IDENTIFYING GENES UNDERLYING ADAPTIVE TRAITS OF BULBING AND BOLTING IN ONION (*ALLIUM CEPA* L.)

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Abstract

Onion (*Allium cepa* L.) is a biennial plant and completes its life cycle in two growing seasons i.e., forms bulbs in the first year and flowering and seed production occurs in the second year. The poor adaptation of onion cultivars at different latitudes results in substantial losses due to reduced yield and precocious flowering. Onions form bulbs when grown under inductive daylength and an adequate amount of far-red light. Onion cultivars grown at particular latitudes require minimum critical day length to form a bulb.

At the physiological level, bulb initiation in temperate regions is regulated in a similar manner to the photoperiodic flowering of *Arabidopsis* (Mettananda and Fordham, 1997; Taylor, 2010; Lee *et al.*, 2013). The first aim of this study was to identify photoperiodic pathway genes responsible for the adaptation of onion under different daylength. The expression of photoperiodic pathways gene was measured under the long-day and short-day condition in DH2150 (long-day onion) and Albasile (short-day onion) over a 24 hours period using RT-qPCR. Two *FT* genes (*AcFT1* and *AcFT4*) regulate bulbing in long-day and short-day onions but respond differently under different daylength. Consistent with their proposed roles, the diurnal experiment suggested that *AcFT1* and *AcAP1* are only expressed in bulbing, whereas *AcFT4* is expressed in non-bulbing population. The photoreceptor, circadian clock, and output pathway genes follow diurnal expression patterns peaking at different times of the day and these genes were not altered in bulbing and non-bulbing populations indicating that they are not involved in the adaptation of two onion cultivars studied here.

To determine the functional role of genes is very challenging in bulb onion due to lack of an efficient genetic transformation method (Eady, *et al.*, 2000; McCallum, 2007). The second aim of this study was to develop an efficient and reliable protocol for generating transformed onion callus cells from shoot tip tissue of germinating seed. Callus was induced efficiently from the two onion cultivars: PLK (Pukekohe long keeper) and DH2107 using *Agrobacterium tumefaciens* strains GV3101 and EHA105.

Premature flowering before bulb development is an undesirable trait and reduces the quality of the bulbs. Baldwin *et al.*, (2014) found *AcBlt1* locus on chromosome 1 which is responsible for bolting. Khosa (2018) found *AcVRN1* gene is important for onion flowering. *AcVRN1* gene turns on by vernalization but optimum temperature for

vernalization varies. The third aim of this study was to develop a molecular marker for the *AcVRN1* gene to identify which onion cultivar are susceptible to bolting at early stage of onion development. TAIL-PCR was used to amplify the unknown promoter and intron 1 region. No polymorphism was found in the promoter and intron 1 region of *AcVRN1* gene in all the seven varieties studied here.

Overall this study will help to understand molecular and genetic difference between LD and SD onion cultivars, which is important for adapting new onion cultivars at different latitudes.

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List of Abbreviations

˚C	Degrees Celsius
µg	Micrograms
µL	Microliters
µM	Micromolar
µmol	Micromole
2,4-D	2,4-Dichlorophenoxyacetic acid
35S	35S promoter of the cauliflower mosaic virus
5'UTR	5' untranslated region
A	*Allium*
Ac	*Allium cepa*
AD	Arbitrary degenerate
AFLPs	Amplified fragment length polymorphisms
ALB	Albasile
AP1	*APETALA1*
At	*Arabidopsis thaliana*
Bar	*Bialaphos acetyltransferase*
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
C terminal	Carboxyl terminal
CCA1	*CIRCADIAN CLOCK ASSOCIATED1*
CDF1	*CYCLING DOF FACTOR1*
CDL	Critical Day Length
cDNA	Complementary deoxyribonucleic acid
CIM	Callus induction media
CO	*CONSTANS*
COP	*COP CONSTITUTIVELY PHOTOMORPHOGENIC*
ddH2O	Double-distilled water
DH	Double haploid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNP	Day-neutral Plant
dNTP	Deoxynucleotide triphosphate
eGFP	*Enhanced green fluorescent protein*

ER	Endoplasmic reticulum
FAC	Florigen activation complex
FAO	Food and Agricultural organization of the United Nations
FD	*FLOWERING LOCUS D*
FKF1	*FLAVIN-BINDING, KELCH REPEAT, F-BOX 1*
FLC	*FLC FLOWERING LOCUS C*
FR-HIR	Far-red high-irradiance response
FT	*FLOWERING LOCUS T*
g	Grams
gDNA	Genomic DNA
GFP	Green fluorescent protein
GI	*GIGANTEA*
GW	Gateway cloning
ID	Intermediate-day
ITS	Intergenic transcribed spacer
LAI	Leaf area index
LB	Lysogeny Broth
LD	Long-day
LDP	Long-day plant
LFR	Low fluence response
LUX	*LUX ARRYTHMO*
M	Molar
Mg	Milligrams
mL	Millilitre
mM	Millimolar
MS	Murashige and Skoog salt
Ng	Nanograms
NGS	Next generation sequencing
OCS	*octopine synthase*
OD	Optical density
PCR	Polymerase chain reaction
PEBP	Phosphatidylethanolamine binding protein
Pfr	Phytochrome far-red
PHR	Photolyase homology region
PHYA	*PHYTOCHROME A*
PHYB	*PHYTOCHROME B*

PHYC	*PHYTOCHROME C*
Pr	Phytochrome red
PRRs	PSEUDO-RESPONSE REGULATOR
Psi	Pound per square inch
QTL	Quantitative trait locus
RFLPs	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
RVR1	*REPRESSOR OF VERNALIZATION 1*
SAM	Shoot apical meristem
SD	Short-day
SDP	Short-day plant
SE	Somatic embryogenesis
SNPs	Single nucleotide polymorphisms
SOC1	*SUPPRESOR OF OVEREXPRESSION OF CONSTANS 1*
SP	Specific primer
SSRs	Simple sequence repeats
TAIL-PCR	Thermal Asymmetric Interlaced PCR
UV-B	Ultraviolet B
VLFR	Very low fluence response
VRN1	*VERNALIZATION* 1
ZT	Zeitgeber time

Chapter 1 Introduction

The Onion (*Allium cepa* L.), which is also known as a bulb onion, common onion, and garden onion is the most widely cultivated species of the genus *Allium*. It is a monocotyledonous bulbous plant, cultivated mainly as a biennial, but some wild onions exist as perennials (Platt, 2003). It is grown under a wide range of climates from tropical to temperate and is of great economic importance (Brewster, 1994; McCallum *et al.*, 2001). Onions are varying in size, shape, colