gene's important role in onion bulb formation. It may play a key part in the process of how leaf cells swell and expand to form a bulb.

RPL34 is related with ribosomal protein, an intricate ribonucleoprotein complex. The primary regulatory mechanisms coordinating r-protein synthesis in animals have been widely identified (McIntosh and Bonham-Smith, 2006), while the mechanisms governing the coordination of plant r-protein expression remain largely unexplored. The *rpL34* gene, which encodes a cytoplasmic ribosomal protein with a high homology to the rat 60S r-protein L34, was firstly isolated from tobacco by Dai (1996). His experiments with promoter fused transgenic plants showed that mechanical wounding increased the *rpL34* promoter activity about five times compared to untreated plants. Besides, the transgenic plants showed the *rpL34* promoter activity is high in actively growing tissues, including various meristems, floral organs, and developing fruits (Dai et al., 1996). In this experiment, *RPL34* was expressed only in onion bulbs, the result is consistent with literature suggesting the gene exists in meristems.

TUBA2 α-tubulin is one of the basic subunits of all microtubules together with β-tubulin. The two polypeptides coassembles the heterodimer of specialized

microtubules, which act as the major structural elements of the cytoskeleton, and the mitotic and meiotic spindles. In Arabidopsis, *TUBA* was expressed primarily in pollen (Carpenter et al., 1992), while the same pollen expression pattern also found in rice (Yoshikawa et al., 2003). Study in *Populus* indicate *TUBA* homologs were abundant in xylem and pollen (Oakley et al., 2007). These data suggest *TUBA* has been linked to secondary cell wall thickening in plant development. In this experiment, *TUBA2* was expressed throughout the plant under any daylengths, suggesting its foundation role in primary plant development.

Protein histone ***H2A*** type 1 is a core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin and further packaged into genomes. The incorporation of histone variants into nucleosomes and the addition of post-translational modifications to histones can alter the physical properties of nucleosomes and thereby serve as a mechanism for regulating DNA exposure. New findings by Su et al. (2017) revealed phosphorylation of histone *H2A* promotes flowering time in Arabidopsis thaliana. *H2A* was found to be bulb-specific in this experiment. The result may correspond with Su's paper which states *H2A* promote flowering time in Arabidopsis, it may also have a potential up regulation in bulb formation in onion.

3.4.3 *Spatial gene expression in Renate leaves*

The genes selected from Arabidopsis flowering pathway for this experiment all showed the expected spatial patterns. And most of the novel bulb-specific sequences also showed the spatial patterns which can be reasonably linked to their predicted functions.

For the genes involved in circadian regulation, *AcFKF1* showed a more leaf-specific expression. The result is similar to previous study (Taylor et al., 2010) which found *AcFKF1* had leaf-specific expression in LD and bulb-specific pattern in SD. In *Arabidopsis* photoperiod pathway, the nuclear protein *CO* is produced in leaves, of which the activity acts upstream of a graft-transmissible signal (Notaguchi et al, 2008). This experiment showed consistent result which onion *AcCOL2* was expressed only

in the leaf. The *AcFT1* and *AcFT4* leaf-specific spatial pattern is also consistent with *FT* in Arabidopsis, of which RNA expression is in leaf then the protein transfers to a distant site of shoot apex, and controls the timing of flowering (Navarro et al, 2015). *AcFT5* and *AcFT6* both showed highest expression in transition parts of the plant. The similarity of the two genes may be due to the interactions with other *FT* genes, and imply their unknown function in *FT*s signal translocating and plant development. *AcLFY* was mostly expressed at the base in this experiment, near apical meristem. The result corresponded with the apex specific expression of *AcLFY* in Arabidopsis, which trigger the expression of the floral homeotic genes at the floral apical meristem and cause flowering (Weigel et al., 1992). The *LFY*'s function in causing a group of undifferentiated cells (meristems) to develop into flowers instead of leaves associated with shoots can also be applied to onion, making leaves swell and develop into bulbs. The result implies the gene is an important element of the transition from the vegetative growth to the bulb formation in onion.

For the genes related to onion quality traits such as carbohydrates and organosulphur content, *DFS*, *GCL* and *LFS* were expressed only in bulbs as expected. *MADS* was expressed mostly in leaf in this experiment. The spatial pattern is consistent with former tissue-specific gene expression experiment but not the onion transcriptome database. Further evidence can be found with Arabidopsis leaf tissue, where *MADS* box transcription factor family is found and proposed to have function in integrating the floral induction and floral organ formation (Folter et al., 2005).

3.5 Conclusion

Bulb initiation in *Renate* F1 onions controlled by photoperiod is discussed in this chapter. The data presented is consistent with data from field experiences that LD onion varieties require LDs throughout the period of bulbing to reach their maturation. Bulbing is also a reversible process. *Renate* F1 onions respond quickly to a transfer to non-inductive conditions.

Twenty-four novel sequences were selected from the onion transcriptome database for their putative functions in circadian regulation, bulb initiation, plant development, or nutrition level, and were identified in *Renate* onion SL, SB, LL and LB materials. The experiment showed supportive results of the onion transcriptome data base for some candidates, while other candidates showed different expression patterns. For the sequences selected based on their putative differential expression between SD bulb VS. LD bulb, *DFS*, *GCL* and *PER12* showed consistent results with the onion transcriptome. *DFS* and *GCL* had LD bulb-specific pattern, while *PER12* showed SD bulb-specific. Other targets *MADS*, *PHYA*, *TIC* and *POLYCOMB* did not support what was found in the RNA-seq study. The SD-bulb and LD-bulb differences were not significant, but all these genes showed highest expression in LD-leaf materials. For the sequences selected based on their putative bulb-specific expression pattern, all the candidates showed consistent results with the onion transcriptome data. These genes were expressed higher at basal tissue as expected, but with more detailed information in their differential expression between SD and LD growth conditions. *LFS* showed significant LD bulb expression pattern, whilst *ALL* and *AHCYL1* showed SD bulb-specific. These observations serve as a framework for the future characterization and functional assessment of onion genome although bioinformatics analysis is yet not complete. Moreover, it adds insights to the understanding of the distribution and diversity of these sequences in onion. The role of these genes will provide valuable information for the plant genetic engineering in the future.

A group of key genes involved in onion day length dependent bulb initiation and onion quality trace were selected from past literature or the onion transcriptome database, and assayed by RT-PCR amplification using more precise leaf section samples. *FT1* and *FT4* are both expressed in the leaf. *FT5* and *FT6* were expressed throughout the

leaf while showing highest expression at transition parts, indicate their unknown function, if any, in response to photoperiod. *COL2* and *FKF1* had significant higher expression in the leaf, while *GI* was expressed constantly from base to blade. *DFS* existed in all bulb samples but showed no expression in leaf nor transition parts. *MADS* showed low expression at the base but started its expression from transition and peaked in green parts of the plant. The result confirmed its pattern found in Tissue specific gene expression experiment, although inconsistent with the onion transcriptome. *LFS* and *GCL* showed expression only in the middle part of the bulb, whilst *PER12* showed contrast pattern to the onion transcriptome or tissue specific gene expression experiment, peaked in the middle of the leaf with no expression in the basal tissue. These expression data are more precise and accurate compared with the predicted pattern in the database. Moreover, the expression sites of the circadian regulated genes in onion are found to be consistent with those in Arabidopsis. The observed spatial expression patterns provide the basis for further study of the expression of these putative photoperiod response genes.

4 GENE EXPRESSION IN THE LD VARIETY *RENATE* AND THE SD VARIETY *HOJEM* UNDER INTERMEDIATE DAYLENGTHS

4.1 Introduction

The circadian clock is an endogenous timekeeping mechanism which is controlled by various feedback loops (Jackson, 2009). This allows the clock to continue to cycle under constant conditions. The basis of this physiological and behavioural circadian rhythms is the periodic expression of clock genes. Circadian control of transcription is widespread and the list of plant genes regulated by the circadian clock is extensive. There is conservation of the genes of photoperiod response in many plant species, whereas different regulation of their homologues has also been reported in various plants. *Arabidopsis thaliana* is one of the plant models for the basic circadian clock regulation genetic network. The mechanism underlying the photoperiodically control of flowering in Arabidopsis is proposed to be conserved across plant species, including onions. Different varieties of onion have different daylength requirements, but mechanisms are still not understood. One possibility is differential expression by photoperiod related genes. Studies on the SD plant *Pharbitis nil* (Liu et al., 2001) and rice (Kojima et al., 2002), as well as LD plant barley (Kikuchi and Handa, 2009) and sugar beet (Chia et al., 2008), all have shown both conservation and variation of the functional genes in different photoperiod responses. This leads to the hypothesis that the same genes are also involved in the photoperiodic control of bulb initiation in onion. To understand the molecular mechanisms of Arabidopsis flowering, it is important to integrate the knowledge obtained from particular plant species and compare it with the model plant Arabidopsis. Considering the diversity in flowering mechanism under inductive and non-inductive conditions together with altered regulation between SD and LD plant species, we used different onion varieties and various daylength conditions in the experiments. As the circadian clock is entrained by day light in 24-hour cycles (Michael et al., 2003), a diurnal experiment studying the rhythmic expression of these genes was conducted and will be described in this chapter.

This chapter describes experiments designed to quantify the diurnal expression in LD and SD onion, of key genes identified as particularly linked to circadian regulation in

Arabidopsis. A previous group member Rashid (2020) compared diurnal expression in both SD and LD onion varieties at 8h and 16h, but these treatments give the same bulbing responses to both (bulbing in 16h and non-bulbing in 8h). Experiments for this chapter therefore were carried out looking at diurnal gene expression at intermediate daylengths (10-14h) where there was a differential bulbing responses between the varieties (LD variety *Renate* not forming bulb until 14h while SD variety *Hojem* bulbs under 12h). The major objective of these experiments was to compare the circadian expression patterns of these genes in different onion varieties under various daylengths using qRT-PCR. A primary goal was to determine whether the functional clock genes in the Arabidopsis flowering pathway have a diurnal rhythmic expression pattern within the onion varieties. Furthermore, to characterise the photoperiod response of these clock genes to different daylengths in different onion varieties.

4.2 Plant material and methodology

4.2.1 Plant material

For the diurnal time-course in **LD variety *Renate***, onion plants were grown in a photoperiod -controlled glasshouse compartment in the PBF at natural LD condition during the period from 18th March to 25th May 2016 when daylight was 12h 3min to 16h 12min, respectively. Initially, onion seeds were sown in modular trays and after 4 weeks plants were potted up into 9 cm pots containing Levington M2 compost. At 69d from sowing, at the time of expected bulb initiation, all the plants were separated randomly and transferred into three SANYO 2297 controlled environmental cabinets for 14 days daylength treatments of 10h, 12h and 14h, respectively with fluorescent supplemented with incandescent lamps. The beginning of photoperiod was set at 8:00am (ZT0) for all cabinets, and lasted for 10h, 12h and 14h for each cabinet. Other growth environment parameters of day/night temperature and percentage of CO_2 and relative humidity remained the same as the PBF glasshouse compartment (Figure 4-1).

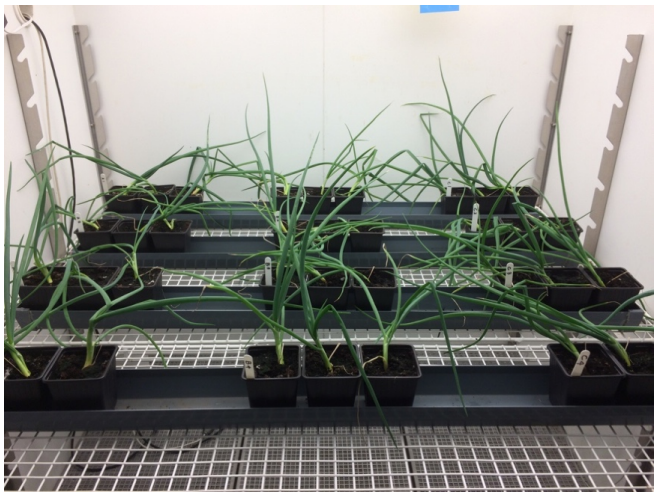


Figure 4-1 Renate plants grown in controlled environment SANYO Cabinet (10h of daylight). A same design was employed for plants grown in 12h and 14h cabinets.

After 14 days of differential daylength treatment in controlled environment cabinets, when the plants were supposed to be coped with the provided environment, harvesting of leaf materials was scheduled at 3h intervals starting from 8:00am (ZT0), and

covered two consecutive days. Six time points were set for sampling from ZT0 to ZT15 for each day (Table 4-1). For each timepoint, three plants were selected randomly from each cabinet (using Completely Randomised Design). 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.

Table 4-1 Sampling timepoints for Renate plants. Clear cells without shades indicate illuminated parts of a day, Lt Trellis shades indicate dark period. Six samplings each day for consecutive two days, from ZT0 to ZT15 at 3 hours intervals.

Sampling time		Sampling timepoints for 1st day						Sampling timepoints for 2nd day					
		8:00	11:00	14:00	17:00	20:00	23:00	8:00	11:00	14:00	17:00	20:00	23:00
ZT time		ZT0	ZT3	ZT6	ZT9	ZT12	ZT15	ZT0	ZT3	ZT6	ZT9	ZT12	ZT15
Number of plants harvested & pooled	**10h**	3	3	3	3			3	3	3	3		
	12h	3	3	3	3	3		3	3	3	3	3	
	14h	3	3	3	3	3	3	3	3	3	3	3	3

For diurnal time-course in **SD variety *Hojem***, a similar experimental design was employed. *Hojem* seeds were planted on 27/05/2017, and plants were transferred into larger pots 26 days later and kept in PBF natural conditions from 22/06/2017 to 03/08/2017 when daylengths were 16h 48min to 15h 24min, respectively. At 69 days from sowing, when bulbing had been initiated, plants were separated and transferred into three controlled SANYO cabinets with the same settings as used for the *Renate* experiment, providing 10h, 12h and 14h of intermediate daylengths (Figure 4-2).

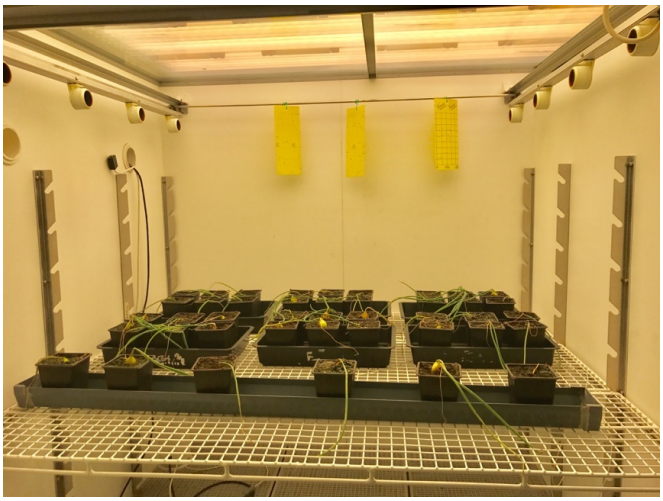


Figure 4-2 Hojem plants grown in controlled environment SANYO Cabinet (10h of daylight). A same design was employed for plants grown in 12h and 14h cabinets.

Hojem plants were kept for 14 days in the cabinets before harvesting for molecular analysis. Similar sampling timepoints of that of *Renate* were conducted except for one additional timepoint at ZT21 for each day (Table 4-2).

Table 4-2 Sampling timepoints for Hojem plants. Red shades indicate illuminated parts of a day, grey shades indicate dark period. Seven samplings each day for consecutive two days, from ZT21 to ZT15 at 3 hours intervals.

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

4.2.2 Methodology

Extraction of total RNA, DNase treatment and synthesis of cDNA using 2 µg total RNA was followed by the protocol as described in Chapter 2. Then followed by the relative expression of the key genes examined by qRT-PCR, which was carried out using the CFX384 Touch™ Real-time PCR machine from BioRad as described. PCR

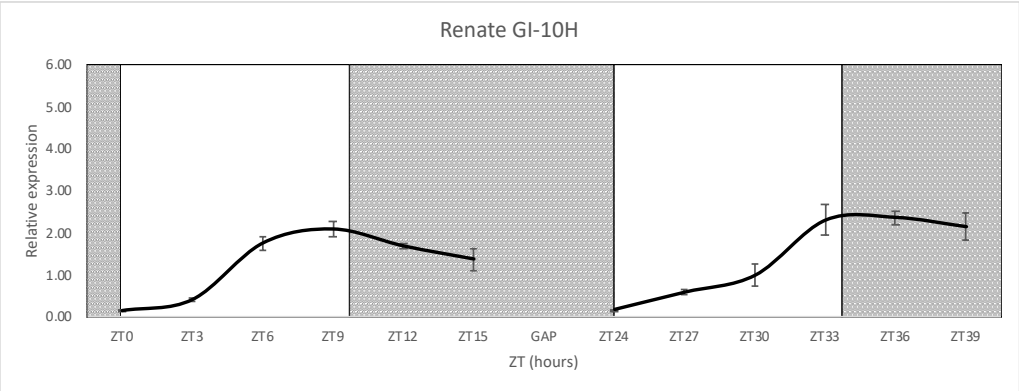
conditions together with designing and testing qRT-PCR primers (forward and reverse) were also conducted as mentioned in General Methods in Section 2.3.8. Eight reference genes were examined for each variety with its pooled leaf samples from all timepoints, and three with the best performance were selected for *Renate* and *Hojem* independently (*Renate* and *Hojem* used a different set of reference genes, see Appendix). Samples from all three daylength conditions within the variety were carried out in one RT-PCR run for each gene. Standard curves (using 10-fold serial dilutions) were plotted using cDNA synthesised from approximately 2 µg of total RNA extracted from leaf material harvested at various time-points.

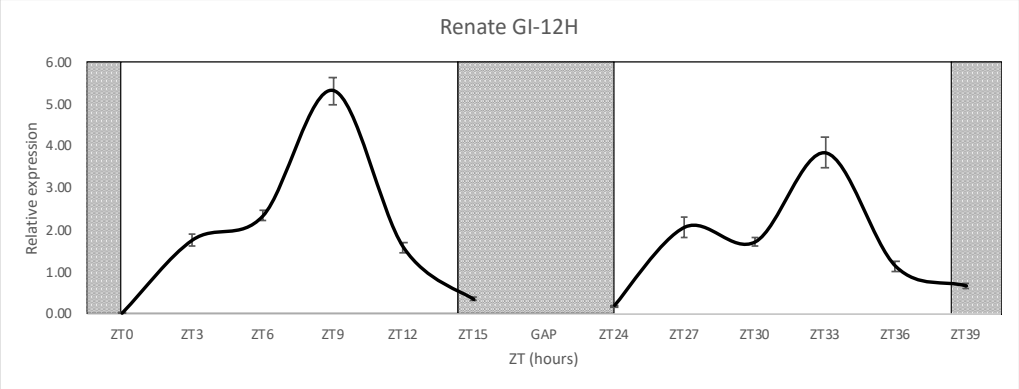
At the end of PCR run, the qRT-PCR data were analysed and normalised against expression levels of the reference housekeeping genes (Appendix V) achieved by using Biogazelle qBase+ software as described for each sample (www.biogazelle.com). qbase+software based on the geNorm (Vandesompele et al., 2002) and qBase technology (Hellemans et al., 2007). Forty-eight-hour averages of expression were calculated and standard errors included. Normalisation was achieved by dividing the expression of the gene of interest at a specific time-point by the expression of the mean of three reference genes at that same time-point.

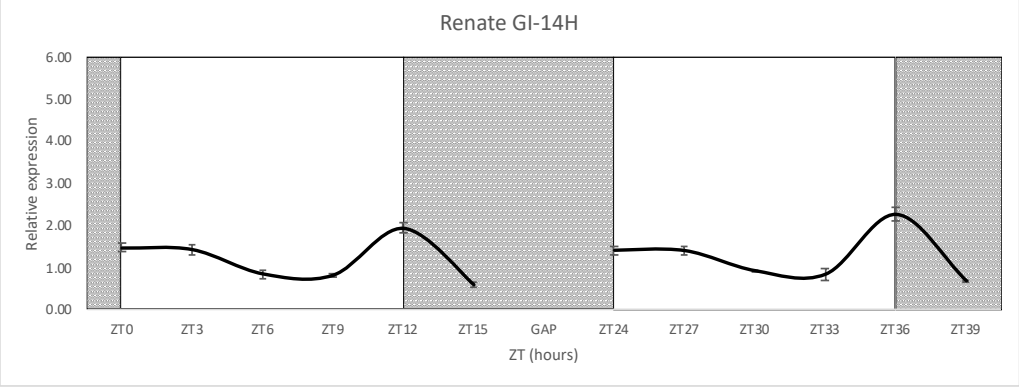
4.3 Results

4.3.1 Average diurnal expression of AcGI in LD (Renate) and SD variety (Hojem)

An average relative expression was obtained using RT-PCR (Figure 4-3). The expression of *GI* in both varieties showed obvious diurnal pattern which peaks at the latter part of the day, with the peak time moving to ZT12 with longer daylengths for both varieties. The result is similar with Fowler et al. (1999) which *GI* mRNA shows a peak in expression around 8-10 hours after dawn. The expression pattern in which peak time occurs around ZT9 under 10h and 12h, and later at ZT12 under 14h is similar for both varieties. There was no obvious difference between LD and SD varieties. The expression pattern is similar to that of Arabidopsis *GI*, which peaks at ZT8 in 8h of SDs and ZT10 in 16h of LDs (Fowler et al., 1999). Also, the result is consistent with Taylor et al. study (2010) suggesting that the expression pattern of *AcGI* is very similar among onion varieties, which responds to different daylengths, and the peak time is later with longer days.


Renate GI-10H
Relative expression
6.00
5.00
4.00
3.00
2.00
1.00
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)


Renate GI-12H
Relative expression
6.00
5.00
4.00
3.00
2.00
1.00
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)


Renate GI-14H
Relative expression
6.00
5.00
4.00
3.00
2.00
1.00
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)

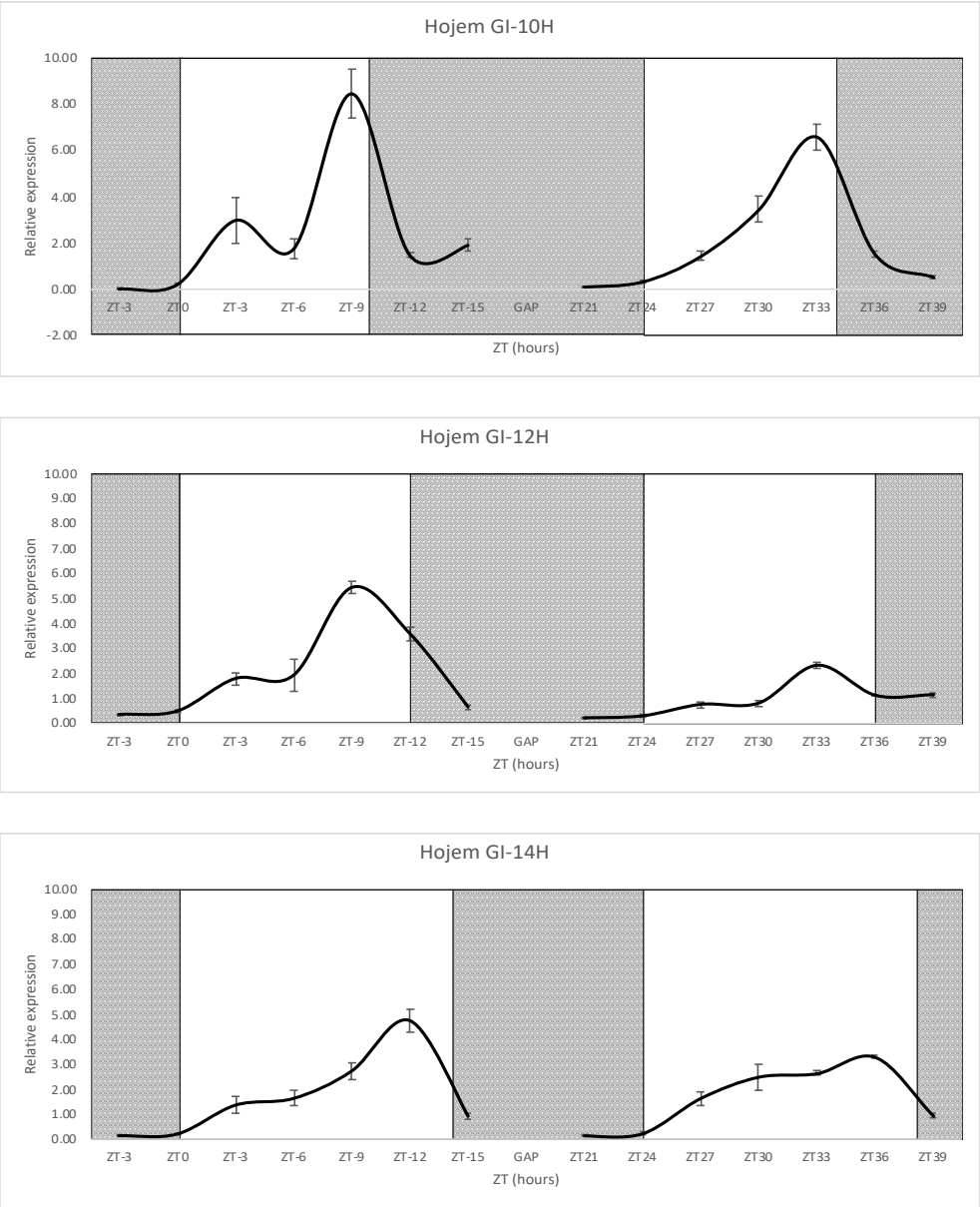
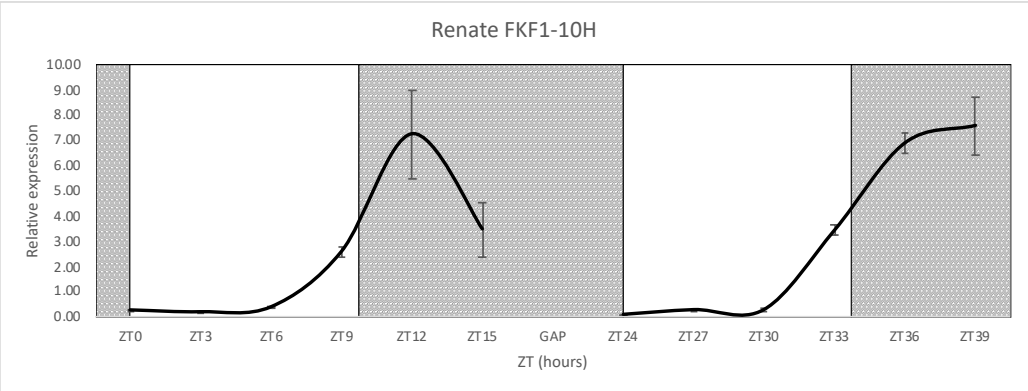


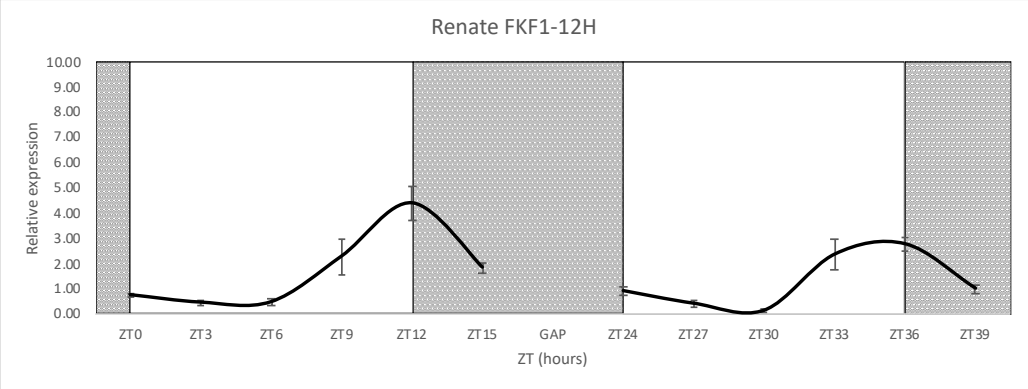
Figure 4-3 Expression of AcGI in long-day (Renate) and short-day (Hojem) varieties of onion over a 48 -h period using qRT-PCR, relative to PP2AA3, PP2A1 and TIP41. Light and dark shades denote light/dark cycles. Error bars represent the SEM.

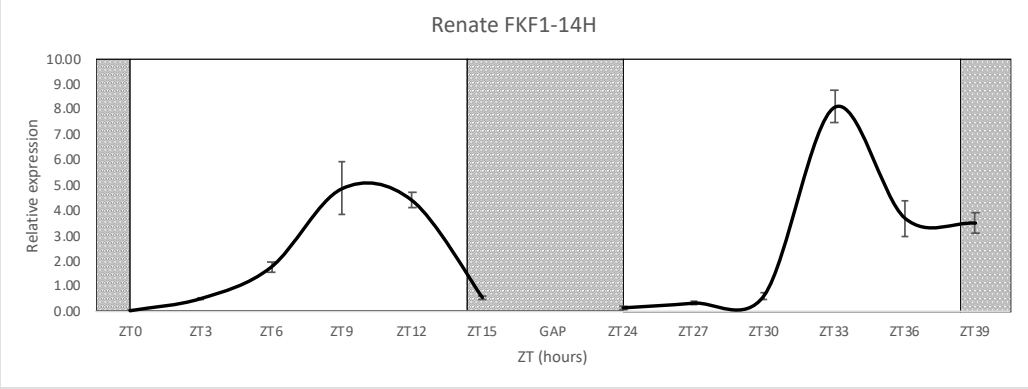
4.3.2 Average diurnal expression of AcFKF1 in LD (Renate) and SD variety (Hojem)

Quantitative real-time PCR provided detailed information of the diurnal expression of *AcFKF1* in different light/dark cycles (Figure 4-4). Both LD and SD varieties showed obvious diurnal expression patterns under all intermediate daylengths with transcripts peaking in the evening. For LD variety *Renate*, the expression profile showed peaks moving from ZT12 to ZT9 with the increase of the daylengths (ZT12

for 10h and 12h, and ZT9 for 14h). For SD variety *Hojem*, the peak time remained at ZT9 for all intermediate daylengths. The result for LD *Renate* was non-relative to *Arabidopsis FKF1* which peaks around ZT7 in SDs and ZT10 in LDs (Imaizumi et al., 2003). It is also different from a previous study by Rashid (2020) suggesting *FKF1* steadily peaks at ZT8 for both varieties under 8h and 16h. In addition, Taylor et al. (2010) examined the gene with peak time at ZT10 for a LD variety under 8h and 16h, ZT7-8 for a SD variety under 8h and 16h, and ZT7-8 under 8h / ZT9 under 16h for an ID variety. It should be noted, however, that the peak times must be taken as approximate as the sampling points are 3 h apart. Thus, the experiments would not differentiate between a peak time of e.g. ZT9 and ZT10. Bearing that in mind, there is some consistency in that *Hojem* showed a diurnal expression pattern unaffected by daylength in this experiment. Therefore, there is a distinct difference between the timing of the peak of expression between varieties: the LD variety showed different daylength responses, whilst SD variety showed the same peak time regardless of daylength conditions.


Renate FKF1-10H
Relative expression
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)


Renate FKF1-12H
Relative expression
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)


Renate FKF1-14H
Relative expression
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)

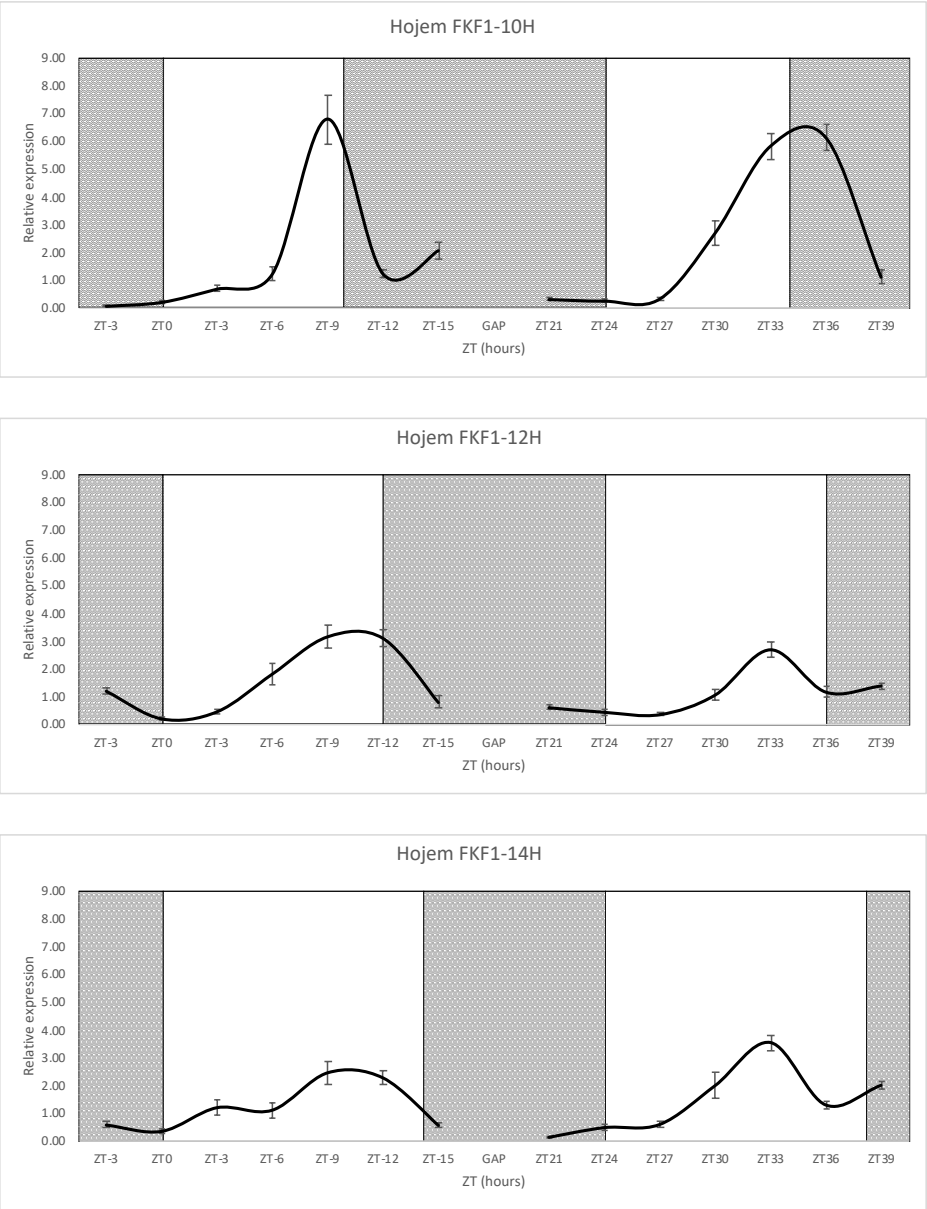
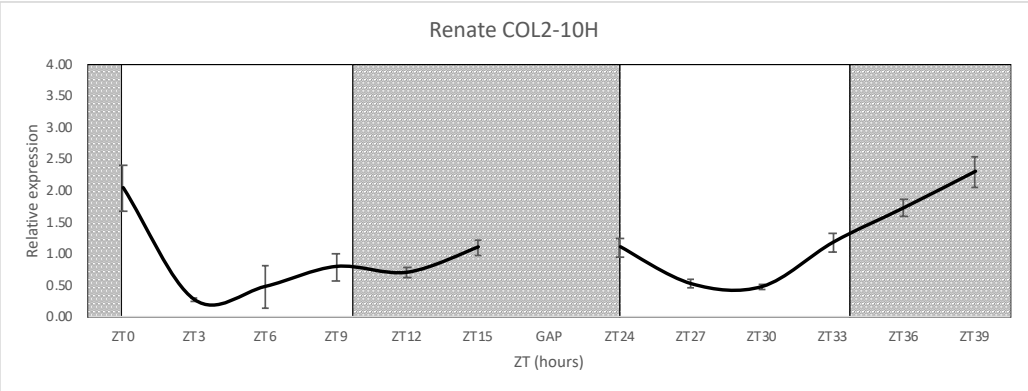
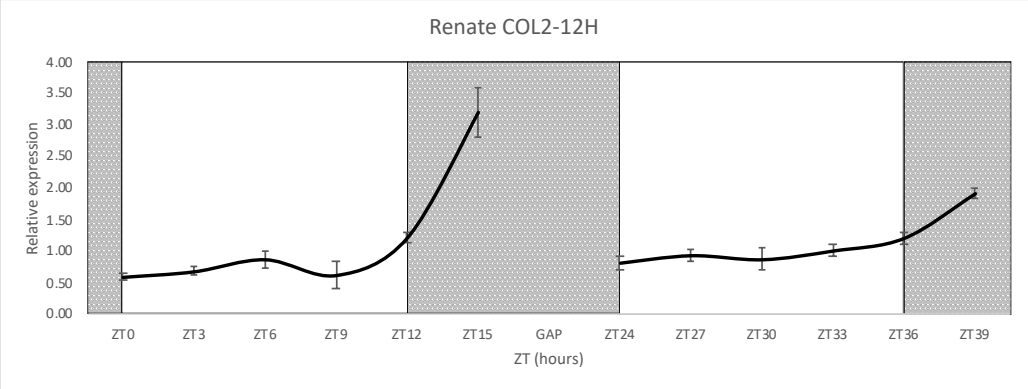


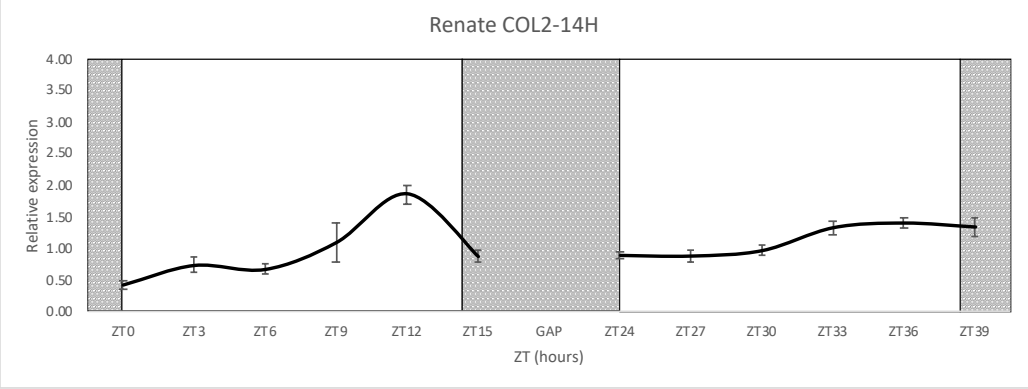
Figure 4-4 Expression of AcFKF1 in long-day (Renate) and short-day (Hojem) varieties of onion over a 48-h period using qRT-PCR, relative to PP2AA3, PP2A1 and TIP41. Light and dark shades denote light/dark cycles. Error bars represent the SEM.

4.3.3 Average diurnal expression of AcCOL2 in LD (Renate) and SD variety (Hojem)

AcCOL2 showed a similar diurnal expression pattern under all intermediate daylengths in both *Renate* and *Hojem* (Figure 4-5). Although no peaks were detected (except *Renate* 14h) due to the lack of sampling times at late night, a clear pattern of increasing to the highest point at the end of a day can be observed. Both varieties showed low *COL2* expression level during the day from ZT0 to ZT9, and began to increase around ZT9-ZT12. Under 10h and 12h of daylight, *COL2* expression peaks in the darkness, whereas under14h peaks towards the end of the day, for both varieties. This is very similar to the diurnal expression pattern of Arabidopsis *CO*, which is at its highest towards the end of a long day and in the dark if grown under a short day (Suárez-López et al., 2001). In addition, their study suggested that *CO* expression in Arabidopsis was seen to peak around ZT 16 in LDs and ZT 20 in SDs (Suárez-López et al., 2001). However, these putative two peak times were not included in the sampling timepoints for this experiment (the gap in sampling time was ZT16-ZT23 for *Renate*, and ZT16-ZT20 for *Hojem*). As the expression pattern was similar between varieties, there was no decisive factor for a role of *COL2* mRNA in onion's ability to bulb under longer daylengths. This suggests that there are other genes playing important roles in this daylength dependent bulb formation downstream, or there are other unknown homologous *CO*-like genes with a role in the daylength response.


Renate COL2-10H
Relative expression
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)


Renate COL2-12H
Relative expression
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)


Renate COL2-14H
Relative expression
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)

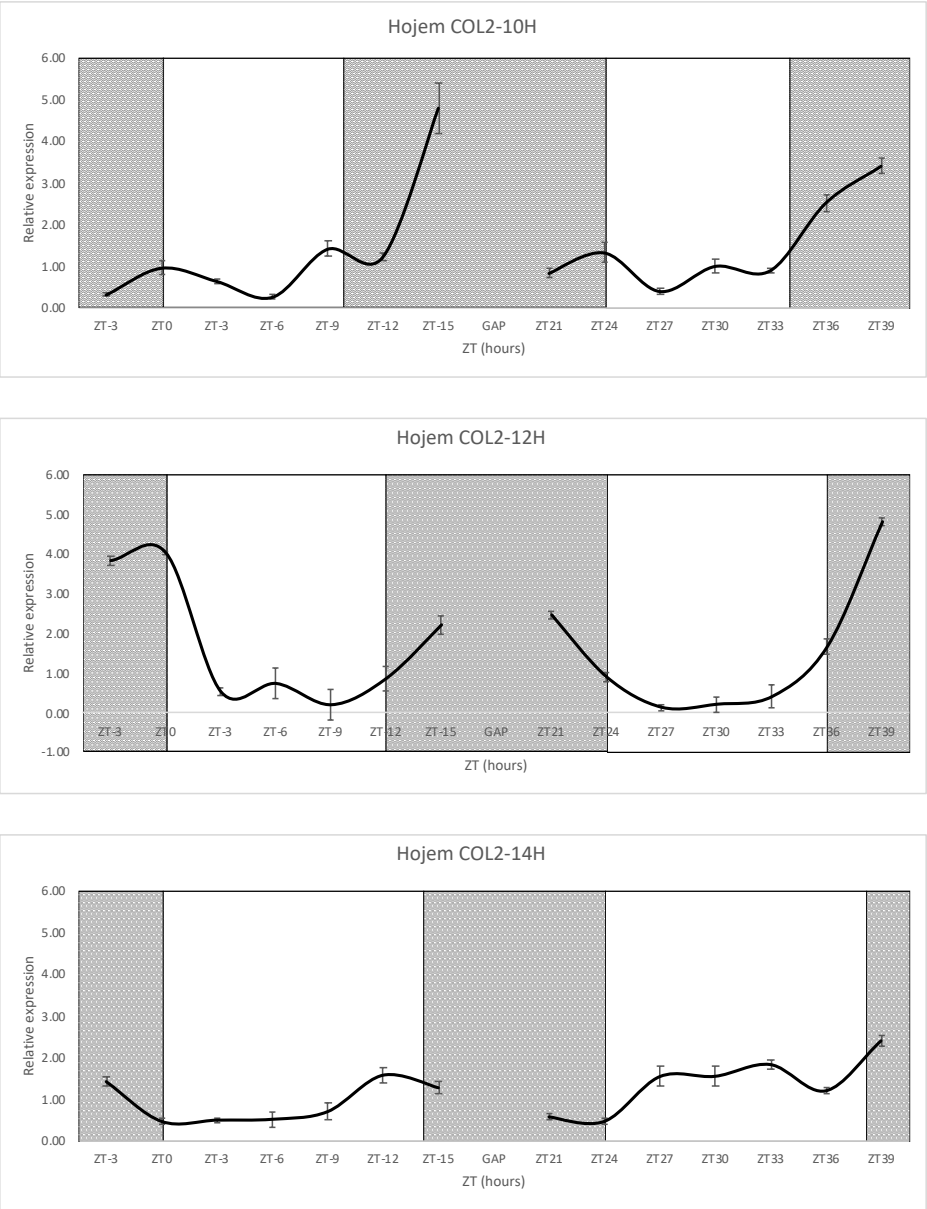
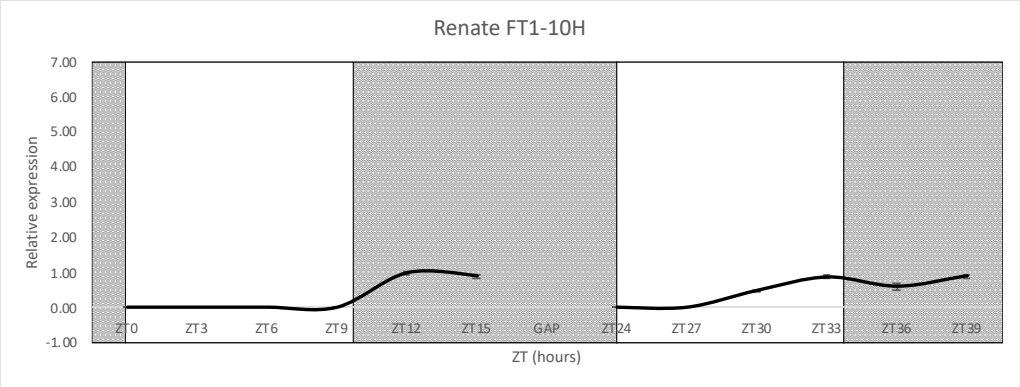
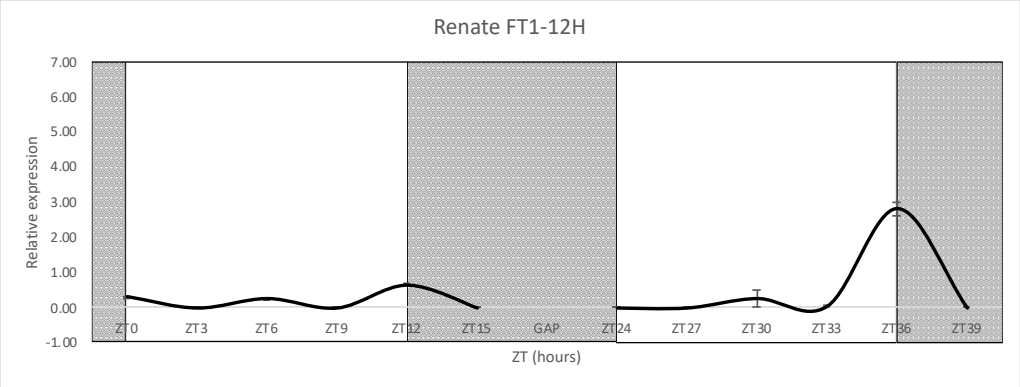


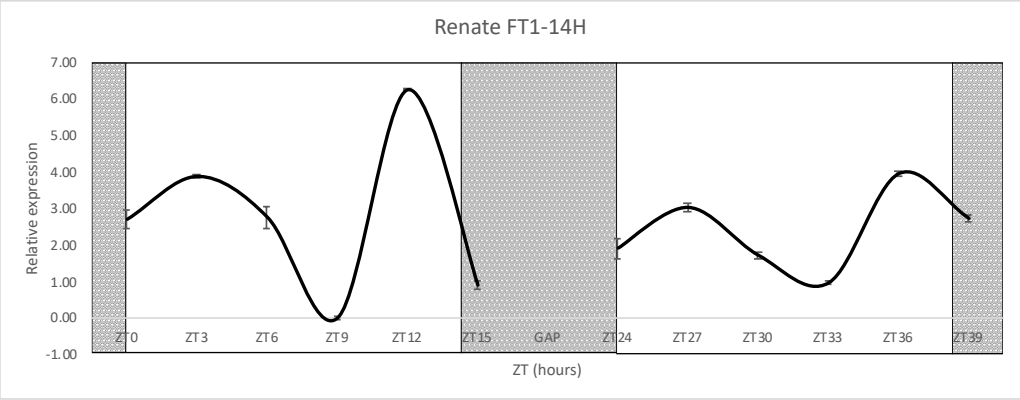
Figure 4-5 Expression of AcCOL2 in long-day (Renate) and short-day (Hojem) varieties of onion over a 48 -h period using qRT-PCR, relative to PP2AA3, PP2A1 and TIP41. Light and dark shades denote light/dark cycles. Error bars represent the SEM.

4.3.4 Average diurnal expression of AcFT1 in LD (Renate) and SD variety (Hojem)

In the previous study Rashid (2020) found *AcFT1* had no expression under a SD of 8h, but a distinct diurnal pattern if under a 16h LD, for both *Renate* and *Hojem*. This experiment of *FT1* showed a consistent result (Figure 4-6). The daylength response was similar between varieties, showing higher expression levels with increased daylengths. Both showed very low expression under 10h of daylight, with a visible trend of increasing in the dark at the end of the day (ZT12). When days were longer, the gene produced more transcripts, together with a clearer diurnal pattern peaking at ZT12, for both *Renate* and *Hojem*. 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), indicating *AcFT1* could have the equivalent role to Arabidopsis *FT* in mediating the response to daylength.


Renate FT1-10H
Relative expression
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
ZT 0
ZT 3
ZT 6
ZT 9
ZT 12
ZT 15
GAP
ZT 24
ZT 27
ZT 30
ZT 33
ZT 36
ZT 39
ZT (hours)


Renate FT1-12H
Relative expression
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
ZT 0
ZT 3
ZT 6
ZT 9
ZT 12
ZT 15
GAP
ZT 24
ZT 27
ZT 30
ZT 33
ZT 36
ZT 39
ZT (hours)


Renate FT1-14H
Relative expression
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
ZT 0
ZT 3
ZT 6
ZT 9
ZT 12
ZT 15
GAP
ZT 24
ZT 27
ZT 30
ZT 33
ZT 36
ZT 39
ZT (hours)

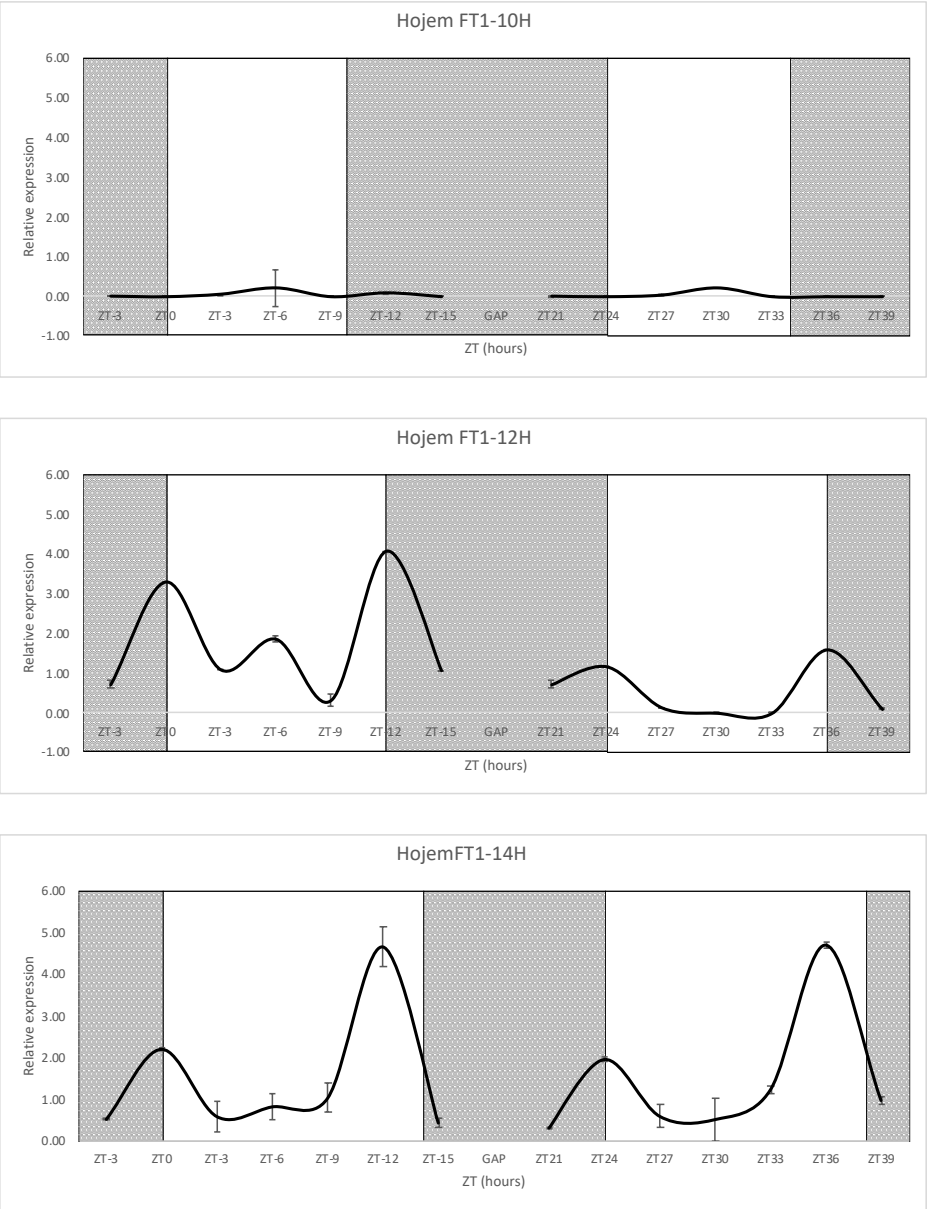
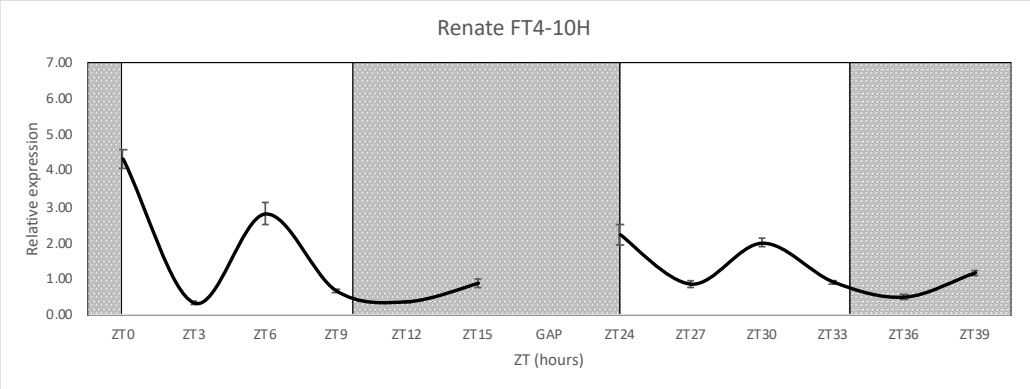
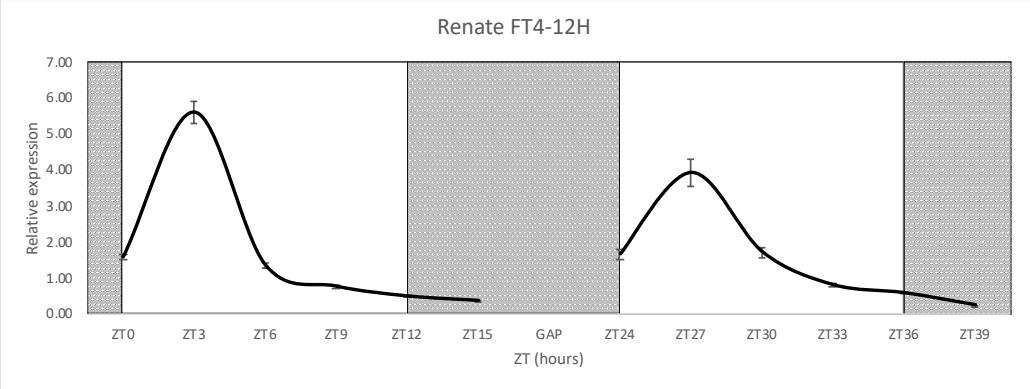


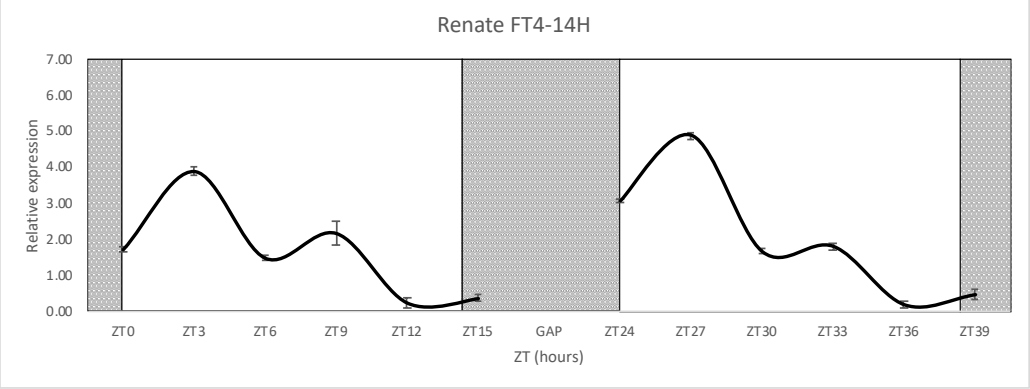
Figure 4-6 Expression of AcFT1 in long-day (Renate) and short-day (Hojem) varieties of onion over a 48 -h period using qRT-PCR, relative to PP2AA3, PP2A1 and TIP41. Light and dark shades denote light/dark cycles. Error bars represent the SEM.

4.3.5 Average diurnal expression of AcFT4 in LD (Renate) and SD variety (Hojem)

Quantitative real-time PCR provided the diurnal expression pattern of *AcFT4* in different intermediate daylength cycles for both LD and SD varieties (Figure 4-7). In contrast to *FT1*, *FT4* showed expression under all intermediate daylengths, though the diurnal pattern only appeared with longer daylengths. For *Renate*, *FT4* had no obvious pattern under 10h, but showed clear diurnal expression, peaking in the early part of the daylight at ZT3 under 12h and 14h. For *Hojem*, the gene showed no pattern under 10h and 12h. When the daylight reached 14h, a same diurnal pattern as *Renate* was seen in *Hojem*. Additionally, for SD *Hojem*, the afternoon peak at ZT9 seemed to decrease longer daylengths. Furthermore, *AcFT4* showed a reverse diurnal expression pattern to *AcFT1* under 14h, where *AcFT1* peaks at ZT12 in the evening, and *AcFT4* peaks at ZT3 at dawn.


Renate FT4-10H
Relative expression
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)


Renate FT4-12H
Relative expression
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)


Renate FT4-14H
Relative expression
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)

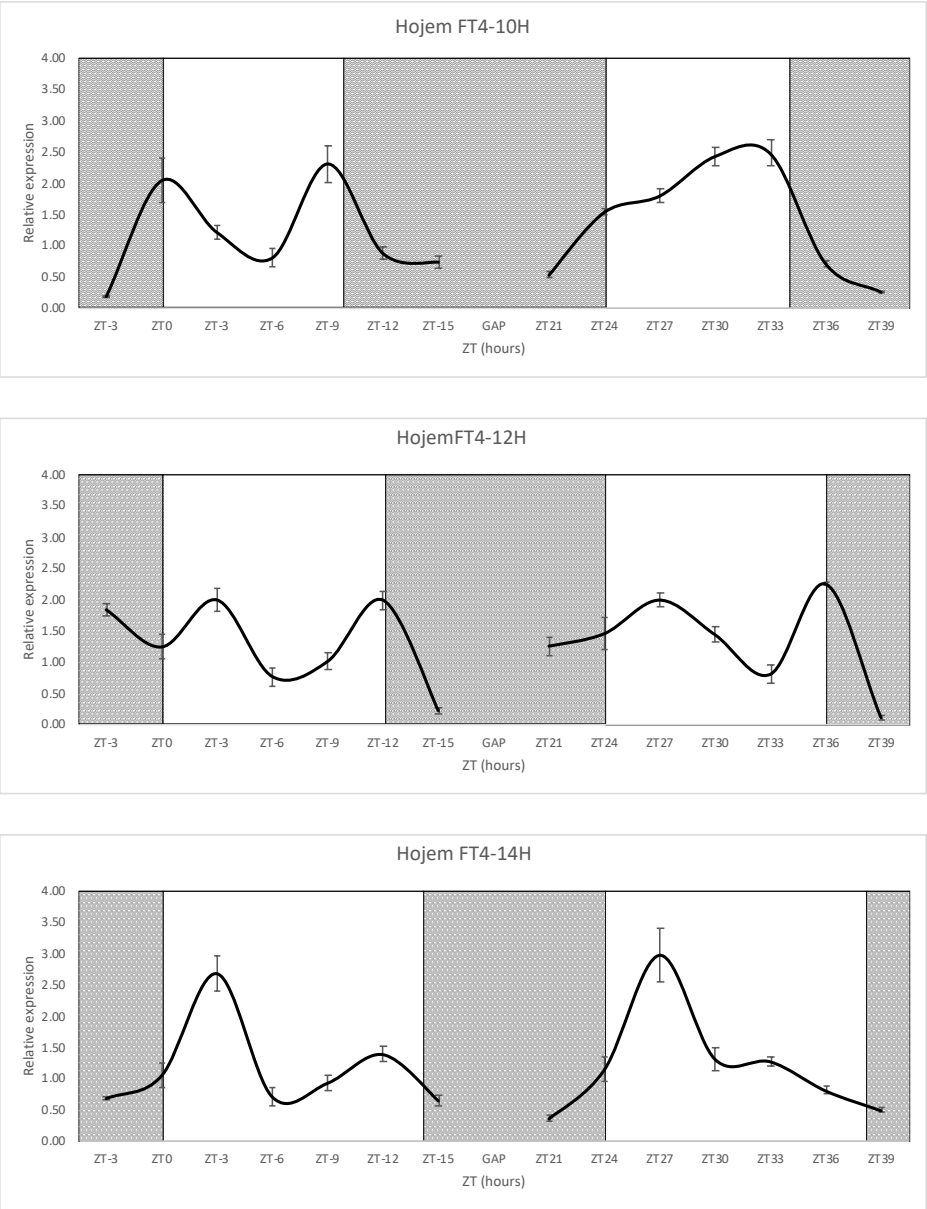
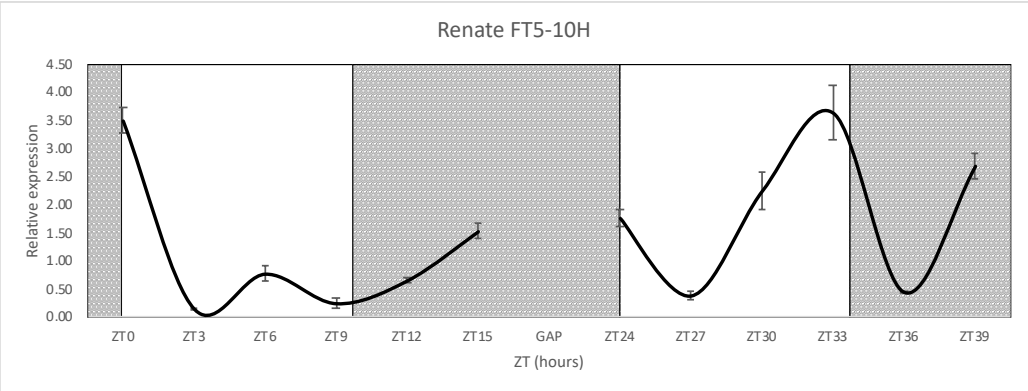
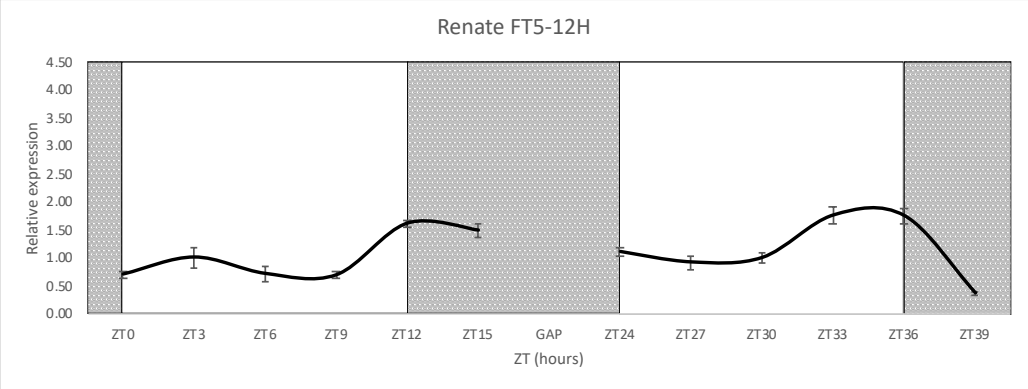


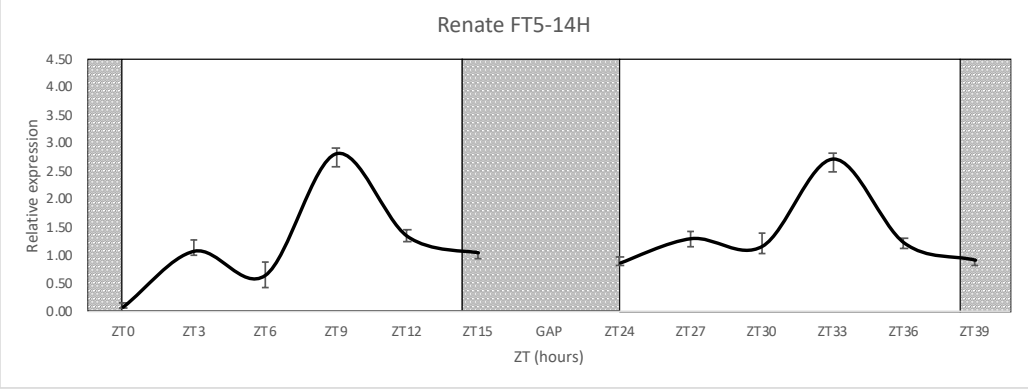
Figure 4-7 Expression of AcFT4 in long-day (Renate) and short-day (Hojem) varieties of onion over a 48 -h period using qRT-PCR, relative to PP2AA3, PP2A1 and TIP41. Light and dark shades denote light/dark cycles. Error bars represent the SEM.

4.3.6 Average diurnal expression of AcFT5 in a LD (Renate) and SD variety (Hojem)

The average relative expression was investigated using RT-PCR for *AcFT5* (Figure 4-8). Generally, the gene was expressed in all samples with no constant diurnal pattern. The broad expression level was not affected by different daylengths. However, it is worth noting that *AcFT5* began to show a distinct and repeatable (data not shown here) diurnal pattern only under 14h for both varieties, which was not seen in 10h or 12h. Under 10-12h, the expression profile for this gene shows no obvious trend and various peaks in both varieties. The expression pattern in the first day is also different from that of the second day, suggesting no circadian or diurnal regulation of the expression of this gene. However, when daylength reached 14h, *FT5* appeared to have clear and repeatable diurnal pattern for both LD and SD plants, though the peak time was different for varieties (ZT9 in *Renate* and ZT3 in *Hojem*).


Renate FT5-10H
Relative expression
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)


Renate FT5-12H
Relative expression
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)


Renate FT5-14H
Relative expression
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)

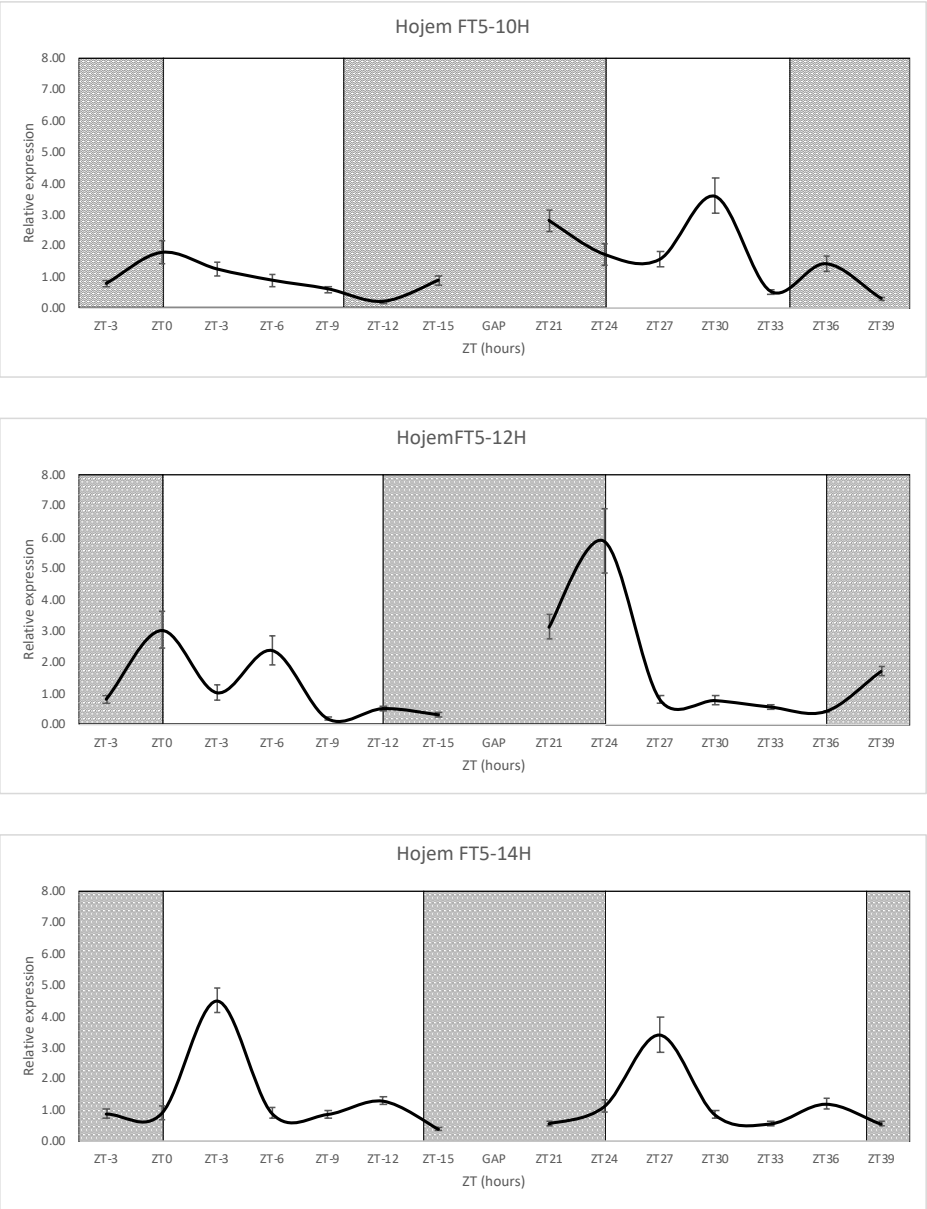
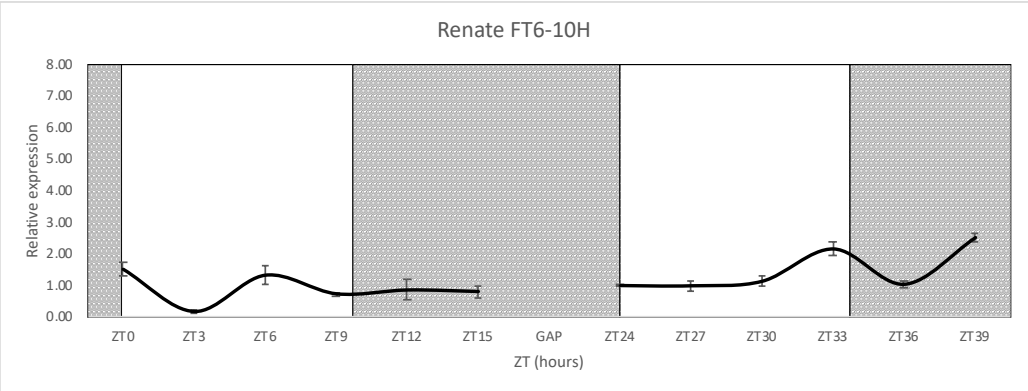
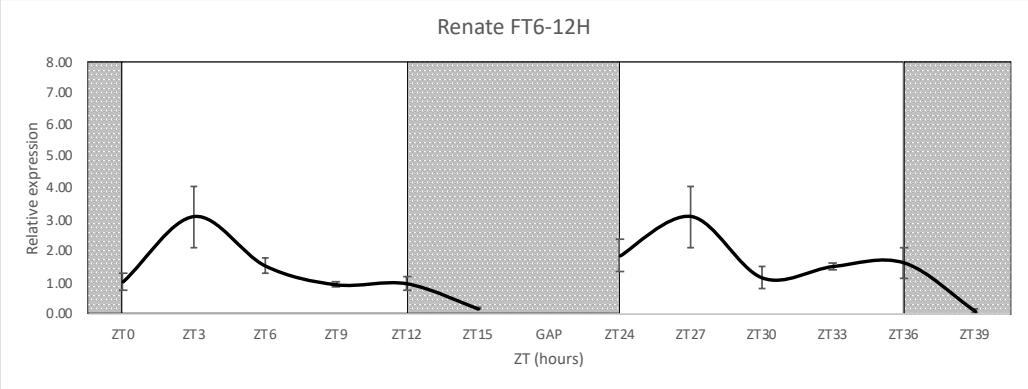


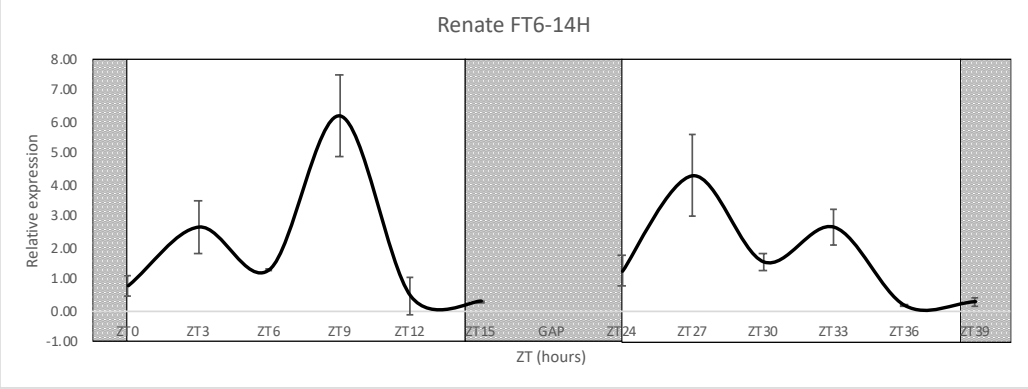
Figure 4-8 Expression of AcFT5 in long-day (Renate) and short-day (Hojem) varieties of onion over a 48 -h period using qRT-PCR, relative to PP2AA3, PP2A1 and TIP41. Light and dark shades denote light/dark cycles. Error bars represent the SEM.

4.3.7 Average diurnal expression of AcFT6 in a LD (Renate) and SD variety (Hojem)

The quantitative expression of *AcFT6* was examined using LD and SD onion under three different intermediate daylengths (Figure 4-9). Unlike *FT5* which showed no difference in expression level with different daylengths, *FT6* was seen to have more transcripts with longer days. The expression tends to increase with daylengths for both varieties. For *Renate*, *FT6* showed very low expression under 10h and 12h with no obvious trend, but a higher expression level can be seen in 12h than 10h. When the daylength reached 14h, a clear rhythm of expression with peak time at ZT9 appeared in *Renate*, together with a weaker peak at ZT3. For *Hojem*, there was no expression of *FT6* in 10h plant. For 12h and 14h, the gene showed distinct rhythmic expression peaking at ZT6, and a lower peak at ZT12. The clearer and more consistent diurnal pattern in *Hojem* under intermediate daylengths (namely 12h) indicates this SD variety copes better with this daylength then LD variety *Renate*.


Renate FT6-10H
Relative expression
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)


Renate FT6-12H
Relative expression
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)


Renate FT6-14H
Relative expression
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
ZT0 ZT3 ZT6 ZT9 ZT12 ZT15 GAP ZT24 ZT27 ZT30 ZT33 ZT36 ZT39
ZT (hours)

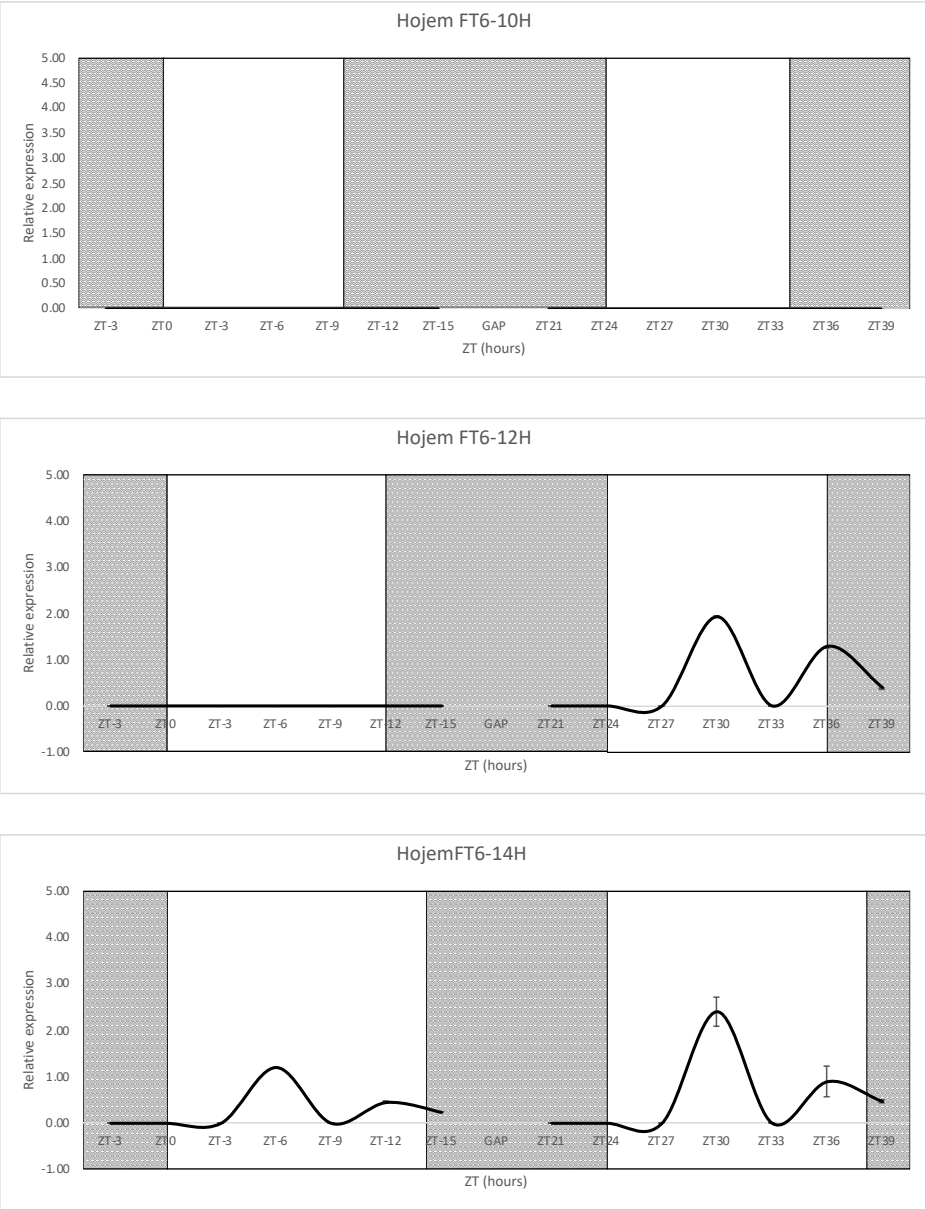


Figure 4-9 Expression of AcFT6 in long-day (Renate) and short-day (Hojem) varieties of onion over a 48 -h period using qRT-PCR, relative to PP2AA3, PP2A1 and TIP41. Light and dark shades denote light/dark cycles. Error bars represent the SEM.

4.4 Discussion

The conservation and variation of the circadian-regulated functional genes among higher plant species has been studied intensively. A *CO* orthologue (*PnCO*) has been identified in the model SD plant *Pharbitis nil* with a circadian pattern of expression, along with its *CO* mutant of *Arabidopsis* exhibited a shortening the time to flower (Liu et al., 2001). This indicates a conservation of circadian functional genes between LD and SD plant. In rice, which is a typical SD plant, the photoperiod pathway genes have been shown to be strongly conserved, despite that the flowering response is only induced by SDs but not LDs. Similar to Arabidopsis, it is suggested that *Hd3a* (the rice *FT* orthologue) promotes flowering under inductive SD conditions (Kojima et al., 2002). However, if under non-inductive (LDs), *Hd1* (the rice *CO* orthologue) inhibited the expression of *Hd3a*. In this way, flowering is inhibited in rice in LDs (Hayama et al., 2003). In other words, although Arabidopsis and rice use the same gene set of *CO*-*FT*, and both function as an important promoter for flowering under inductive conditions, the role of *Hd1* acting as a repressor under non-inductive conditions is not found in Arabidopsis *CO*. This indicates the possibility of altered function of these photoperiodical genes. In barley (which is more closely related to rice than to Arabidopsis, but is a LD plant like Arabidopsis), the cascade is different from either Arabidopsis or rice. A study by Kikuchi and Handa (2009) showed that even under non-inductive SD conditions, there was still floral promoting pathway by *HvFT*s, partially explaining why the LD plant barley can still induce flowering even under SD conditions. This case highlights the fact that some differences exist in the genetic control of photoperiod response. Despite that, it seems that many other photoperiod response genes, *GI* for instance, are still conserved in barley (Dunford et al., 2005). In another LD plant sugar beet, the *CO* homologue maps to a separate location to the *B locus* (a dominant early-bolting gene), and shows a different expression pattern (dawn-phased diurnal pattern of transcription) to Arabidopsis *CO* (Chia et al., 2008). In potato, in which photoperiod dependent tuberisation takes place under SDs, a study provided evidence that floral and tuberisation transitions are controlled by two different *FT*-like paralogues (*StSP3D* and *StSP6A*) that respond to independent environmental cues (Navarro et al., 2011). *StSP6A* was identified as a major component of tuberigen as its overexpression induced early tuberisation (Teo et al., 2016). Interestingly, it has been found that an overexpression of Arabidopsis

CO in potato would delay tuber formation under short days (Martínez-García et al., 2002). Despite that, detailed evidence in potato tuberisation regulation suggested a strong conservation with Arabidopsis flowering pathway. A potato transcription factor member of *CDF* (*StCDF1*) interacts with *StGI1* and *StFKF1*, and represses *StCO1/2* and activates *StSP6A* (Kloosterman et al. 2013).

This chapter compared the circadian expression patterns of these photoperiod related genes in different onion varieties under varied daylengths. The Table 4-3 below shows a brief summary of average relative expression of the target genes. A general concern is that there is a gap of 8 hours for *Renate* and 5 hours for *Hojem* with no data point, also the last and first sampling timepoint of each day are too far apart. Thus, there can be peak times not perceived during this time period. The sampling pattern was limited by the capacity of the cabinet and the need for replication of samples and to sample over two cycles. It was judged that it was more informative to have more frequent sampling during the day than at night.

Table 4-3 Summary of diurnal expression patterns of LD (Renate) and SD (Hojem) varieties under different intermediate daylengths. GAP for Renate is ZT16-ZT23, for Hojem is ZT16-ZT20. Multiple peaks are shown in brackets.

Gene	Relative expression under intermediate daylengths					
	LD variety *Renate*			SD variety *Hojem*		
	10h	12h	14h	10h	12h	14h
AcFKF1	ZT12	ZT12	ZT9	ZT9	ZT9	ZT9
AcGI	ZT9	ZT9	ZT12	ZT9	ZT9	ZT12
AcCOL2	GAP	GAP	ZT12	GAP	GAP	GAP
AcFT1	Low expression	ZT12	ZT12	Low expression	ZT12	ZT12 (ZT0)
AcFT4	Continuous	ZT3	ZT3	Continuous	Continuous	ZT3
AcFT5	Continuous	Continuous	ZT9	Continuous	Continuous	ZT3
AcFT6	Low expression	Continuous	ZT9 (ZT3)	No expression	Continuous	ZT6 (ZT12)

4.4.1 AcGI

The role of *GI* in circadian regulation is discussed in Chapter 1. *GI* mRNA has been shown to be tightly controlled by light/dark cycles in Arabidopsis, which peak levels occur around 8-10 hours after dawn (Fowler et al., 1999). The *GI* protein peaks at a similar time to *GI* mRNA (David et al., 2006). The timing of *GI* expression is critical for the seasonal control of flowering. The circadian regulated *GI* protein accumulation and degradation ensure the *GI* protein is available in the late afternoon under LD conditions (David et al., 2006), so as to form a complex with *FKF1* under blue light. The complex then binds to a repressor of *CO* (*CDF1*) and forms on the *CO* promoter, regulating *CO* expression. This occurs in the late afternoon in LDs, leading to *CO* protein expression and eventually flowering (Sawa et al., 2007). In this diurnal experiment, *GI* showed clear diurnal expression pattern for both onion varieties under all intermediate daylengths, confirming its character in photoperiod response (Jackson, 2008). The expression pattern of *AcGI* was found to be very similar within onion varieties and respond to different daylengths, peaks move to ZT12 with increasing daylengths. It seems the gene's peak time is pushing back with longer daylengths, regardless of varieties. The result suggested that the circadian rhythm component of the photoperiod pathway is active in both LD and SD varieties of onion. The similarity in expression pattern to Arabidopsis *GI* (8-10h after dawn) also indicates a conservation in function in onion bulb formation. Furthermore, the expression pattern of *AcGI* was found no different between SD and LD varieties, implying that if these varieties have a modified photoperiod pathway, the modifications occur downstream of *GI*.

4.4.2 AcFKF1

The role of *FKF1* and its role in circadian regulation is discussed in Chapter 1. In Arabidopsis, *FKF1* plays an important role in the flowering pathway through regulating daytime *CO* expression (Li et al., 2013). In Arabidopsis, *FKF1* shows distinct peaks around ZT7 in SDs and ZT10 in LDs (Imaizumi et al., 2003). The results had some consistency with the onion study by Taylor et al. (2010), which examined the expression of *FKF1* at various points over a 24-hour period and also

suggested that the earliest expression peak was seen in the SD variety. Whereas Rashid (2020) conducted the diurnal experiment that showed *AcFKF1* had a constant peak time at ZT8 under 8h of critical short day and 16h of long day in both SD and LD onion varieties. In this experiment under intermediate daylengths, *FKF1* peaks move from ZT12 to ZT9 in LD variety *Renate*, and remain the same at ZT9 in SD variety *Hojem*. This difference in expression profiles between varieties indicates there is different response of onion bulbing process. Considering what the field work has confirmed that *Renate* forms no bulbs under 10h and 12h, while *Hojem* forms bulbs under intermediate daylengths, it appears *AcFKF1* peaks in the dark period in non-bulbing situations, and in the light period in bulbing situations. It is possible that a peak in the light may allow the gene to function, whereas a peak in the dark period may inhibit function. Besides, considering that *GI* and *FKF1* form a complex to complete the function, a movement in *FKF1* expression will then affect the *GI* response. The data in this experiment showed that *AcFKF1* expression differs between varieties and different daylength responses, whereas *AcGI* remains unchanged. It might be the movement of *FKF1* expression that changes the timing of the *GI*/complex response. At any rate, the expression data strengthens the hypothesis that *AcFKF1* has a role in day-length regulated bulbing initiation.

4.4.3 AcCOL2

The role of *CO* in the photoperiodic flowering pathway is discussed in chapter 1. *CO* gene is an integral part of this pathway and regulates the expression of the floral integrating genes *FT* and *SOC1* (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1) leading to floral initiation. In Arabidopsis, *CO* expression is high at the end of long-days, and the *CO* protein is degraded at night, so light plays important role in controlling the stability of the *CO* protein. In the dark, *COP1* (CONSTITUTIVE PHOTOMORPHOGENIC1) and *SPA1* (SUPPRESSOR OF PHYA-105) proteins promote the degradation of the *CO* protein (Laubinger et al., 2006; Jang et al., 2008), leading to a delay in flowering in SDs. In contrast, if plants are grown under LDs, *CO* transcription and a blue or far-red light signal occur simultaneously, stabilising the protein and promoting the expression of FT, leading to Arabidopsis flowering. Rashid (2020) identified three *CO-LIKE* (*COL*) genes in the

Renate transcriptome, and conducted diurnal experiment under 8h and 16h. He suggested only *COL2* is a candidate for being a homologue of Arabidopsis *CO*, as it was the only one of the three that showed distinct diurnal expression pattern. Therefore, only *AcCOL2* was selected as a candidate in this experiment to examine its rhythmic expression. This diurnal experiment showed a consistent result with Rashid (2020). *AcCOL2* showed a similar diurnal expression pattern for both LD and SD varieties under all intermediated daylengths. It presents its highest expression at the end of the daylight for 14h, and in the darkness for 10h and 12h. The *COL2* expression was generally very low during ZT0 to ZT9, due to the degradation of *CDF* proteins in the afternoon and mediation by *PHYB* in the morning (Valverde et al., 2004). The timing of the peaks in the later part of a day is closely related to the function of this gene within the photoperiod pathway. The consistent expression pattern to Arabidopsis also indicates a conserved role in circadian regulation in bulbing process. Because the expression of *AcCOL2* is seen to peak in the dark in non-inductive conditions, the protein is degraded at night, making it the limiting factor in the photoperiodic control of bulb initiation.

4.4.4 AcFTs

Arabidopsis *FT* promotes flowering in LDs and is described as a floral-pathway integrator gene as it is responsive to signals from several flowering pathways (Jack, 2004; Thomas et al., 2006). *FT* is an early target of *CO*, where its expression is directly regulated by *CO* protein (Kobayashi et al., 1999). Then it acts upstream of *SOC1*, which can induce flowering by long-distance transportation. In Arabidopsis, LD-specific *FT* induction occurs in leaf phloem companion cells and the expression around dusk in long days (Mouradov et al., 2002; Song at al., 2013). In onion, six *FT* genes (*AcFT1-6*) were identified by Lee et al. (2013), and he proposed the downregulation of *AcFT4* and the upregulation of *AcFT1* in onion bulb formation. Lee also reported that the upregulation of *AcFT2* in onion flowering, however, as we only focus on bulb formation in this thesis, *AcFT2* was not selected in this experiment. For the other two *FT* genes, *AcFT5* and *AcFT6*, were also chosen as candidates despite their functions in photoperiodic regulation are still unknown.

Previous Rashid study showed *FT1* had no expression under 8h and clear diurnal expression under 16h (2020), while this experiment showed consistent results. The expression of *FT1* responded to daylengths sensitively. Very low levels of *FT1* mRNA was seen under 10h, and with the daylengths increasing, *FT1* showed its abundance in general expression level and clearer diurnal pattern. Under 12h and 14h, *FT1* showed a significant rhythm in expression with peak time at ZT12 (the later part of the day) for both varieties. The similarity in diurnal pattern to Arabidopsis *FT* (Kardailsky et al., 1999) indicates this *AcFT1* could be a homologue of that of Arabidopsis, and is positively regulated by *AcCOL2*. The result is also consistent with Lee's study (2013) which suggesting *FT1* promotes bulbing the process, and *AcFT1* is only expressed in LD grown plants but is not expressed in SD grown plants. It seems *FT1*'s higher expression level and clearer rhythmic pattern with longer days can be one explanation of onion's ability to bulb under longer daylengths. However, the gene is not solely responsible for determining the onion's ability to bulb. As *Renate* will not bulb until 14 hours of daylight, whereas it still showed distinct diurnal expression of *FT1* under 12h in this experiment. *FT1*'s expression in non-bulbing plants indicates there are other factors involved in this complicated onion bulbing process. The recent study showed the phloem companion cell protein *MRF1* being a floral regulator (You et al., 2019), underlying the important epigenetic modifications regulating this photoperiodically driven process.

In contrast to *FT1*, *FT4* was expressed in both varieties under all intermediate daylengths, although no diurnal pattern was seen under short days (10h in *Renate*; 10h and 12h in *Hojem*). A clear rhythmic expression which peaks in the early part of the day can be seen under 14h for both varieties. The result does not support Lee's suggestion (2013) that *FT4* was expressed at a lower level in long day, neither the gene's function of inhibiting bulbing formation. However, with increasing daylengths, the higher expression of *FT1* in the evening seems to correlate with a decrease the afternoon peak in *FT4*. There may be a down regulation by *FT1* with its expression suppressing *FT4* transcripts. Anyhow, the non-selective generous expression under all daylengths of *AcFT4* may suggest it has a less important role in onion bulb initiation compared to *AcFT1*.

The results showed that *FT5* had peaks occurring at different times in different onion verities. The peak time was only seen under 14h, and was at ZT9 in LD variety *Renate* and ZT3 for SD variety *Hojem*. It is clear that *FT5* only showed clear diurnal pattern under longer daylengths, for both varieties. The result is consistent with previous study by Rashid that *FT5* had higher expression level and better trend under 16h rather than 8h. Although there is still no explanation of the expression pattern of *FT5*, its differential behaviour in varieties under different daylengths indicate that any role it may have in the onion bulbing process is complex.

As with *FT5*, *FT6* showed different peak times between onion varieties. It also had very low expression and no diurnal pattern under daylight of 10h, while higher expression level as well as better diurnal pattern appeared with longer daylengths. *Hojem* was seen to have distinct rhythmic expression peaking at ZT6 at daylengths of 12h and above, whist *Renate* only showed a peak time at ZT3 until 14h. The evidence that *FT6* behaves better in SD variety may be related to *Hojem*'s ability to bulb under 12h. However, future work is required to understand the role of this gene.

4.5 Conclusion

This chapter details the diurnal time-course expression of genes in onion that are linked to circadian regulation in Arabidopsis. All candidates presented clear diurnal expression patterns in both LD and SD variety of onion, indicating their role in daylength dependent bulbing process at molecular level, and confirming the hypothesis that the clock genes in Arabidopsis would be circadian regulated in onion.

For *AcFKF1* and *AcGI*, both genes showed distinct diurnal expression with similar patterns to that of Arabidopsis. The peak time of *FKF1* moves from ZT12 to ZT9 with increased daylengths in *Renate*, and not affected by daylength in *Hojem*. Both varieties showed similar rhythmic expression of *AcGI*, which peaks at ZT9 under 10-12h, and ZT12 under 14h. However, as these genes work as a complex, the shift in FKF timing may be decisive in the timing of their action. *AcCOL2* showed a constant diurnal pattern in both varieties under all daylengths, peaking towards the end of the long day and slightly later into darkness in shorter daylengths. This is very similar to the expression pattern of *CO*, responsible for daylength regulation of flowering in Arabidopsis. This suggests that *AcCOL2* is a candidate for being a homologue of Arabidopsis *CO*. There was no obvious relationship between the timing of *COL2* mRNA expression and the differential response to daylengths at the intermediate daylengths. However, it should be noted that *CO* has been shown to be subject to post transcriptional regulation in Arabidopsis and other species and daylength sensitivity could be established at that level.

All *FT*s showed different diurnal expression patterns peaking at different times of the day. In both *Renate* and *Hojem*, *AcFT1* expression level increases with daylengths. A clear diurnal pattern was seen in the later part of the day in longer daylengths, but very limited transcripts under short days. The result is supportive of *AcFT1* being responsible for the correlation of bulbing under LD conditions (Lee et al., 2013). The similar diurnal expression pattern of *AcFT1* to Arabidopsis *FT* suggests that this gene could be a homologue in Arabidopsis flowering pathway. In contrast, *AcFT4* was expressed generously in all daylengths, suggesting it has a less important role in daylength dependent bulbing process. Under 14h, both varieties showed clear rhythmic dawn expression of *AcFT4*. In the SD variety *Hojem*, the afternoon peak

decreases with longer daylengths. It seems the increasing *FT1* expression at that time may repress the afternoon peak of *AcFT4*. However, future work is required to examine this possible downregulation of *FT1* of *FT4* in the later part of the day. The other two *FT* genes, *AcFT5* and *AcFT6*, showed various peaks at different time between varieties. For both genes, the clearer and repeatable diurnal pattern only appears with longer daylengths for both varieties, suggesting they might be the active components present for circadian or diurnal regulation under LD conditions. In addition, *FT6* presents its diurnal pattern in 14h in *Renate* and 12-14h in *Hojem*. Those daylengths are the same with the varieties' daylength requirement to bulb, therefore *FT6* may have its function in determine onion's ability to bulb under particular daylengths.

5 GENE EXPRESSION IN LEAF AND BULB TISSUES DURING THE DEVELOPMENT OF THE LD ONION VARIETY *MARCO*

5.1 Introduction

Plant development is a complex process that involves coordination of specific developmental events, with each event dependent on the adequate supply of resources. The availability of these biological components in a growing plant affects the timing of developmental programmes, such as flowering and bulbing. Also, the components themselves may exist as crucial nutrients in many crop species. For higher yield and better quality in crop production, it is essential to not only have a high level of photosynthesis but also effective transportation of photosynthetic products. Sucrose is the most important product of photosynthesis and is transported from leaves throughout the plant. It can either be stored or broken down for use in the synthesis of all the other components of living cells. During the process of plant sucrose metabolism, invertases (beta-fructofuranosidase, *FFS*) catalyse sucrose hydrolyzation to produce hexoses (fructose and glucose) available for cellular activity such as growth, development and maintenance, respiration and cell wall synthesis (Benkeblia et al., 2004).

Another important enzyme in sugar metabolism is sucrose 1-fructosyltransferase (*SST1*), which is the key enzyme in plant fructan biosynthesis, since it catalyses fructan synthesis from sucrose. Fructans are built-up fructose residue with a sucrose unit. They are non-structural fructose-based highly water-soluble polysaccharides, and the most widespread reserve carbohydrates in higher plants (besides starch) (Hellwege et al., 1997). For many crop plants, for instance, wheat and barley, fructans are major non-structural storage carbohydrates in their vegetative tissues (Hendry, 1993). In these plants, fructan metabolism is crucial in the photosynthetic partitioning process, and ultimately determines yield (Wiemken et al., 1995; Pollock et al., 2003). Onion is one of the most highly fructan-synthesizing plants. Bulbs of onion store fructans, about 80% of bulb dry matter consists of non-structural carbohydrates (Darbyshire & Henry, 1978). The predominant components include glucose, fructose, sucrose and low-molecular weight fructans, while starch and raffinose are absent (Darbyshire & Henry, 1981; Benkeblia et al., 2002). Henry and Darbyshire (1980)

isolated fructan fructosyltransferase from the inner leaf bases of bulbing onion plants, and found a high concentration of trisaccharide produced. Fructans is one of the major nutrients in onion crops for its function in lowering blood lipid and insulin levels (Ritsema and Smeekens 2003).

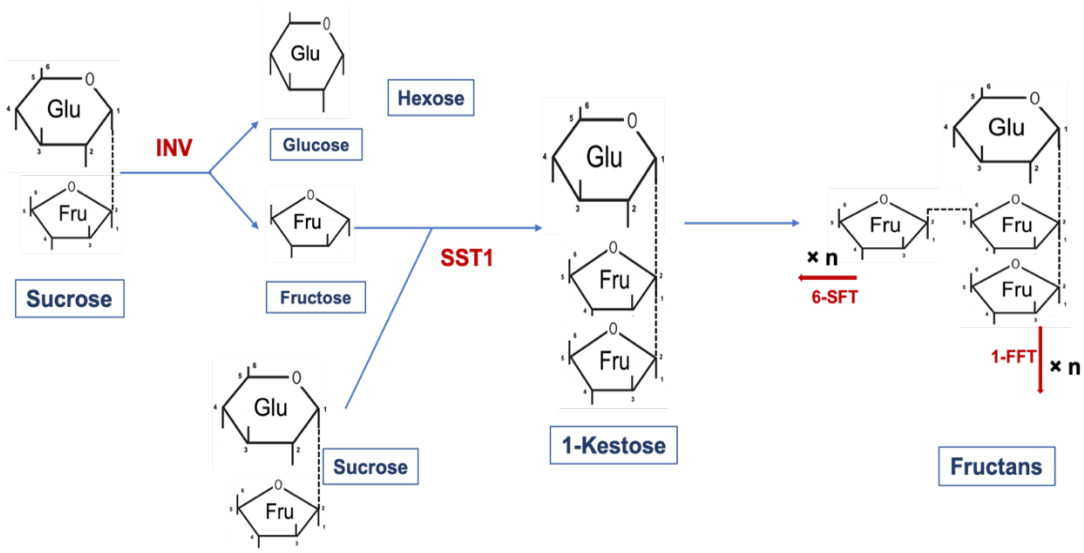


Figure 5-1 Schematic drawing of the activities of invertase (FFS) and the fructosyltransferase SST1.

Invertases (*FFS*, *INV*) and fructosyltransferases (*SST1*) are functionally related (Fig.5-1). Sucrose is split into glucose and fructose. After the release of glucose, fructose is also released via hydrolysis in the case of invertase (INV/*FFS*). However, fructose may be bound to another sucrose molecule, catalysed by *SST1*, and form 1-kestose, the shortest-chain fructan (fructo-oligosaccharide) (Vijn et al., 1998). When the fructose units continue to be linked, the linear fructans are formed. The elongation of the fructan chain is either catalysed by *1-FFT* (fructan:fructan 1-fructosyltransferase) or *6-SFT* (fructan:fructan 6-fructosyltransferase) activities depending on the attaching position of the fructose on the glucose. In onion, the major fructan content is the inulin neo-series, in which beta (1–2)-linked fructose chains can be attached to the fructose C1 or the glucose C6 of the sucrose starter unit (Darbyshire and Henry 1978; Ernst et al. 1998; Vijn et al. 1997). The predominant bulb fructo-oligosaccharides type differs in different cultivars, whist all fructan biosynthesis is initiated by the enzyme *SST1*, which catalyses the formation of 1-kestose from sucrose (Vijn et al., 1998).

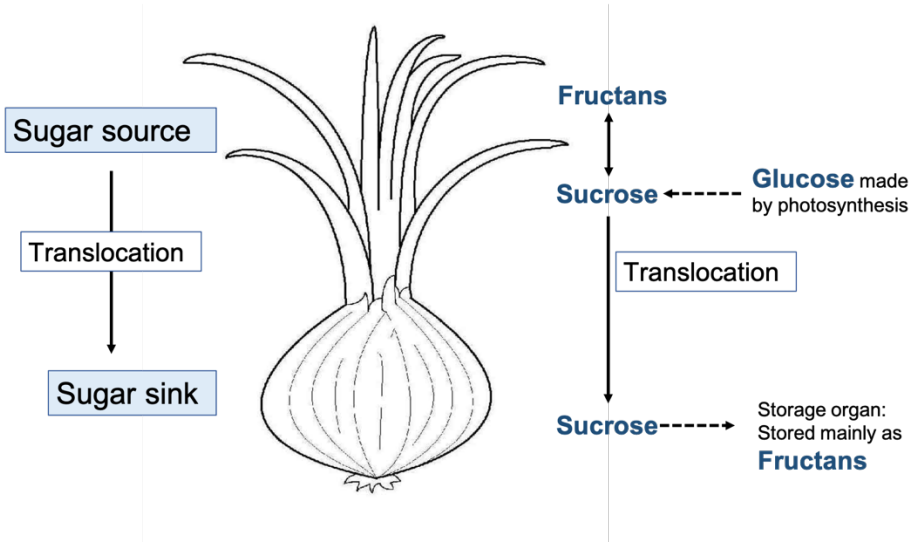


Figure 5-2 Translocation of sugar in an onion plant.

The physiology of onion with source-to-sink carbohydrates transition is shown in Fig. 5-2. Photosynthesis provides sugar source (mainly glucose), and new growth (bulb) is the sugar sink (mainly glucose, fructose, sucrose and low-molecular weight fructans). The source for onion bulb is the fructans produced from sucrose and fructose and accumulated in sinks, including basal scales during bulbing (Pak et al. 1995). There are also fructans in leaf tissues, thus fructans may be translocated to the sink tissues directly through the phloem, or be hydrolysed first, then transported and resynthesized (Sharma et al., 2015).

Another sugar metabolic enzyme of interest is trehalose-6-phosphate synthase (*TPS*). Trehalose is a disaccharide sugar found widely in invertebrates, bacteria, algae, plants and fungi. It is formed by the dimerization of glucose. The most widely distributed pathway for trehalose biosynthesis is a two-step process, which trehalose-6-P (*T6P*) synthase synthesizes *T6P* from UDP-Glucose and Glucose-6-P, followed by dephosphorylation to trehalose by *T6P* phosphatase (*TPP*) (Fig. 5-3).

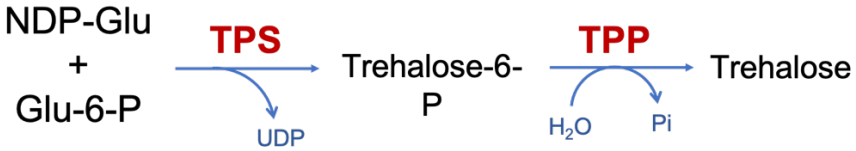


Figure 5-3 Pathways for the synthesis of trehalose. TPS represents trehalose-6-phosphate synthase; TPP represents trehalose-6-phosphate phosphatase

In some desiccation-tolerant “resurrection plants”, trehalose plays an important physiological role as an abiotic stress protectant and serves as osmoprotectants. Transgenic plants with overexpression of TPS displayed increased resistance to abiotic stress (Holmström et al., 1996; Romero et al., 1997; Pilon-Smits et al., 1998), particularly an increased dehydration tolerance (Nelson et al., 2004). However, in other non-resurrecting plants, trehalose exists at an almost undetectable level and does not accumulate, indicating the role in anti-stress function in insufficient, but may play a regulatory role and act as signalling metabolite in these plant species (Goddijn and Smeekens, 1998).

To understand the complex development, we must unravel the molecular basis for differential gene expression. This chapter aims at understanding how the regulation of gene expression contributes to plant development on transcription level. The chapter describes the developmental time-course expression of the genes in onion that are particularly involved in photoperiodic regulation, carbohydrate accumulation and onion quality traits. The experiment was conducted for measuring bulbing ratio and generating materials for molecular analyses using LD onion cultivar under SD and LD conditions. Candidates include some key genes in the Arabidopsis flowering pathway, as well as sequences selected from the onion transcriptome RNA-seq database with putative function in carbohydrate accumulation and onion quality traits.

5.2 Materials and methods

5.2.1 Plant material (Onion Marco under SD and LD)

As *Renate* seed was no longer available after preliminary experiments in Chapter 3, we selected onion variety *Marco* as the plant material for this experiment. It is a robust variety with a good LD bulbing response. The F1 Hybrid seeds were used (S.E. Marshall & Co Ltd, Cambridgeshire, UK). The critical daylength requirement of this variety is suggested to be roughly 15 daylight hours (Auntie Dogma's Garden Spot). Initially seeds were sown on 16/12/2017 in modular trays as described in Chapter 2 and kept under natural conditionals in a common PBF chamber. At 22/01/2018 (37 DFS) plants were potted up into 9cm pots as described in Chapter 2. From 37-100 days from sowing, plants were kept in a controlled temperature compartment with supplementary lightning extension time from 02:00 to 18:00. After 63 days (28/03/2018, 100 DFS), plants were separated into two groups. Half of the plants were transferred to LD provided by a NC compartment with 16h of supplementary light (400 W SONT lamps) from 03:00 to 19:00. Plants were kept there for 42 days until the experiment finished, the natural daylight varied from 12h 42min to 15h 24min, during the LD treatment. The other half of the plants were transferred into a compartment fitted with photoperiod blackout blinds to give an 8 h daylength from 8:00 to 16:00, during which period they received supplementary light from 400 W SONY lamps. Plants in SD compartment were also kept for 42 days for sampling.

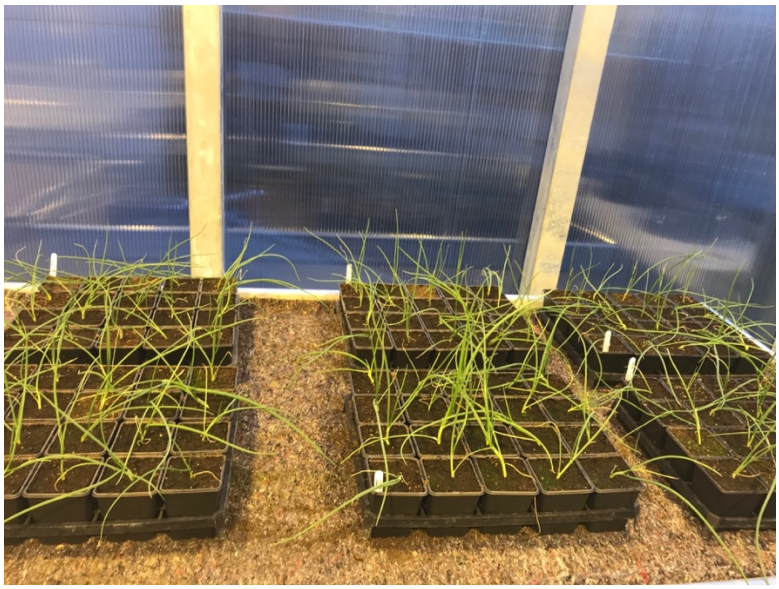


Figure 5-4 Growth of Marco plants before transfer to LD and SD compartments

Samples were taken from two days after the transfer date at weekly intervals from 102 to 114 DFS (7 sampling times in total). For each sampling time, five plants were taken from both LD and SD compartments at ZT10 (LD compartment at 13:00 and SD compartment at 18:00). Plants were selected for harvesting using a random number generator (Haahr, 2006). Measurements of bulb and neck diameter were conducted using slide callipers and visible leaf number was counted for each plant. Harvesting of both leaf and bulb tissues were carried out as described in Chapter 2 before storing at -80°C. The harvested materials were used for molecular analyses and the 'Bulbing ratio' was calculated as described. For the plants sampled at the last time point (144 DFS) only measurements of bulbing ratio and leaf number were made as the differences in bulbing responses were already well established.

Table 5-1 Sampling timepoints and sample names for Marco Developmental Experiment

Sampling date	28/03/2018		4/04/2018		11/04/2018		18/04/2018		25/04/2018		02/05/2018		09/05/2018	
Days from sowing	102		109		116		123		130		137		144	
Days from transferring	2		9		16		23		30		37		44	
Sample name	LB1	LL1	LB2	LL2	LB3	LL3	LB4	LL4	LB5	LL5	LB6	LL6		
	SB1	SL1	SB2	SL2	SB3	SL3	SB4	SL4	SB5	SL5	SB6	SL6		

5.2.2 Gene selection

For *Marco* developmental experiment under SD and LD, the identification of the genes was obtained in two ways. First batch of genes were the *Arabidopsis* photoperiodic flowering homologs chosen from past papers, including *COL2* (Rashid, 2020) and *FT*s. Others were selected from the onion transcriptome database (Thomas Group) based on their differential expression between leaf and bulb provided by the RNA-seq data. The candidates were chosen especially for their putative function in quality traits such as carbohydrates and organosulphur content.

Table 5-2 Target genes selected for Marco Developmental Experiment. Eight of them were supposed to be bulb-specific and one to be leaf-specific, according to the onion transcriptome.

Target Name	Relative expression in the transcriptome		Predicted Function
	leaf	bulb	
FFS-1	0.2	**9.2**	beta-fructofuranosidase 1-like [Brachypodium distachyon]
FFS-2	2.4	**9.7**	beta-fructofuranosidase, insoluble isoenzyme 7-like isoform X2 [Setaria italica]
SST1-B1	0.4	**5.4**	sucrose 1-fructosyltransferase-like [*Setaria italica*]
SST1-B2	17.3	**144.0**	sucrose 1-fructosyltransferase-like [*Setaria italica*]
SST1-L	**231.4**	19.1	sucrose 1-fructosyltransferase-like [*Setaria italica*]
TPS	0.0	**0.4**	trehalose-6-phosphate synthase, putative [*Ricinus communis*]
DFS	64.8	**595.7**	defensin J1-2-like [*Cucumis sativus*]
LFS	110.6	**1931.9**	lachrymatory factor synthase [*Zea mays*]

Selected novel sequences shown above were BLASTed with other plant species obtained from the publicly available NCBI database. 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 their homologue genes in other plant species were also calculated. Sequences were aligned and phylogenetic trees were constructed using predicted amino acid sequences.

5.2.3 Quantification of mRNA expression

The identification and isolation of the genes of interest in onion was followed using the techniques as described in Chapter 2. Primer pairs for candidates associated with photoperiodic regulation (namely *COL2* and *FT*s) remained the same as previous chapters. Primers (Forward and Reverse) for new target genes were designed and synthesised using Primer 3 plus as described in Chapter 2. The mfold web server was used to check primer characteristics in order to avoid unwanted secondary structures in the PCR product. Primers used for obtaining the full-length of target genes in onion are presented in the Appendix. Extraction of total RNA, DNase treatment and synthesis of cDNA using 2 µg total RNA was followed by the protocol as described. The expression of the key genes was initially examined in pooled cDNA samples of Renate bulbs at 12 h and *Marco* leaf and bulb samples under LD and SD conditions, respectively. Gradient PCR was conducted to obtain optimum annealing temperatures. The relative expression of the target genes was further examined by qRT-PCR carried out using the CFX384 TouchTM Real-time PCR machine from BioRad as described. Optimisation of PCR conditions for each candidate were followed as mentioned in Chapter 2. Three replications were carried out for each sample and the average CT value calculated. qRT-PCR data were analysed after completion of each PCR run and data indicated as means and normalized against expression levels of the reference genes for each sample. Normalisation was achieved by using Biogazelle qBase+ software based on the geNorm and qBase technology. The average expression level was calculated and standard errors were included. Standard curves (using 10-fold serial dilutions) were plotted using cDNA synthesised from approximately 2 µg of total RNA extracted from pooled Renate and *Marco* materials, respectively. Reference genes for Renate Developmental Experiment were *UBC9*, *UBQ1* and *PP2AA3*. Reference genes *Marco* Developmental Experiment were *AcTUB*, *PP2A1* and *UBL* (Appendix).

5.3 Results

5.3.1 Bulbing ratio and leaf number

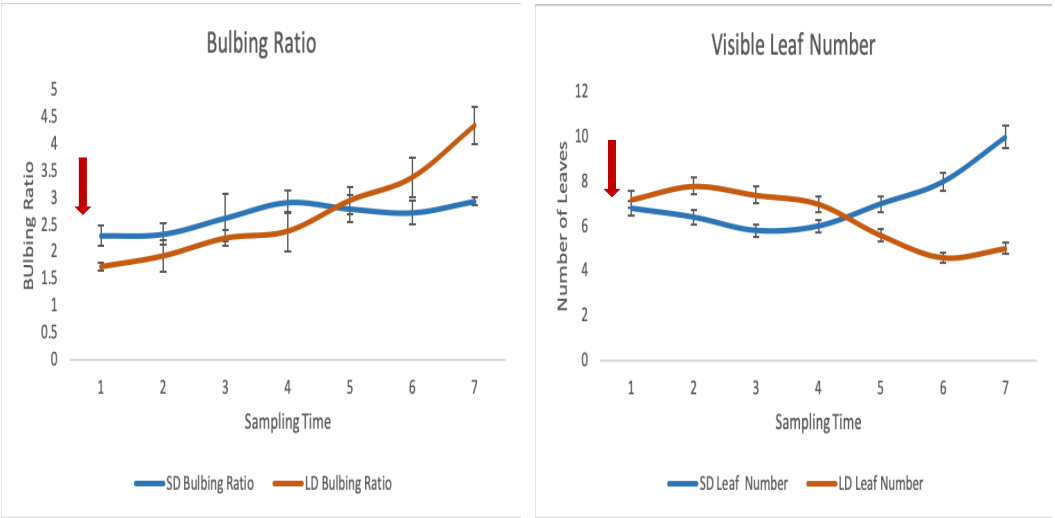


Figure 5-5 Bulbing ratio and visible leaf number after plants transfer. The red arrow indicates the transferring point (2 days before the first sampling date). Error bars represent the SEM. Five plants were used as technical replicates.

Figures above show the bulbing ratio and visible leaf number during plant development in *Marco* grown in LD and SD (Figure 5-5). There were slight differences at the starting point for both figures due to the randomization when separating the plants. Plants in which bulbing was just beginning to take place, as judged visually, were preferentially placed in SD. Plants where no sign of bulbing was apparent were preferentially placed in LD to make any changes due to the subsequent transition to bulbing in the LD treatment clearer. For plants grown in 8h of SD, there was no obvious change in bulbing ratio following the transfer date but after 4 harvests, an increase in visible leaf number can be seen, indicating an interrupted bulbing process followed by a restart of leaf production. For the LD plants grown under natural daylength with 16h of supplementary lighting, there was a clear increase in the bulbing ratio. Leaf production ceased. The apparent reduction in leaf number is because the oldest leaves had senesced and died back. The bulbing ratio for LD plants was less than that of SD plants at the beginning of the sampling timepoints, but progressively increased after around 127 days from sowing. The reverse was true

for leaf number and crossover of the values for visible leaf number between SD and LD occurred at approximately the same time as the bulbing ratios.

5.3.2 Relative gene expression – photoperiod genes

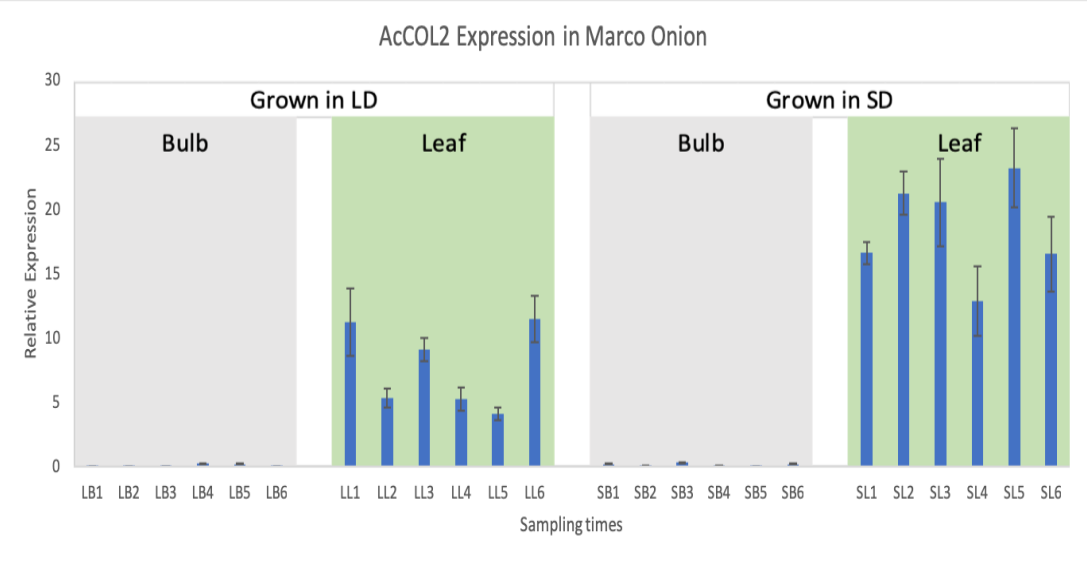


Figure 5-6 Relative expression of AcCOL2 in Marco leaf and bulb tissue under SD and LD. Error bars represent the SEM.

AcCOL2 showed specific leaf expression, which is consistent with the results of the Spatial experiment in Chapter 3. The expression of *COL2* in SD leaf is higher than that of LD. Apart from that, the gene showed a similar level of expression throughout the plant development in both LD and SD, suggesting *COL2* was expressed constantly in *Marco* leaves (Figure 5-6).

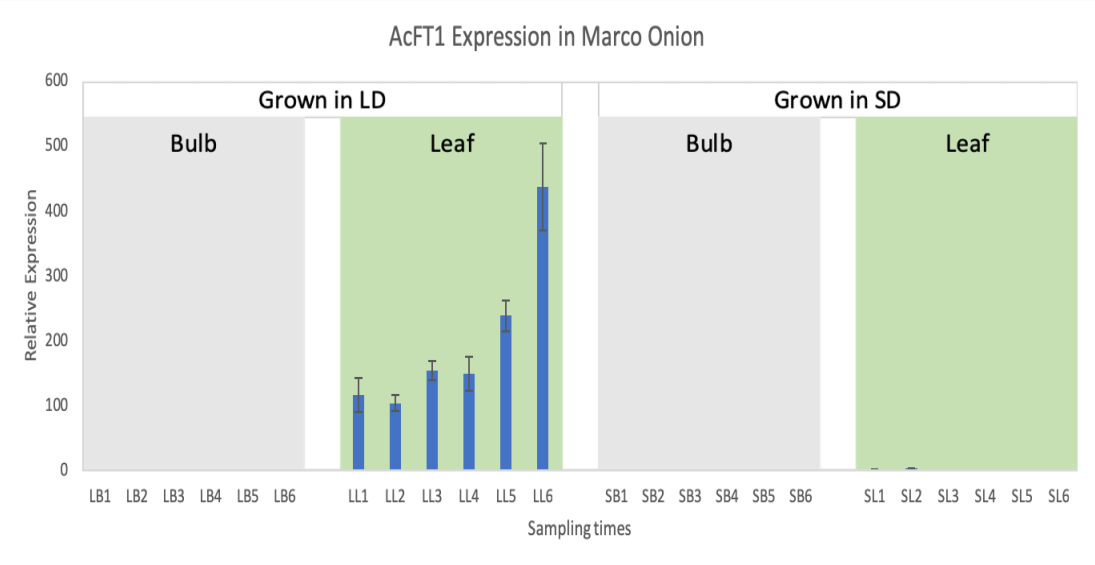


Figure 5-7 Relative expression of AcFT1 in Marco leaf and bulb tissue under SD and LD. Error bars represent the SEM.

AcFT1 was expressed only in LD leaf (Figure 5-7). The gene showed almost no transcripts in bulb material for both day-lengths, and very low expression in SD leaf material although foliage leaves continued to be produced. Moreover, the number of days had significant effect on *FT1* expression, as the amount of mRNA was detected to increase along with plant development. The result is consistent with former spatial experiment that *FT1* was expressed mainly in the leaf blade, as well as the diurnal experiment that *FT1* showed higher expression with longer daylengths. In addition, this experiment showed the increasing expression pattern of *FT1* during plant development under long days, indicating its role in promoting onion bulbing process under inductive daylength.

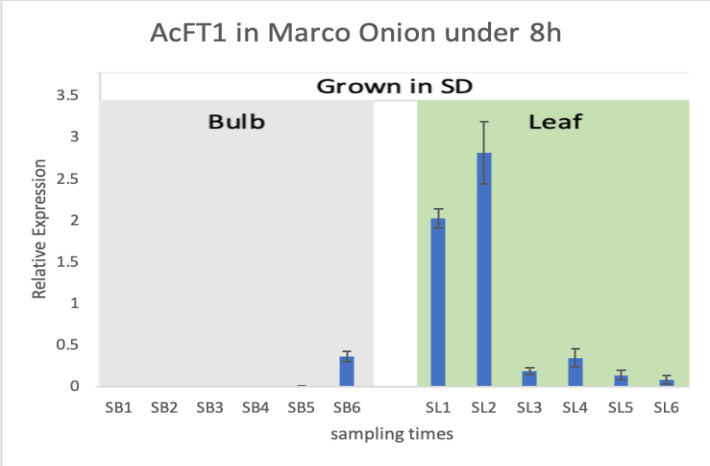


Figure 5-8 Relative expression of AcFT1 in Marco leaf and bulb tissue under SD on an expanded scale. Error bars represent the SEM.

In addition, in Figure 5-8, which plots the SD data on an expanded scale, it can be observed that there was some expression of *FT1* in the newly transferred material that was just beginning to show signs of bulbing but this was sharply reduced after the plants were transferred from LD to SD. The rapidly down-regulated expression of *FT1* under short days may further support its role as promoter in onion bulb formation.

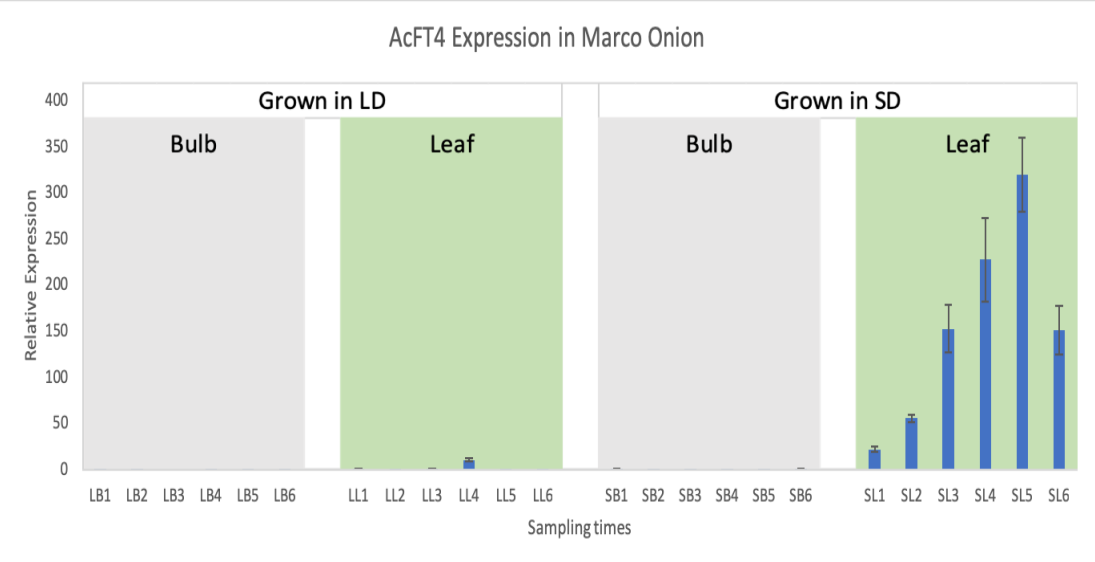


Figure 5-9 Relative expression of AcFT4 in Marco leaf and bulb tissue under SD and LD. Error bars represent the SEM.

By contrast, ***AcFT4*** was only expressed in the SD leaf with a rising trend along with *Marco* development (Figure 5-9). There was not expression detected in the basal tissue, which is consistent with Spatial experiment in Chapter 3. The expression level was also very low in the leaf grown in LD. The generous expression and the increasing trend of *FT4* in SDs can be related with its putative role as inhibitor in onion bulb initiation.

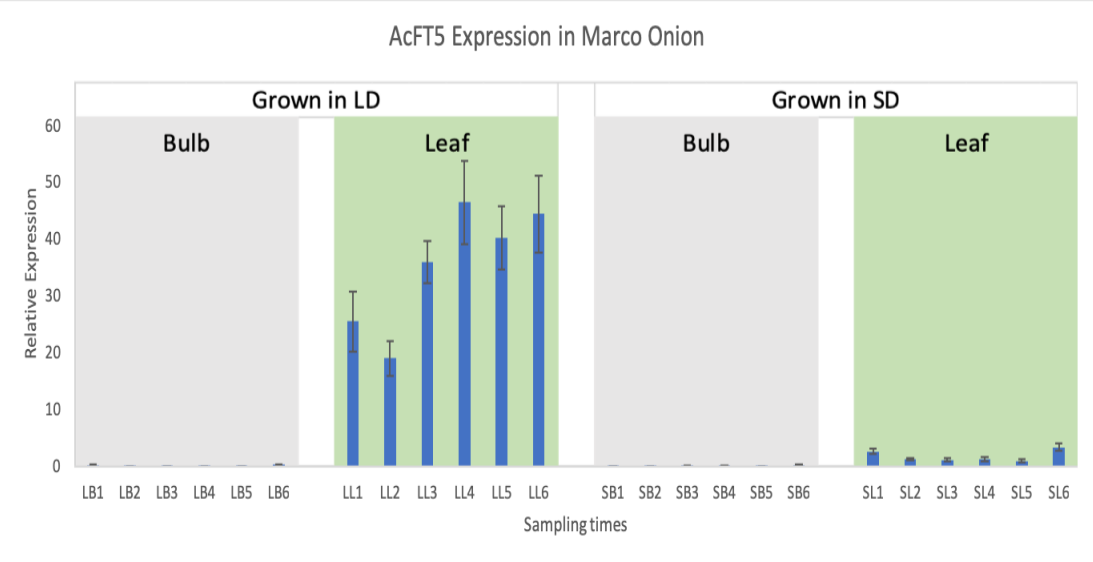


Figure 5-10 Relative expression of AcFT5 in Marco leaf and bulb tissue under SD and LD. Error bars represent the SEM.

AcFT5 showed its abundance in LD leaf material, together with very limited expression in SD leaf (Figure 5-10). There was barely expression in bulb tissues. The result is consistent with previous diurnal expression experiment in Chapter 4 that *FT5* showed higher expression and clearer pattern with longer daylengths. However, this result is inconsistent with Lee's study (2013), where the authors proposed *AcFT5* expression did not appeared to be strongly affected by daylengths. The significant high expression in LD leaf of *FT5* indicates its role in inductive green source part of the plant, as well as its unknown function in the daylength dependent bulbing process.

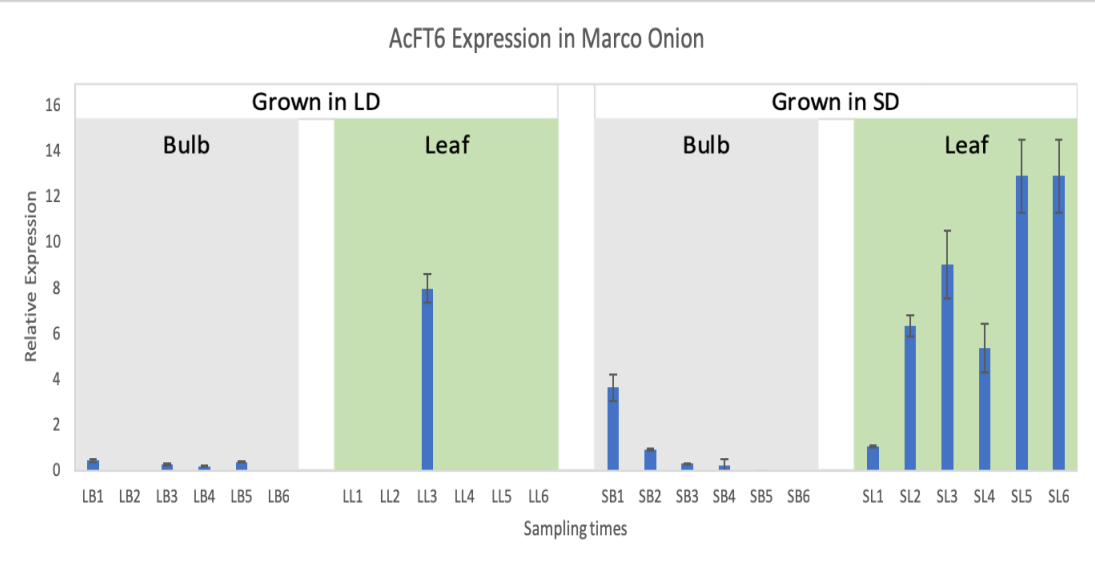


Figure 5-11 Relative expression of AcFT6 in Marco leaf and bulb tissue under SD and LD. Error bars represent the SEM.

AcFT6, by contrast, was expressed mostly in the SD leaf material, combined with an increasing pattern during the plant development. The expression of *FT6* was not detectable in LD leaf material (exception of one sampling point). Also, very low level of transcripts was presented in basal tissue for both daylengths. There is a reducing expression pattern in *FT6* mRNAs in SD bulb material after the plants being transferred from LD (Figure 5-11). The possible negative correlation between bulb and leaf of *FT6* expression under SD implies the gene's unknown function in bulbing process under short daylengths.

5.3.3 *Relative gene expression – carbohydrate and quality trait genes*

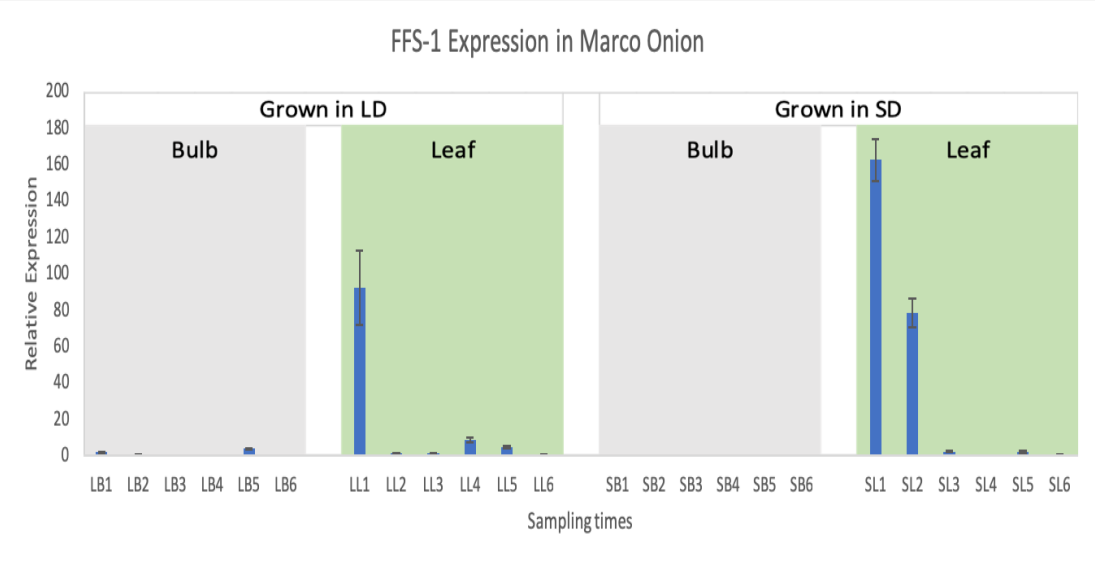


Figure 5-12 Relative expression of FFS-1 in Marco leaf and bulb tissue under SD and LD. Error bars represent the SEM.

The expression of ***FFS-1*** was mostly detected in leaf materials (Figure 5-12). The result is not consistent with its description in the onion transcriptome RNA-seq database which suggested *FFS-1* was a bulb-specific sequence. A decrease in expression level can be seen under both daylengths in the leaf during plant development.

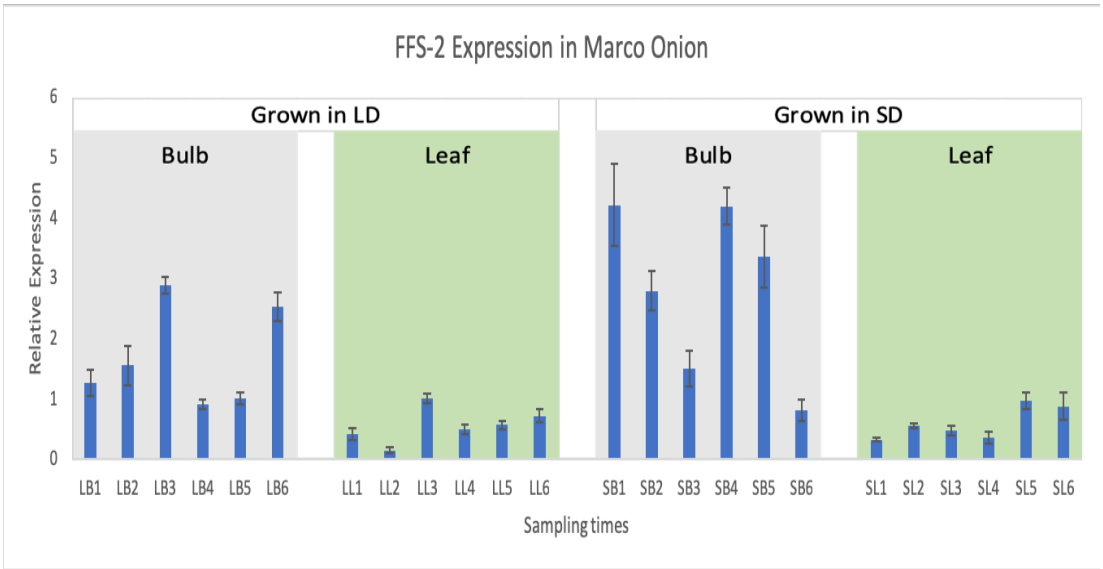


Figure 5-13 Relative expression of FFS-2 in Marco leaf and bulb tissue under SD and LD. Error bars represent the SEM.

The mRNAs of ***FFS-2*** was detected throughout the plant under both daylengths in plant growth (Figure 5-13). The expression in bulb was higher than in leaf, which is consistent with the onion transcriptome database. No obvious trend can be seen over time. As *FFS-2* was predicted to code insoluble invertase, its generous expression in onion bulbs may be related with the thickening of cell walls in sink tissue during bulb formation.

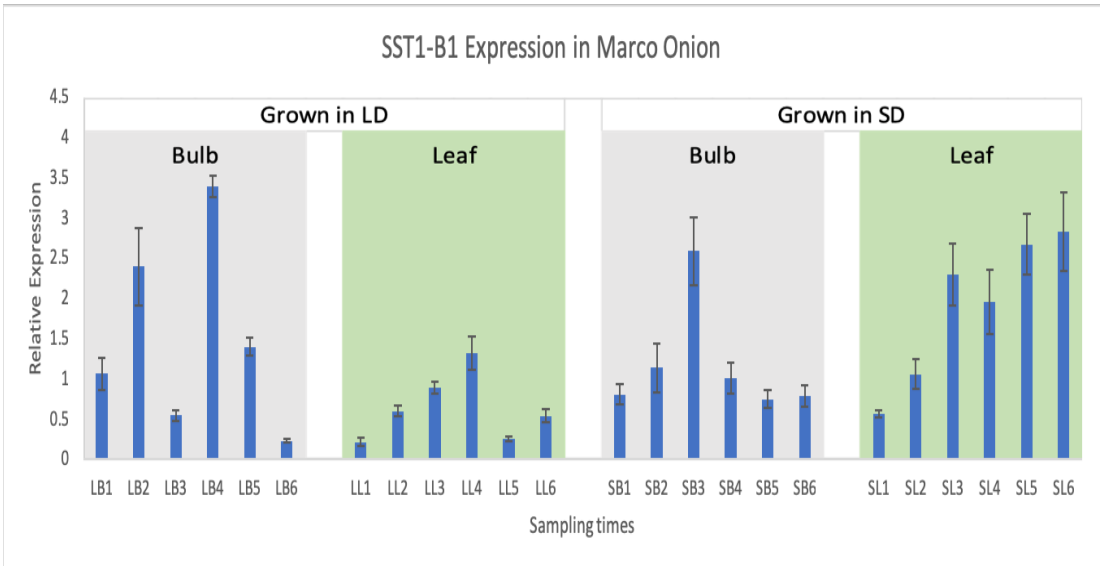


Figure 5-14 Relative expression of SST1-B1 in Marco leaf and bulb tissue under SD and LD. Error bars represent the SEM.

SST1-B1 was expressed generously throughout the developmental stage in all tissues under both daylengths. Under long days, the gene showed more bulb-specific expression pattern as described in the transcriptome database. Under short days, the expression level was slightly higher in leaf material. An increasing level of *SST1-B1* expression can be seen in leaf blade under SD treatment (Figure 5-14).

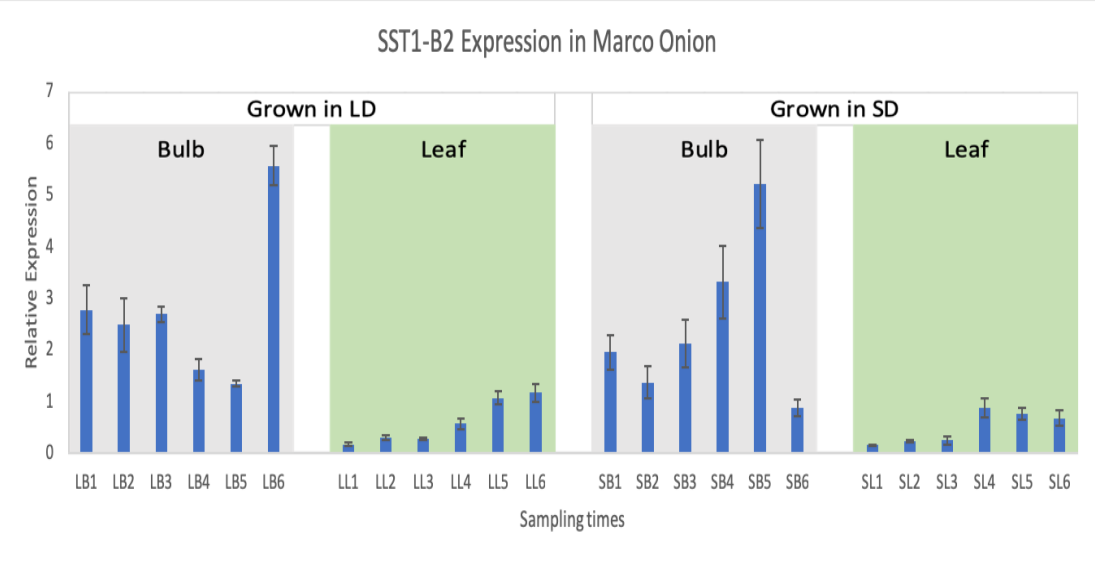


Figure 5-15 Relative expression of SST1-B2 in Marco leaf and bulb tissue under SD and LD. Error bars represent the SEM.

SST1-B2 showed greater abundance in onion bulb materials under both daylengths, which is consistent with its description in the transcriptome database. There was no obvious differential expression in response to daylengths. In addition, the gene seemed to have an increasing number of transcripts in all samples over time, indicating it may have a role in fructan accumulation in both source and sink materials (Figure 5-15).

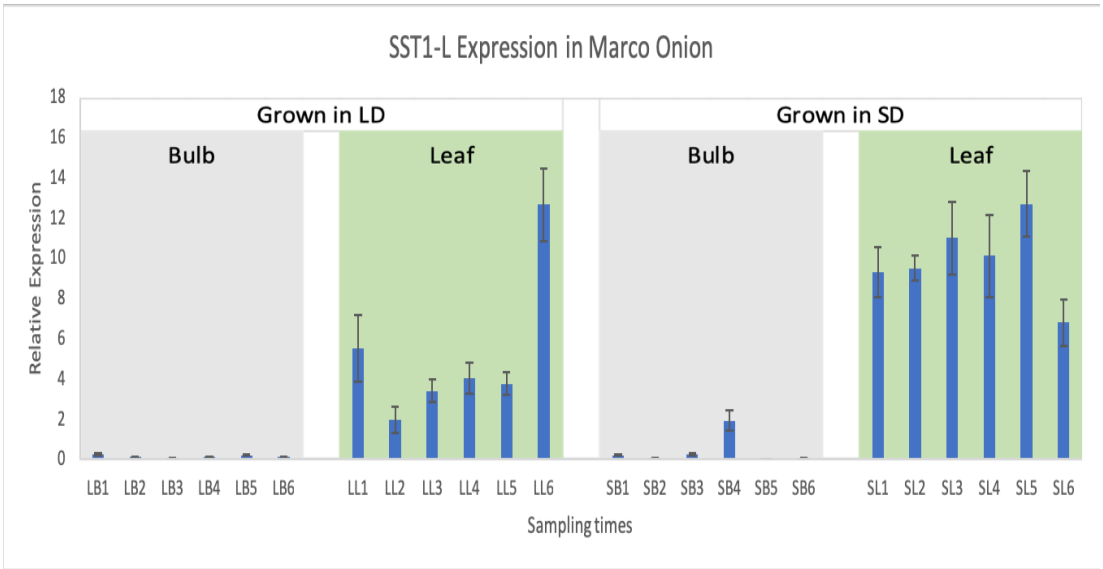


Figure 5-16 Relative expression of SST1-L in Marco leaf and bulb tissue under SD and LD. Error bars represent the SEM.

SST1-L exhibited a significant leaf-specific pattern with very limited mRNAs detected in the basal tissue (Figure 5-16). The result is supportive of its leaf-specificity in the onion transcriptome database. The expression level is higher in SD compared of that in LD.

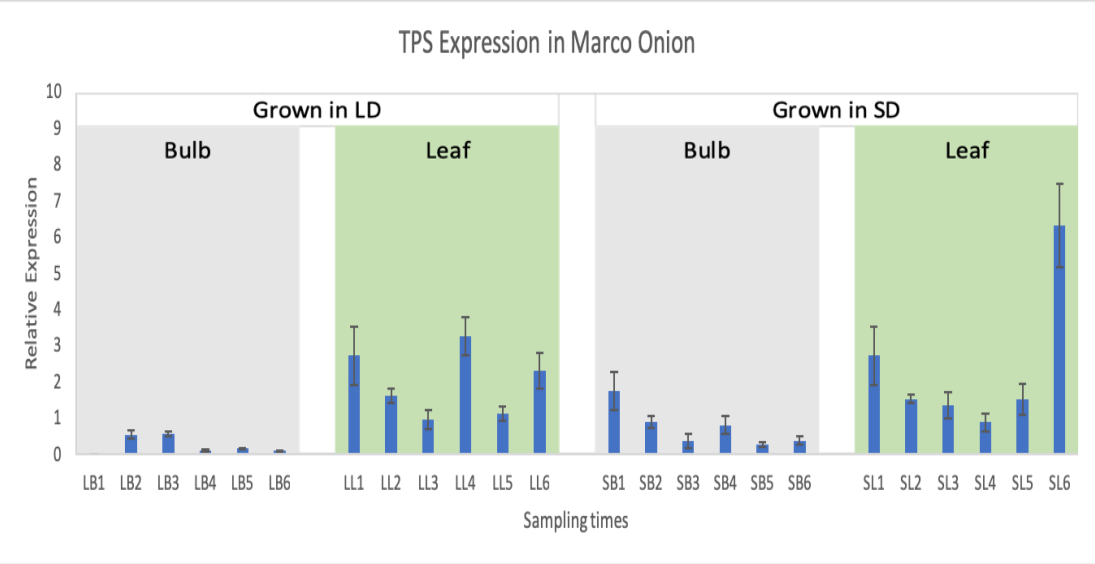


Figure 5-17 Relative expression of TPS in Marco leaf and bulb tissue under SD and LD. Error bars represent the SEM.

TPS was expressed in all materials, with more abundance in leaf materials. The result is inconsistent with its description of bulb-specific in the onion transcriptome database. The expression in SD bulb material seemed to have a decreasing trend (Figure 5-17). The non-selective expression of *TPS* over developmental time course indicating the gene has a wide role in plant growth.

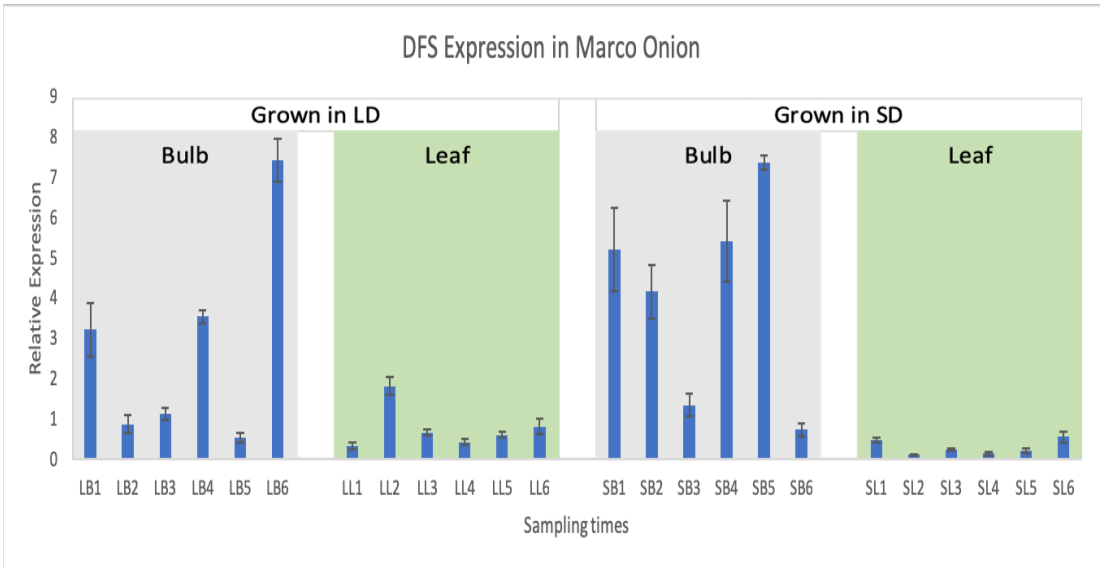


Figure 5-18 Relative expression of DFS in Marco leaf and bulb tissue under SD and LD. Error bars represent the SEM.

DFS presented clear bulb-specific pattern, which is supportive result of the transcriptome as well as spatial experiment in Chapter 3. The transcripts were also detected in leaf material but much lower than that in bulb. There was no noticeable difference in expression between daylength treatments, nor an accumulation in transcription level was detected (Figure 5-18).

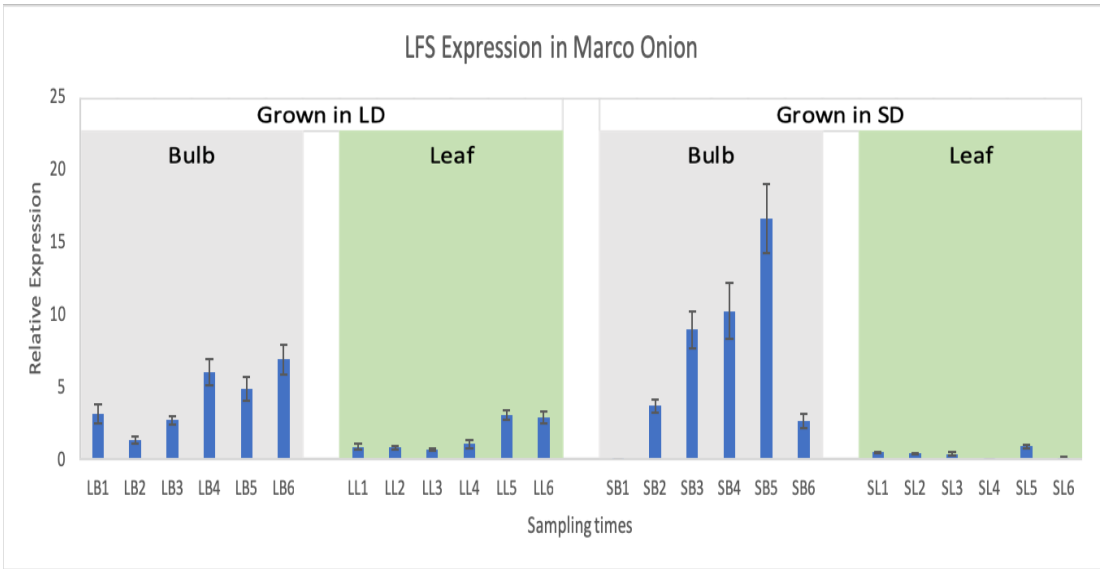


Figure 5-19 Relative expression of LFS in Marco leaf and bulb tissue under SD and LD. Error bars represent the SEM.

LFS mRNAs were evident throughout basal tissues for both SD and LD. The expression was also detected in leaves but much lower than that in bulb (Figure 5-19). The bulb-specific pattern is consistent with former spatial experiment in Chapter 3 as well as the onion transcriptome RNA-seq database. Additionally, a clear increasing trend can be seen in both bulb materials over time, which may indicate an accumulation of its functional protein existing in this part of the plant.

5.4 Discussion

5.4.1 Photoperiod genes

For the genes of Arabidopsis flowering pathway, all the targets showed leaf-specific pattern, indicating their role in light perception as well as sugar source organs. The results were also consistent with previous Spatial experiment in Chapter 3, but with more details in expression of daylength response and developmental time course. ***COL2*** was expressed constantly in the leaf blade during the growth, with a slightly higher transcript level in SD. The stable behaviour of COL2 expression throughout onion development and more transcripts under non-inductive daylength suggests the regulation by this gene is not a function of mRNA level. Supporting a putative mechanism that it is the instability of *COL2* protein that leads to the cessation of bulbing under SDs. ***FT1*** was expressed only in the LD leaf material, with a significant increasing pattern during plant development. The result is not only consistent with its leaf-specific character in spatial experiment (Chapter 3) but also with the diurnal experiment (Chapter 4) which the gene presented its abundance with longer daylengths. In addition, this experiment showed the transcripts of *FT1* continued to increase under inductive daylength conditions. In the expanded scale of expression in SD leaf material, a sharply decreased *FT1* mRNA level was observed, indicating the gene was quickly down-regulated after transfer from LD to SD. All these traits of *FT1* expression in this time-course experiment can further support its functional role as a promoter in onion bulbing process.

In contrast to *FT1*, ***FT4*** was expressed only in the SD leaf material, also with an increasing pattern during plant growth. There were barely any transcripts detected under inductive long day conditions. It can be seen that the gene was clearly up-regulated after the plants were transferred from LD to SD, indicating its role in preventing bulb formation in onion development. Moreover, the reverse pattern of *FT1* and *FT4* may suggest there is a negative correlation between the two.

FT5 and ***FT6*** also showed a reverse expression pattern, in which *FT5* was found mostly in LD leaf while *FT6* was only expressed in SD leaf. This is the reverse of what was seen in *Renate* diurnal experiments in the previous chapter. The result is

also inconsistent with previous studies that suggested the genes do not respond to daylength differences (Lee et al., 2013). An increasing number of transcripts of both *FT5* and *FT6* were detected during plant development at the required daylength, indicating a potential role in regulating this photoperiodic driven process, although the functions are still unknown.

5.4.2 Carbohydrate and quality trait genes

In higher plants, the catabolism of sucrose is to allow the subsequent utilization of the component hexoses. This catabolism is mediated by two enzyme activities, invertase and sucrose synthase (ap Rees, 1988). Invertase (β-fructofuranosidase) cleaves sucrose to glucose and fructose. In general, higher plants contain a family of invertases based on their pH optimum, subcellular localization and solubility in buffer (Tymowska-Lalanne and Kreis, 1998). In this experiment, ***FFS-1*** and ***FFS-2*** were selected from the onion RNA-seq database. The former ***FFS-1*** is predicted to be soluble acid invertase which means the enzyme functions in the vacuole. Not consistent with its description in the transcriptome database, the experiment showed the transcripts for *FFS-1* was predominantly detected in the leaf and the mRNA level reduced with plant maturity. The reason for this leaf-specific pattern of *FFS-1* may be that there is a requirement for sucrose catabolism to support heterotrophic tissues (e.g. epidermal cells) or to provide respiratory substrates at night in leaves. Besides its function in sucrose metabolism in heterotrophic tissues (epidermis and vasculature), Kingston-Smith et al. (1999) suggested the possible sensing role of invertase in leaves. The mRNA abundance of *FFS-1* is sensed and transduced into altered patterns of gene expression, and further alter patterns of the genes modulating sucrose catabolism in sink tissues.

FFS-2 was predicted to be an insoluble isoenzyme, meaning it is one type of cell wall-bound invertase. The experiment showed its transcripts mainly in the bulb material, which is a consistent result of the transcriptome database. The bulb-specific pattern of this gene indicates its role in carbohydrate accumulation in onion bulb initiation. Strong correlations have been established between sink activity and increases in enzyme mRNA abundance (Koch et al., 1996). Invertase tends to cause an

accumulation of hexoses, which will be continuously supplied to the meristematic tissues, and providing substrates for growth of onion bulb tissues. It has been suggested in various studies that invertase is closely correlated with growth (Arai et al., 1991) and expansion in many sink tissues (Schaffer, 1986).

The organ-specific expression pattern of invertase seen in this experiment has been found in many other plant species. In *poplar*, there are six *PtCWINVs* differentially expressed in leaf, stem and root tissue (Chen et al., 2015). In carrots and tomatoes, some cell wall or vacuolar invertase genes showed markedly different organ specific expression patterns with plant development (Sturm et al., 1995; Godt and Roitsch, 1997). This experiment also showed tissue specific invertase activity of *FFS*s, revealing that these genes play an important role in their respective organs in terms of providing carbohydrates for growth and development, and provides a basis for understanding the function of invertases in onion.

Fructosyltransferases transfer fructose from sucrose to the growing fructan chain. According to the classical model of Edelman and Jefford (1968), two enzymes (*SST1* and *FFT1*) are involved in the synthesis of the simplest form of fructan, and this experiment only focused on the first enzyme *SST1*s. Three *SST1*s were selected from the onion transcriptome RNA-seq database, and all of them showed consistent results in their tissue-specific expression sites. The homologs were specialized in storage bulb organs or in photosynthesizing leaves or both. ***SST1-B1*** was expressed generously in all materials throughout the developmental stage, together with an increasing trend in mRNA level in leaf blade. ***SST1-B2*** was preferentially expressed in bulb material while ***SST1-L*** only showed its transcripts in the leaf as expected. In addition to its role as a major reserve carbohydrate, fructan synthesis might have a multifunctional role in plant sugar metabolism and development. Vijn et al. (1997) cloned *SST1* from onion, and found if placing onion leaves under continuous illumination, the excessive Suc content would induce fructan synthesis and *SST1* mRNA accumulation. This experiment provided a supportive result showing an increasing number of transcripts of *SST1-L* in LD leaf materials. However, Van den Ende et al. (2000) isolated the cDNA for *SST1* from *Taraxacum officinale*, and the data showed the mRNA concentrations of *SST1* were very low in leaves but abundant in young roots. Fructans are often stored in different specialized organs, for example

in the taproot of chicory *(Cichorium intybus*), the tubers of dahlia (*Dahlia variabilis*), and the bulbs of tulip (*Tulipa gesneriana*). In this experiment, both *SST1-B1* and *SST1-B2* also were abundant in bulb materials as seen in tulip. The more active fructosyltransferases in these storage organs firstly indicate a higher level of fructan nutrients in those parts, and further might be related with the plant's regrowth when sprouting in the spring (Ritsema and Smeekens, 2003). Fructans can also play a role in the expansion of flowers. Studies showed petals of daylily (*Hemerocallis*) and *Campanula rapunculoides* rapidly degraded fructan during flower opening stage (Bieleski, 1993; Vergauwen et al., 2000). In cereals, fructans temporarily accumulate in stems and early in seed development (Van den Ende et al., 2011; Joudi et al., 2012) as well as in reproductive organs (Ji et al., 2010). Another, different, role in the protection of plants during drought tolerance (Pilon-Smits et al., 1995) and cold spells (Konstantinova et al., 2002) was also suggested for fructans. However, the physiological role of fructans in plants is not fully understood. The reason for multi-functions of fructan in plants could be based on different physiological needs, or might be the consequence of the different evolutionary origins of fructan biosynthesis in different families.

TPS was mainly expressed in leaf material in this experiment, which is not consistent with the onion transcriptome RNA-seq database. The leaf-specific expression of this gene may indicate it has a role in source organs. The result may support the study in rice (Garg et al., 2002) which showed the increased trehalose accumulation correlated with an elevated capacity for photosynthesis. In tobacco, expression of Escherichia coli *TPS* also results in increased photosynthetic activity (Paul et al., 2001; Pellny et al., 2004). It is worth noting that there is increasing evidence suggesting that, instead of trehalose, the *TPS* coding trehalose-6-phosphate may be a key regulator of metabolism. In Schluepmann's study with Arabidopsis, pleiotropic effects were observed as a result of *TPS* expression in growth and development, however, the authors found these effects are not due to increased trehalose accumulation, but rather to an increase of the intermediate trehalose-6-P, which may act as an important signalling molecule (Schluepmann et al., 2003). Van Dijken's (2004) study also showed the absence of trehalose-6-phosphate synthase in Arabidopsis plants precluded transition to flowering, indicating the gene is important for vegetative growth and transition to flowering (van Dijken et al., 2004). In this experiment, *TPS*

was expressed generously throughout the onion development with no obvious differences between SD and LD. Although it has a higher expression in photosynthesis leaves, the gene seems not sensitive to daylength in the onion bulbing process. The role of *TPS* in modulating sugar sensing and carbohydrate metabolism is still not understood and future work needs to be done.

In terms of onion quality trait genes, both ***DFS*** and ***LFS*** showed bulb-specific expression pattern, which is consistent with both the onion transcriptome database and previous tissue-specific experiment in Chapter 3. The higher level of transcripts of these genes in the bulb materials confirming these healthy phytochemicals accumulate in onion's basal part. Furthermore, *LFS* transcripts showed a clear increasing trend in bulb materials, suggesting the sulphur-related compound of lachrymatory factor is accumulating over time during bulb development.

5.5 Conclusion

The data presented in this chapter details the gene expression in leaf and bulb tissues during the development of *Marco* under SD and LD. The relatively developmental and daylength-induced changes of some key genes was examined at the mRNA level based on quantification RT-PCR results. Variety *Marco* was firstly used in experiments and data obtained suggested it was a robust variety with a good LD bulbing response. This chapter firstly detailed the characterisation of some onion putative photoperiod response genes. All the candidates in this group showed leaf-specific expression, while some of them also presented daylengths sensitive expression pattern. *COL2* was expressed constantly in leaf blade during the plant growth with no obvious SD/LD differences. *FT1* was expressed in LD leaf whilst *FT4* was in SD leaf, though both genes showed significant increasing patterns during plant development. The data can further support the role of *FT1* as a promoter and *FT4* as inhibitor in onion bulb formation, and there seems a negative correlation between these two genes. A clear down-regulated *FT1* was seen when plants were transferred from LD to SD. *FT5* and *FT6* also presented a reverse expression pattern, with *FT5* existing mostly in LD leaf while *FT6* was in SD leaf, both showed an accumulation in mRNA level. The second group of genes were selected for their putative role in onion carbohydrate accumulation and sugar metabolism. Two *FFS*s of invertase, three *SST1*s of fructan synthesis and one *TPS* of trehalose metabolism were isolated. *FFS-2* showed bulb-specific expression consistent results with the onion transcriptome while *FFS-1* showed the opposite leaf-specific pattern. No daylength sensitivities were seen in these two genes. The different tissue specific invertase activity reveals the diversity of invertase-related genes and their specific role in respective organs. The tissue specific expression pattern of three *SST1*s were all consistent with the onion transcriptome database, with *SST1-B1* generously expressed in all materials, *SST1-B2* preferentially expressed in bulb materials, and *SST1-L* only in the leaf blade as expected. The results indicate that fructans are stored in different specialized organs in onion, which has been seen in diverse plant species. Apart from its role as a major reserve carbohydrate, fructan synthesis may also have multifunctional roles in plant sugar metabolism and development. *TPS* showed more abundance in leaf material, which in inconsistent with its description in the onion transcriptome. No daylength response or developmental trend were seen, indicating

the gene may have a wider role in plant growth. Although the functions of these genes are currently unknown, this experiment still provided some insights into the relationships between these carbohydrate genes, and set up a system for characterising onion sugar metabolism genes in the future. The last group consist of *DFS* and *LFS*, both are onion nutrient related genes. The two genes showed significant bulb-specific expression pattern as expected, and *LFS* showed additional increasing number of transcripts over time. Overall, this chapter described here allowed a system to be set up in order to study the expression of not only the photoperiod response genes, but also other genetic regulations in understanding the nature of onion bulb initiation.

6 GENERAL DISCUSSION AND CONCLUSIONS

6.1 General discussion

The main aim of this work was to gain a further understanding of the molecular mechanism involved in daylength-dependent bulbing response for onions. Specifically, to test how the candidate genes interact with different photoperiods in different onion cultivars to control the initiation of bulbs. A series of physiological and molecular experiments were carried out throughout the project. Genes including Arabidopsis flowering time genes as well as some bulbing-related novel sequences were identified and isolated in onion.

6.1.1 The spatial distribution of bulbing-related genes in onion

The spatial distribution of the onion homologues putatively in the Arabidopsis flowering pathway, including *AcGI*, *AcFKF1*, *AcCOL2*, *AcFTs* and *AcLFY,* showed consistency in spatial distribution with those of Arabidopsis: whether in the site of perception (green leaf), or in the site of response (basal leaf).

All onion leaves develop from a basal meristem, cells move through the basal region into a transition zone during leaf growth, and turn into the photosynthetic green part (leaf blade). By investigating the expression of the genes at different sites along the length of the leaf, it can be corelated to their functional regions, as well as giving a picture of how expression changes in cells of the increasing plant age (older away from base). A spatial mRNA and quantitative gene expression analysis was carried out using the leaf sections of the LD onion cultivar *Renate* grown in inductive long-days. Homologues of Arabidopsis flowering time genes were isolated and characterised in onion along with their spatial expression patterns. Consistent with Arabidopsis, *AcFKF1*, *AcCOL2* showed their major expression in green parts of the leaf (Chapter 3/Figure 3-8). The leaf-specific expression of *AcFT1* and *AcFT4* could also a the parallel to Arabidopsis *FT* (Chapter 3/Figure 3-7). The preferential expression of these genes in the photosynthetic parts of the leaf indicates their role in daylength perception in the bulbing process. Both *AcFT5* and *AcFT6* presented most

of their transcripts in the transition parts of the plant (Chapter 3/Figure 3-7). The similarity of the two genes may be due to the interactions or correlations with other FT genes, and imply an unknown function in FTs signal translocating and plant development. In Arabidopsis, *LFY* has been suggested to be a meristem-specific gene that positively correlates with flower induction (Smart and Roden, 2015). In this report, the basal meristem specific expression of *AcLFY* in the first-year onion bulbs can correspond with Arabidopsis *LFY* which presents highest expression level in apical inflorescence meristem (Chapter 3/Figure 3-8) (Yang et al., 2016). It can be suggested the gene's function of causing undifferentiated cells (meristems) to develop into flower parts instead of leaves may also be applied to the bulb initiation onions.

In brief, the spatial distribution of bulbing-related genes in onion is consistent with their identified expression sites in Arabidopsis, supporting the hypothesis that bulb formation in response to daylength is similar to the daylength regulation of flowering. The results allow for further parallel genetic studies on the photoperiodic control of bulbing to be undertaken.

6.1.2 The interaction of FTs and other circadian genes in different daylength types and different photoperiods to control the bulbing response

The conservation and variation of the circadian-regulated functional genes among higher plant species under inductive and non-inductive daylength conditions highlights the fact that some differences exist in the genetic control of photoperiod response in different species. For better understanding of the mechanisms of different daylength requirements for different onion varieties, a set of diurnal and developmental time-course expression was conducted and combined to examine the differential expression of these photoperiod related genes. The diurnal expression in onion leaves was set using LD (*Renate*) and SD (*Hojem*) varieties grown under three different intermediate daylength conditions (10h, 12h and 14h). The tissue specific developmental time-course expression was set with another LD cultivar (*Marco*) grown under inductive (16h) and non-inductive (8h) daylength conditions. In general, all candidates with the exception of *LFY* presented clear rhythmic expression in diurnal patterns, together with leaf-specific expression either in short-day or long-day during onion development. The results fundamentally confirmed the hypothesis that

the clock genes in Arabidopsis flowering pathway would be also circadian regulated in onion.

With the increase of daylength, *AcGI* showed similar rhythmic expression among varieties (Chapter 4/Figure 4-3), whilst *AcFKF1* showed a shifted peak time in *Renate* but not in *Hojem* (Chapter 4/Figure 4-4). As the two genes work together as a complex, the shift in *AcFKF1* timing may be decisive in the timing of their action and affect the bulbing process negatively under short days.

AcCOL2 was expressed constantly in *Marco* onion leaves with a similar level in both long- and short-days (Chapter 5/Figure 5-6). In *Renate* and *Hojem*, *AcCOL2* showed a constant diurnal pattern under all intermediate daylengths, peaking towards the end of the long day and slightly later into darkness in short day (Chapter 4/Figure 4-5). The consistent behaviour of *AcCOL2* expression regardless of daylength and cultivars suggests this gene does not function in mRNA level, corresponding the putative mechanism that it is the lack of *COL2* protein causing the growth cessation of bulbing under non-inductive daylengths. Further, the mechanism of its post transcriptional regulation in onion bulb initiation could be established.

With *Marco* development under long and short days, *AcFT1* was only expressed in the LD leaf with the transcripts accumulating during plant growth (Chapter 5/Figure 5-7). The pattern is supportive with its putative role as promoter in the onion bulbing process. Moreover, a sharply decreased *AcFT1* mRNA level was observed in the leaves of plants transferred to SD when the data were plotted on an expanded scale, indicating that the gene was quickly down-regulated after transfer from LD to SD (Chapter 5/Figure 5-8). Diurnal expression with *Renate* and *Hojem* under intermediate daylengths also showed the gene with higher abundance in longer daylengths (Chapter 4/Figure 4-6). A clear rhythmic expression peaking in the latter part of the day could be observed with an increase of daylengths for both varieties. This diurnal pattern of *AcFT1* is consistent with the behaviour of Arabidopsis *FT*, indicating the gene could be a functional homologue of Arabidopsis flowering pathway.

In contrast, *AcFT4* was only expressed in the SD leaf material in *Marco* development, though also with a rising trend indicating the accumulation of the transcripts (Chapter

5/Figure 5-9). The generous expression and the increasing trend of *AcFT4* in SD can be related with its putative role as inhibitor in onion bulb initiation. In the diurnal experiment, *AcFT4* was expressed generously in both varieties under all daylengths (Chapter 4/Figure 4-7). The non-selective generous expression of *AcFT4* may suggest a less dominate role in the predicted negative correlation of *FT1* and *FT4*. In addition, under longer days, the clearer diurnal expression of *AcFT4* peaking in the early part of the daylight seemed to be a reverse diurnal pattern to *AcFT1*. More interesting, *FT1* in the evening seems to correlate with a decrease the afternoon peak in *FT4*. The peak of *FT4* transcripts in the evening under shorter days was suppressed once *FT1* expression increased under longer daylengths (*Hojem*). Anyhow, more insights were added in the proposed role of *FT1* and *FT4* in onion formation (Lee et al., 2013), there may be negative correlation between the two, with possible dominant role of *AcFT1*.

In *Marco* development, *AcFT5* existed mostly in LD leaf while *AcFT6* only in SD leaf (Chapter 5/Figure 5-10, Figure 5-11). Two genes seem to have a reverse role in daylength response. This contradicts the previous study suggesting the two genes do not response to daylength differences (Lee et al., 2013). An accumulation of transcripts of both genes was detected in leaves during *Marco* development, indicating their role in regulating bulb initiation. In *Renate* and *Hojem* under intermediate daylengths, *AcFT5* and *AcFT6* showed various peaks at different timepoints, and a repeatable diurnal pattern appeared with longer daylengths (Chapter 4/Figure 4-8, Figure 4-9). However, the diurnal or the developmental expression data for these genes is compromised, as their site of expression is mostly in the transition area, whereas the tissues were sampled from the middle of the leaf and the base. Although we may have detected some active components of *AcFT5* and *AcFT6* in this report, future experimental work needs to be conducted with transition tissues where the genes are most active.

6.1.3 Genes differentially expressed in bulb tissues during bulb formation

Additionally, a series of novel sequences with large differential expression in LD Vs. SD or leaf Vs. bulb in the onion transcriptome database were also identified in this project. These candidates putatively play a role in either circadian regulation, onion carbohydrate metabolism, onion sulphur metabolism or other aspects in plant

development (e.g. leaf morphology, cell expansion etc.). A tissue-specific expression pattern or a developmental time-course expression pattern was found for each of the candidates, adding a more widespread characterisation of these novel genes.

Circadian regulation

For the sequences putatively related to circadian regulation, *PHYA* was generally expressed more highly in the bulb than the leaf, while the most of the transcripts appeared in the long-day leaf tissue. The high presence in LD-leaf indicates the role of *PHYA* as basic photoreceptors to perceive daylight (Franklin and Whitelam, 2007). However, its abundance in bulb tissues indicates there are also sensory photoreceptors detecting light in basal parts although the function not in the plant circadian system. *TIC* showed most of its transcripts in LD-leaf materials whist no amplification in SD-leaf (Chapter 3/Table 3-5), consistent with the role of *TIC* for proper clock activity and altered leaf morphology under inductive long days in onion development (Duc et al., 2009).

Sulphur metabolism

For the genes related to sulphur metabolism and onion bulb traits, all candidates (*LFS*, *ALL*, *DFS*, *GCL*, *GST*, *ECT* and *AHCY1*) showed their major expression in bulb materials as expected (Chapter 3/Table 3-5, Table 3-6). Plant defense and nutrition level are highly interrelated, for the health benefits of some mineral nutrients are originally working as natural crop defense in response to the action of pathogens (Garcia-Mina, 2012). Many biochemical pathways and mechanisms involved in natural plant defense system are mainly due to antioxidants that respond against the attack of pathogens, and vice versa, the improved nutrition level also strengthens the antioxidants to improve the defense system of plant. The bulb-specific expression of these plant defense genes indicates these phytochemicals existing in the basal part of the plant, firstly to protect onion bulbs against predation and biotic stress especially over winter, plus add health beneficial effects to edible bulbs for humans. *LFS*, *DFS* and *GCL* presented a LD-specific expression pattern, suggesting the sulphur-related compounds would be preferentially synthesised under inductive long days as the bulbs increased in size (Chapter 3/Figure 3-9) (Chapter 5/Figure 5-18). In the *Marco* developmental time-course study, an accumulation of *LFS* transcripts was observed in bulb materials over time, indicating an accumulation of the functional proteins

(Chapter 5/Figure 5-19). The characterisation of these plant defense system genes could help investigate the genetic pathways of some unique phytochemicals in onion crop, and contribute to onion breeding programmes for “tear-less” or “high nutrition” varieties.

Carbohydrate metabolism

For the genes associated with onion carbohydrate metabolism, two *FFS*s of invertase, three *SST1*s of fructan synthesis and one *TPS* of trehalose metabolism were isolated. None of them showed daylength sensitivities in this study. The physiology of onion with source-to-sink carbohydrates transition is showed in Chapter 5. The carbon captured by photosynthesis is released from chloroplasts in the sugar source organs (leaf) and converted to sucrose which is then used to make fructans. Fructans can either be stored in leaves for use of living cells, or be hydrolysed into sucrose and fructose by invertase (*FFS*) and transported over long distances to the sugar sinks (bulbs). The released fructose units then can be assembled to make reserve fructans by *SST1*. In this project, we found *FFS-2* with bulb-specific expression, which is consistent with the onion transcriptome, while *FFS-1* showed the opposite i.e. a leaf-specific pattern (Chapter 5/Figure 5-12, Figure 5-13). The different tissue specific activity of *FFSs* suggests that the sucrose is hydrolysed in respective organs. Homologs of *FFSs* not only have a role in the storage sink bulb scales, but also exist in leaves to meet the sucrose catabolism requirement for much wider roles (Benkeblia et al., 2004). The diversity was also found with *SST1*s. *SST1-B1* was generously expressed in all materials, *SST1-B2* preferentially in bulb scales with an accumulation of transcripts, and *SST1-L* only in the leaf blades (Chapter 5/Figure 5-14, Figure 5-15, Figure 5-16). The different tissue specific activity *SST1s* indicates that fructans are synthesised and stored in both source and sink organs. Fructans may be translocated to the sink tissues directly through the phloem, or be hydrolysed first, then transported and resynthesized (Sharma et al., 2015). *TPS* showed higher expression in leaf materials, indicating the gene may play a wider a role in the non-resurrecting onion plant (Chapter 5/Figure 5-17). The result may support *TPS* possible role in source organs to elevate capacity for photosynthesis (Garg et al., 2002).

Onion development

For the genes involved in onion development and leaf morphology, a few of them showed inconsistent expression sites with the onion transcriptome RNA-seq database, or even shifted spatial expression pattern in different experiments, implying their possible multifunctional roles in plant development. *MADS* and *POLYCOMB* were detected mainly in LD-leaf material (Chapter 3/Table 3-5) (Chapter 3/Figure 3-9). *SAUR*, *RPL34* and *H2A* were found in all bulb tissue with no obvious daylength sensitivity (Chapter 3/Table 3-7). *TUBA2* was expressed generously throughout the plant without significant tissue preference (Chapter 3/Table 3-7). *PER12* was characterised as SD-bulb-specific in the onion transcriptome, and the preliminary gene expression experiment provided the consistent expression pattern (Chapter 3/Table 3-5). However, the gene showed contrast pattern in *Renate* spatial expression study, peaking in the middle of the leaf blade while no transcripts in the basal tissue (Chapter 3/Figure 3-9). The complexity of expression sites of this gene may indicate that it has multiple roles at different stages of plant development.

Although the functions of these novel sequences are currently unclear, we still provided some insights with the distribution and diversity of these genes. These studies help set up a system for characterising the relationships between these genes, and could be combined to develop a wider scheme for future onion genetic studies.

6.2 Recommendations for future experimental work

The rationale behind this project was that gaining a greater knowledge of the daylength response in onion is important for adapting new varieties for growth at different latitudes. This extends to the choice of which current varieties will perform best at different latitudes. This piece of work will provide a base for future work towards a thorough characterisation of the photoperiodic control of bulb initiation and bulb traits.

Future work should be centred on *AcFT*s as these genes appear to show differential expression in onion varieties with different daylength responses. Following this, it may be possible to perform genetic screens on onion varieties in order to predict which

varieties will perform the best at different latitudes, as well as a better understanding of the genetic network in higher plants as a whole.

Firstly, it would be useful to confirm the potential negative correlation of *AcFT1* and *AcFT4* in the bulbing process. Transformation and complementation studies for these two genes using respective Arabidopsis mutants would help to functionally characterise their potential interactions and correlations. The mutant lines could be transformed to over-express or knock out *AcFT1* and *AcFT4* and the phenotypes assessed in both LD and SD conditions. Possible RNAi technology (Eady et al., 2008) or virus induced gene silencing (Lu et al., 2003) could be advantageous.

Over expression and knockouts in LD and SD onion would also be useful, particularly to test whether mutual cross-regulation of FTs occurs. However, onion transformation is technically difficult and time consuming, while still has been achieved by few laboratories (Eady, 1995; 2002); Buitveld (1998); and Kik (2002).

Repeat experiments of functional complementation of onion *COL2* in Arabidopsis *CO* could be done. To investigate the effect of *AcCOL2* on flowering time in Arabidopsis *co-2* mutant and Ler wild-type plants, we attempted to obtain Arabidopsis transformants by floral dip using *AcCOL2* cloned vector. Although there was one seedling survived the BASTA herbicide selection, the DNA gel blot analysis for its T1 plant demonstrated that the surviving plant was not actually transgenic. However, we did not repeat the floral dip and BASTA selection due to insufficient time in the project.

Additional developmental and diurnal experiments for *AcFT5* and *AcFT6* need to be conducted with the transition tissue of onion plants. The experiment with onion leaves has been carried out in this project whereas leaf may not be their functional site. Again, overexpression or knockouts in onion would be valuable in testing their putative functions.

Molecular interaction studies could be carried out, possibly using Chip-seq or a Yeast Two-Hybrid system (Gietz et al., 1997) for different circadian regulated onion homologues (*AcCOLs* and *AcFTs*). This would help to identify whether a particular

gene activates or supresses the transcription of others. Complementation studies using Arabidopsis mutants may help further functionally characterise these onion homologues. A positive result (functional complementation) would show an involvement of this gene in the photoperiod response.

It would also be useful to carry out some bioinformatic analyses for those quality trait genes such as those involved in carbohydrate and sulphur content. Mapping of a gene involved in onion bulb fructans content has been previously reported and mapping of other putative genes could follow a similar protocol (McCallum et al., 2006).

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APPENDICES

Appendix I. Onion gene sequences

AcFKF1

GenBank Accession: GQ232754

AcGI

GenBank Accession: GQ232756

AcCOL2

GenBank Accession: KY012331

AcFT1

GenBank Accession: KC485348

AcFT4

GenBank Accession: KC485351

AcFT5

GenBank Accession: KC485352

AcFT6

GenBank Accession: KC485353

AcLFY

GenBank Accession: JX275963

PHYA

CACACAAACGAAACAACCGCATAACCATAAATTTCAAACGTGAACATAA
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DFS

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LFS

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ALL (designed primers for target gene)

CGCGGGGACTGAAAGTTGAATAAATACTAATAAGTAATAGTCCCATATA
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GCL

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GST

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MADS

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PER12

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POLYCOMB

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AHCYL1

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ECT

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H2A

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RPL34

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SAUR

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TUBA2

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FFS-1

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FFS-2

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SST1-B1

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SST1-B2

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CTTGAGCATCCATGGCGGGTATGTCAACCGCCGGTGAGTGAAAGGATAT
GTGTTTTAGTGAATAGACCTACAAATCGTGCTGCAACTTTTATAGTGCTA
CGGAAGTAAAGTGATGTTATGCGTGGGGTGACG

SST1-L

CAAACATTCAAACAACGTTTATATTCATTTCATCGTTCATTTTTTCAGAC
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CTCCCTTATCTTCCCTATCCCCTCTCCTTTCTCCCGAAGAATCCGCTCCCC
GGCAAGCCAAAAAGGCATACTTTGTATCGGTCGCTCTTATGCTTCTGATG
CTTGCAGGCTTGGCTGCGTTCGTTTTCGTTTCGGGTACGGATGTAACGGG
TTGGGATCCGATGCATCGGGACCCGATCGTGGTGGATCGAGGTGTGAAA
GCGGGAGTGTCGATGAAGGGTTCGGGTATAGGTACGGGTATGTTGAAAG
TGGGTTCGTATCCGTGGACTAATCAGATGCTGTT**GTGGCAGAGAAGCG**
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CCAATGTATTACAATGGATGGTACCATTTCTTCTACCAATACAACCC
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TCCTACGACGCAGACGGTGTATGGACTGGTTCAGCCACAATTTTACCCG
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GTTCAAAACATCGCAGTTCCAGCAAATTTGTCCGATCCACTGCTGCTAGA
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GTCGGTGCTGACGACTTCAGAGACCCCAGCACTGCATGGTTTGAACCCT
CCGACTCCACATGGCGCGTCACCATTGGTTCAAAAGACGCATCCAATAG
CGGTATCGCTCTAGTGTACAGTACCAAGGACTTCAAAACCTTCACGCTCC
TTCCCAACACTTTACACGCAGTTGCAAAAGTAGGTATGTGGGAGTGTAT
CGATTTCTACCCTATTGCCACCTCTGAGGCCGAAGCAAACAAGGGCCTG
GACCTGTCCGAAGGCCCGAGTTTGAAAACAAAGCATGTGCTTAAGGCTA
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GAGGTTAAGCAAAGAAGGATTTTATGGGGTTGGGTTAAGGAGGCCGATA
GTGAGAGCACCGATGTTACCAAAGGCTGGGCTTCCCTTCAGGGAATTCC

TCGAACAGTGTTGTACGATTTGAACACGAAGACACACCTATTAACATGG
CCAGTAGAGGAGGTGGAGAGTTTAAGAACAGAACACAGGGATTTCAGC
GGAATTACAGTGGATGCTGGGAAAACTATGGAGCTCAATGTAGGTGGTG
CTGCACAGTTGGATGTTGAAGTTGAATTTACAATCGAGGAAAAGGCGTT
AGAATTGGCGATTGAAGAAGACGTTGAGTATGAATGCAACACAAGTAGC
GGTGCAGCACAGCGTGGACTGCTTGGACCGTTTGGATTGCTTGTGCTTGC
CAATGGGGATTTGACTGAACAGACTGCTACTTATTTCTATGTTAGCAAGA
AATCAGACGGCAGTTTCGTTACTCATTTTTGTCAAGATGAATTAAGATCT
TCCAAAGCTAGTGACACTATTACTGAAATCGTTGGGCATACCGTTCCTGT
TCTTAATGGAGAATCCTTCACACTAAGAGTGCTGATTGATCACTCAATCG
TGGAAAGTTTTGCACAAGGAGGGAGAGCTAGTATGACGTCTAGAGTGTA
TCCAACAGAGGCAATTTACAACGATGCACGCTTGTTTGTCTTCAATAATG
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TPS

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CTAGTTCTTGTAAAGTAGTAGACTCCATTGTGTTTCTCAAAGGAAATGTC
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GCACCTAGCAAATTCGACGAAGTTCTTGCAAATGGATGGCTTGATGC
CAT**GCAATCTTCTTCACCGCCTCGTAAGA**GGTCTCTTAGCAAAGATTT

GTCCTTTGGATCCGATTCTGATGAAGCAGATTCTGCTTATAGTGCATGGA
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TCCTATTGTTGATAATCCTGATCGCGCTTTTATGTCCAACGCGATGAGGA
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AGAATATCCCAAGTTGAAACTAACTCATGGCCGGAAGGTTTTAGAAATC
CGACCAATCATTGATTGGAACAAAGGCAAAGCTGTGGAATTTTTGCTTG
AATCTCTTAAATTGAACGATCGTGATGATGTGCTCCCCATATATATTGGA
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GAGGCTATGGCATATTAGTATCTTCCACTTCAAAGGACACAAATGCTTCG
TTTTCTTTAAAAGATCCTTCAGAGGTTAAGGAATTTTTGAAATCATTGGT
GAAATGGCAGAAAACAAAGGCAAACTTTGAATGAAGATGGCAATAGTT
TGGTGGCTGCATTTGTATACATAGTAACTATATCAGTCAGTTCTTTTTTTA
TGTGAAAAGATTTGTTGAATGATCCCATGTCTTGTTCTTTAGGTTAGAGT
TTGAAATTTTGGTCAAGGAGGTTAATAACTCAATGTGAATTCCTACTGTT
TATTATAATTTCATTTGTTGTATTTTTTGTACCTACAAAATTATATGGGTG

Appendix II. Primers for PCRs used to estimate the expression of genes of interest in onion.

Forward is top in each section and Reverse is below. Primer sequences all displayed 5' to 3'.

qRT-PCR primers obtained from literatures					
Gene	GenBank Accession	Forward (RT-FOR) and Reverse (RT-REV) primer sequences	Annealing temperature (°C)	Product size (bp)	Gene size (bp)
ALL (for primer checking)	L48614.1	TTCAGAAACTAAGAGAGAGGT CTTCCCATTCACACTTCACCC	53	160	1771
AcFKF1	GQ232754	AGGTCGCAATTGCCGATTCTTA AACGTGACCCGTTCATGGAAGT	60	815	2054
AcGI	GQ232756	CACAGATGGATTGCTTGTTGATG ATTGGCTACGAGATGAACTGCTC	61	94	3555
AcCOL2	KY012331	ATTGATCAGGTGCTGAAGGGATTG AATCGTCACCATTAAACTACACTGAAA	65	143	1323
AcFT1	KC485348	AAACCATCACAAATAACTCAGCA GTTTCTCGCCCAAAGTTCG	56	185	726
AcFT4	KC485351	TGAAATAGGAGGTGTACCAAGAAT TTCCGAAACTACCATCCATATTTG	60	143	539
AcFT5	KC485352	GAAATTGGAGGACGCGAC CTTGCATCTTTTGCTTCTGGTA	60	137	604

qRT-PCR primers obtained from literatures					
Gene	GenBank Accession	Forward (RT-FOR) and Reverse (RT-REV) primer sequences	Annealing temperature (ºC)	Product size (bp)	Gene size (bp)
AcFT6	KC485353	TCGTCAATCGATGGTTATAAATCA TTTCCATAACTTGCATCGACTGT	60	180	841
AcLFY	JX275963	AGCGTGCTATCAACCGATAGTAGTGA AGCTTAGTCGGAACATACCAAATGGA	60	108	1119

q-PCR primers designed from onion RNA-seq transcriptome database					
Gene	sseqid	Forward (RT-FOR) and Reverse (RT-REV) primer sequences	Annealing temperature (ºC)	Product size (bp)	Gene size (bp)
DFS	gi|460408989|ref|XP_004249923.1|	ATCCACCACCAAAGCCCTCTTTAGTG AGCGAAGGTATGCGTGTATCCAAGTC	58	101	518
LFS	gi|226504278|ref|NP_001151021.1|	TTATTATCCAGCGCCACCAGCTTCTC GAAGGTGAGGCCAATGTTGTTGGTTG	59	107	815
GCL	gi|255537467|ref|XP_002509800.1|	GGGTGATGCTCAGGTTGTTCTTCT CCAAATCTCCCAACTCCTCAACAC	60	110	1696
MADS	gi|357115238|ref|XP_003559398.1|	TCAGCATCGCAAAGAACAGAAATC CAAAAGTCAATCAATGGGGAGAGG	58	138	1116

q-PCR primers designed from onion RNA-seq transcriptome database					
Gene	sseqid	Forward (RT-FOR) and Reverse (RT-REV) primer sequences	Annealing temperature (°C)	Product size (bp)	Gene size (bp)
POLYCOMB	gi|359490057|ref|XP_002281679.2|	TACTGTCAGTCATCCTCCCTGAAG GTGGGACCCAGGTACAACCAAC	60	188	4838
PER12	gi|470108362|ref|XP_004290489.1|	GCTTTTAAGGCCGTTTTTCTGAGG CATTTCCATGATTGCTTCGTTCAG	58	122	1356
FFS-1	gi|514742652|ref|XP_004960197.1|	TGCCTGAACCCAACGTACCTTTTG TGCACCCGTCATTGCACCATG	58	93	337
FFS-2	gi|449523750|ref|XP_004168886.1|	TGGACTGGACTTGAGACTGCACTTTC TAATATTGTGGCTGACCCGCTCCAAC	58	81	1001
SST1-B1	gi|514707057|ref|XP_004951208.1|	ATGCCGGTGTGTCTACAAAAGGTACG GCAATGACAGCTTCTGGTTTGTCCAC	58	93	639
SST1-B2	gi|514707057|ref|XP_004951208.1|	GGAGCTCTTCGTGGCGCTTAAAAATG CGAGGCAATAAATTCTGCAGCTCGAG	58	141	2307
SST1-L	gi|514707057|ref|XP_004951208.1|	GTGGCAGAGAAGCGGGTTTCATTTTC ATGTTTCCCCATACTGCTGCTTCTGG	58	132	2250
TPS	gi|255539761|ref|XP_002510945.1|	TTGGTTCCATACTCCCCAACATCAGC TCTTACGAGGCGGTGAAGAAGATTGC	59	142	1782

Standard PCR primers designed from onion RNA-seq transcriptome database					
Gene	sseqid	Forward (ST-FOR) and Reverse (ST-REV) primer sequences	Annealing temperature (°C)	Product size (bp)	Gene size (bp)
PHYA	gi|357115238|ref|XP_003559398.1|	AGAAACAGAACGCGAAACATAACACA CTCGCTTCTTCCTCTGTAGCTGGTAG	54	109	4183
TIC	gi|359479681|ref|XP_002272732.2|	TGGTGGTCTGTGCTTAGATCCAGTAA AGACACAGCCCTTCTTCAAATACAGC	56	99	4014
ALL	gi|255566734|ref|XP_002524351.1|	TCACCGAATGTTACCAGCAA TCACGATCTGTCTTGGCTTG	53	500	1771
SAUR	gi|356577485|ref|XP_003556855.1|	CAAAACCACCAACACCATCA CCTAGAACTTACCACAATCAAAACG	53	454	671
ECT	gi|460406228|ref|XP_004248569.1|	TGACGAATTCTTATTCAAGGCATA AGGAACGGGGACAACTCAC	55	342	358
GST	gi|449516399|ref|XP_004165234.1|	TCCCATTGAAAGAGCCAAAG ACAACAACCAAATGCACGAA	51	498	1143
RPL34	gi|449529828|ref|XP_004171900.1|	TATCGCAAGAGGCACAGCTA GCATGCGAAGATTTGTGATG	53	488	672
AHCYL1	gi|460404838|ref|XP_004247887.1|	TCTCTGAAATCGACCCCATC CTCGCCTTCTCATTCCAGAG	55	418	1134
TUBA2	gi|359496623|ref|XP_002263822.2|	ACCAGTGCCTCCACCTACTG GCTGGTATTCAGGTCGGAAA	55	405	956
H2A	gi|502081979|ref|XP_004487029.1|	AAGCGAAGAAGGGGACATCT CACCAAGCAATTTTCCCAAC	53	403	413

Appendix III. Additional data: Table from recently merged onion RNA-seq transcriptome with integrated expression patterns.

Name	all leaf vs. bulb.logFC	all leaf vs. bulb.PValue	bulb SD vs. LD.logFC	bulb SD vs. LD.PValue	leaf SD vs. LD.logFC	leaf SD vs. LD.PValue	LD leaf vs. bulb.logFC	LD leaf vs. bulb.PValue	SD leaf vs. bulb.logFC	SD leaf vs. bulb.PValue
LD-bulb-specific in separate data sheet										
DFS	-11.085	9.83E-16	-4.240	1.05E-15	NA	NA	-13.842	6.15E-65	-6.890	2.19E-09
GCL	-11.561	2.32E-14	-4.489	7.64E-29	NA	NA	-12.546	2.58E-52	-7.977	2.31E-05
MADS	NA	NA	-3.160	4.38E-15	NA	NA	NA	NA	NA	NA
PHYA	-3.100	1.10E-11	NA	NA	NA	NA	-2.322	8.45E-09	-4.462	3.06E-26
TIC	-9.174	3.23E-05	-10.398	7.93E-14	NA	NA	-10.228	9.31E-13	NA	NA
SD-bulb-specific in separate data sheet										
POLYCOMB	NA	NA	2.313	2.28E-07	9.248	2.89E-11	-7.870	4.91E-05	NA	NA
PER12	-3.998	2.78E-08	2.859	8.02E-10	NA	NA	NA	NA	-4.746	1.41E-23
Bulb-specific in separate data sheet										
ALL	-8.373	1.17E-25	NA	NA	NA	NA	-8.064	9.06E-20	-9.332	1.04E-85

Name	all leaf vs. bulb.logFC	all leaf vs. bulb.PValue	bulb SD vs. LD.logFC	bulb SD vs. LD.PValue	leaf SD vs. LD.logFC	leaf SD vs. LD.PValue	LD leaf vs. bulb.logFC	LD leaf vs. bulb.PValue	SD leaf vs. bulb.logFC	SD leaf vs. bulb.PValue
SAUR	-9.090	1.80E-69	NA	NA	NA	NA	-8.219	1.23E-51	-13.586	2.94E-74
ECT	-3.571	1.74E-31	NA	NA	NA	NA	-3.390	8.54E-26	-3.872	2.30E-35
GST	-2.687	1.42E-29	NA	NA	NA	NA	-2.467	2.61E-17	-2.966	4.93E-27
RPL34	-2.022	8.29E-21	NA	NA	NA	NA	-2.090	3.52E-13	NA	NA
AHCYL1	-2.158	7.46E-23	NA	NA	NA	NA	-2.355	8.83E-17	NA	NA
TUBA2	-3.078	4.56E-25	NA	NA	NA	NA	-3.091	1.14E-16	-3.086	6.44E-27
LFS	-4.162	1.35E-16	NA	NA	NA	NA	-4.315	1.57E-25	-3.846	2.16E-39
H2A	-3.688	2.70E-40	NA	NA	NA	NA	-3.310	1.02E-23	-4.107	1.79E-38
DFS	-3.378	1.28E-15	NA	NA	NA	NA	-3.785	1.62E-32	-2.613	2.71E-18
FFS-1	-5.065	1.54E-06	NA	NA	NA	NA	-6.876	0.002492948	-4.602	0.000373885
FFS-2	-2.435	1.13E-06	NA	NA	NA	NA	-2.511	0.000342731	-2.365	0.006870119
LFS	-4.162	1.35E-16	NA	NA	NA	NA	-4.315	1.57E-25	-3.846	2.16E-39

Name	**all leaf vs. bulb.logFC**	**all leaf vs. bulb.PValue**	**bulb SD vs. LD.logFC**	**bulb SD vs. LD.PValue**	**leaf SD vs. LD.logFC**	**leaf SD vs. LD.PValue**	**LD leaf vs. bulb.logFC**	**LD leaf vs. bulb.PValue**	**SD leaf vs. bulb.logFC**	**SD leaf vs. bulb.PValue**
SST-B1	7.364	1.78E-49	NA	NA	NA	NA	7.575	2.28E-30	7.233	2.20E-38
SST-B2	-3.081	2.26E-24	NA	NA	NA	NA	-3.207	1.05E-22	-2.943	1.43E-15
TPS	-5.703	0.008518127	NA	NA	NA	NA	NA	NA	-6.653	0.00428473
Leaf-specific in separate data sheet										
SFT-L	3.398	1.62E-14	NA	NA	NA	NA	4.216	4.42E-29	2.991	2.07E-24

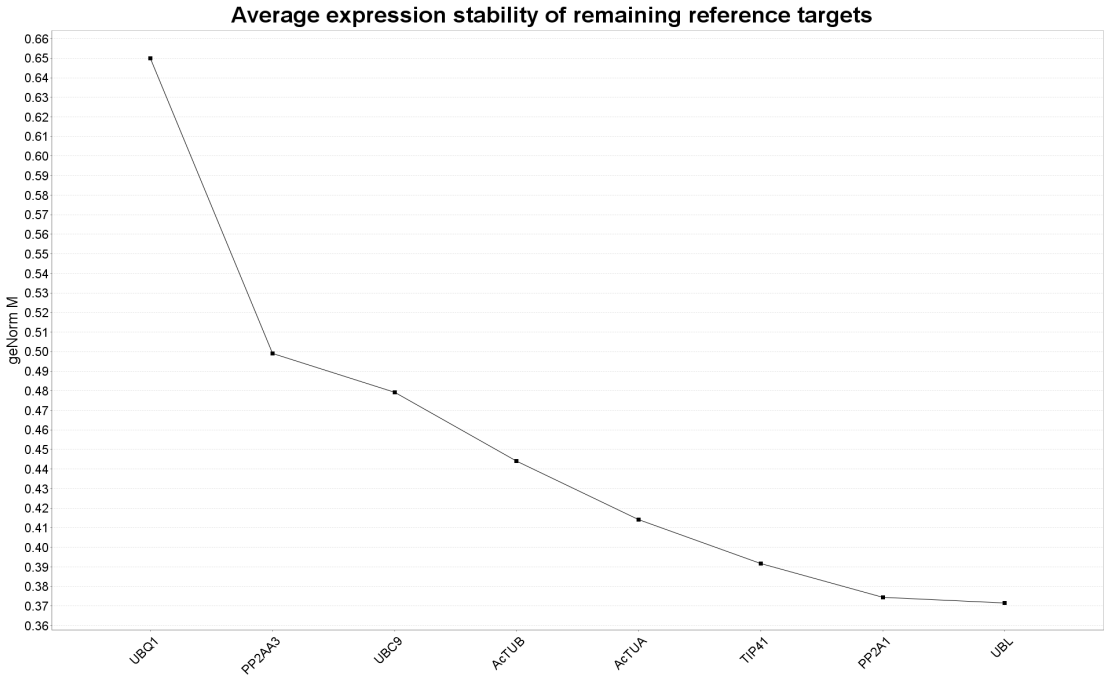
Appendix IV. qRT-PCR primers used to detect reference genes and their similarities with Renate F1 onion transcriptome sequence.

Forward is top in each section and Reverse is below. Primer sequences all displayed as 5' to 3'.

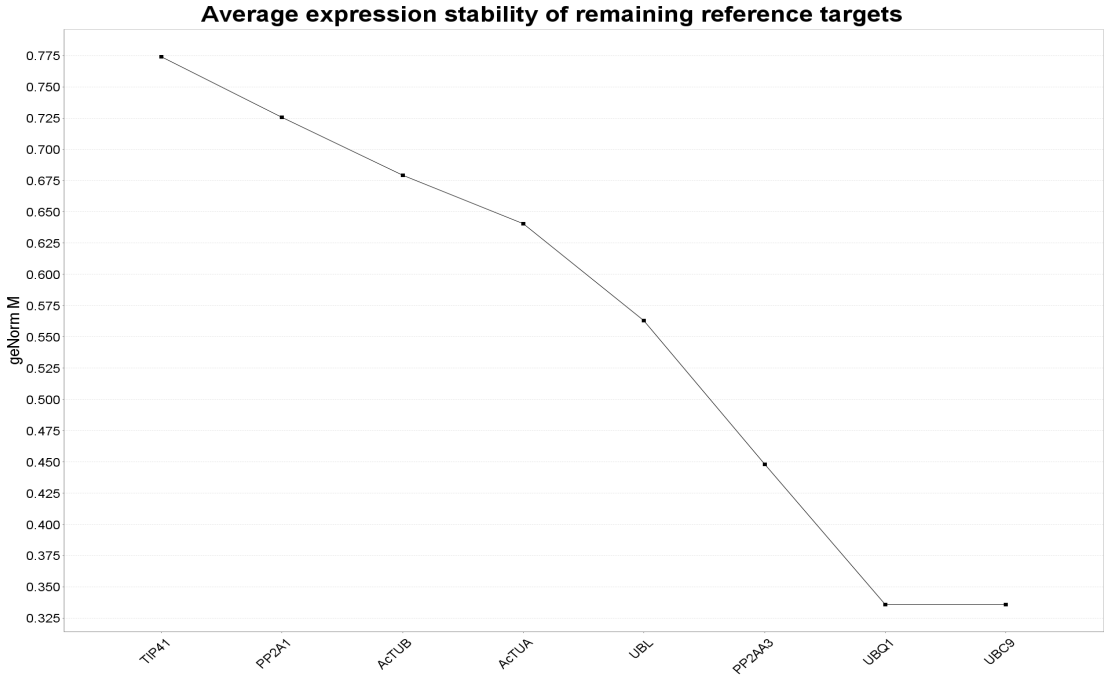
Gene	GenBank ID (*Renate F1* sequence)	GenBank ID (NCBI database sequence)	Forward (qRT-FOR) and Reverse (qRT-REV) primer sequences	Product size (bp)	Nucleotide sequence length (bp)		Similarity between NCBI database sequence and onion transcriptome sequence	
					NCBI Reference sequence	*Renate F1* transcriptome	% Nucleotide	% Amino acid
UBQ1	KY072872	AY059080.1	AATCAGCTAAGGCCGAAGAAGAAGAT TCAATTCAAGATGCAAAGTACACCAA	149	418	751	82	97.7
UBL	KY072874	NM129118.4	CTGTCCTTCATCTTGTGCTTGCTCT CAAGACCGAAACAAACCATCAAATTA	148	712	596	84	94.8
AcTUA	KY072876	BT000718.1	GATTACGAAGAAGTTGGAGCTGAAGG GCACTTCACCAGAACCCACATATACT	106	1646	1665	78	95.1
UBC 9	KY072873	AF325019.2	GAAGCTGGACCCAGAAGTATGCTATG TATAGGGAAGTAGCGAAGCAATGCAC	95	896	972	82	96.6
TIP-41	KY072882	NM119592.4	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
AcTUB	KY072875	AA451549.2	GTCTTCAGAGGCAAGATGAGCAC TCAGTCCAGTAGGAGGAATGTCG	138	1004	1728	98	100

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

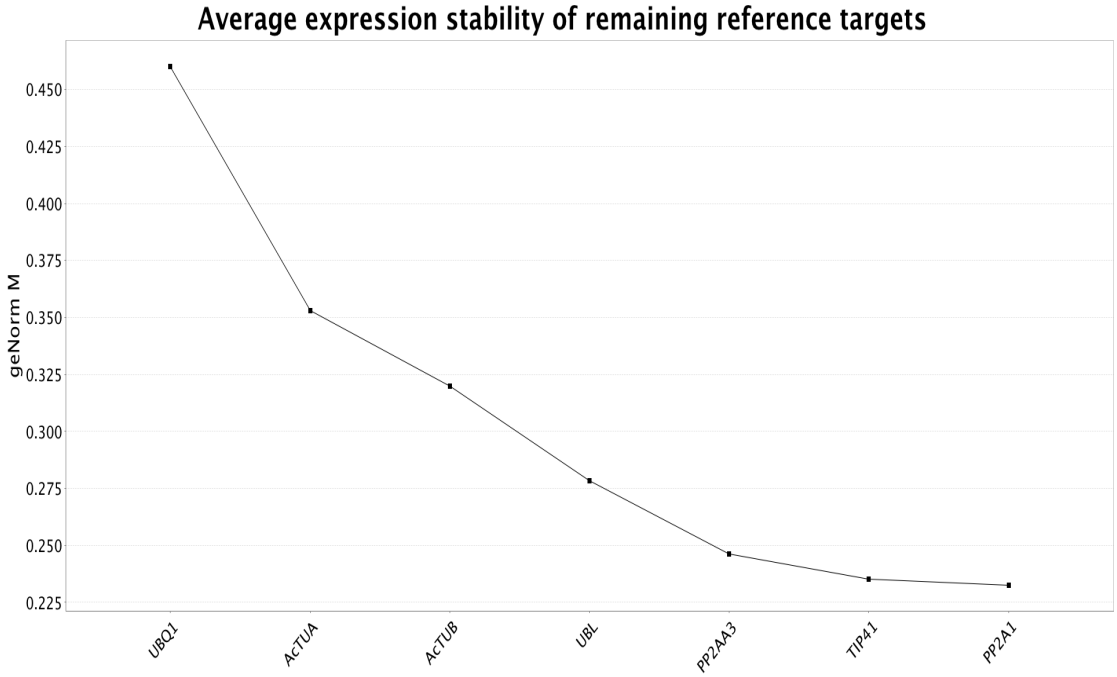
Chapter 3. Spatial gene expression in *Renate* leaves: *UBL*, *PP2A1* and *TIP41*



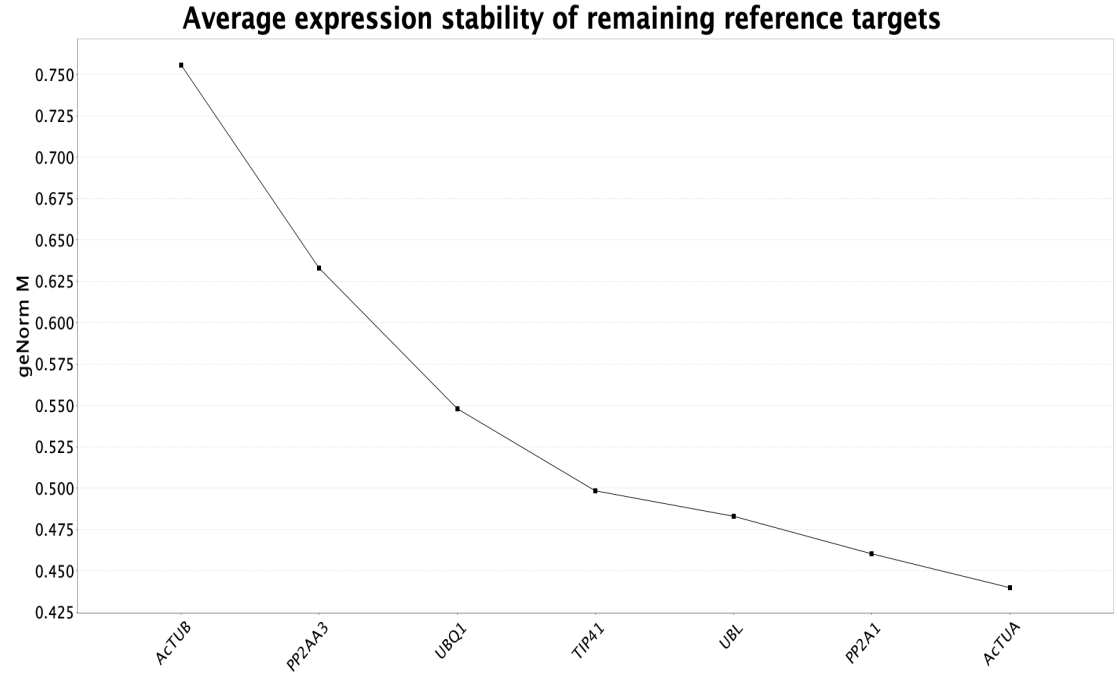
Chapter 4: Diurnal gene expression in *Renate* leaves under intermediate daylengths: *UBC9*, *UBQ1* and *PP2AA3*



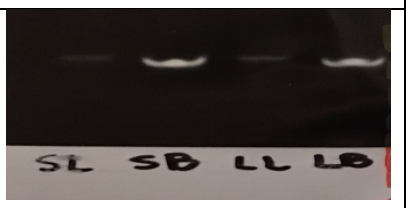
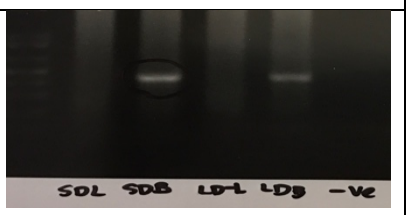
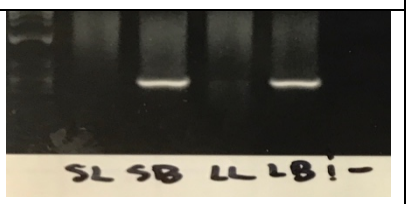
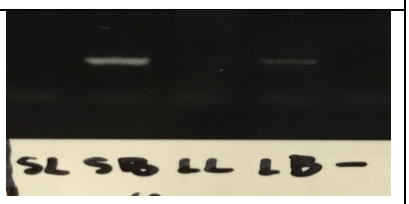
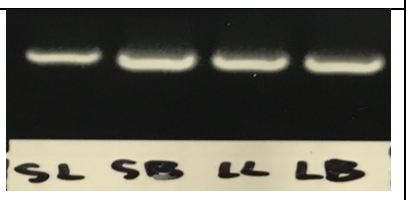
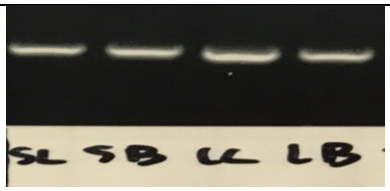
Chapter 4: Diurnal gene expression in *Hojem* leaves under intermediate daylengths: *PP2A1*, *TIP41* and *PP2AA3*



Chapter 5: Developmental gene expression in *Marco* leaf and bulb tissues during the development



Appendix VI. Differential expression of some genes of interest in the individual tissues of Renate onion using gel red stained agarose gel electrophoresis.

Gene	mRNA Expression				Gel Images
	SD-LEAF	SD-BULB	LD-LEAF	LD-BULB	
MLP	\	**	\	**	
LOC: uncharacterized protein	\	**	\	*	
U1: uncharacterized	\	**	\	**	
U2: uncharacterized	\	**	\	\	
U3: uncharacterized	**	***	**	**	
U4: uncharacterized	**	**	***	**	
U5: uncharacterized	*	***	**	**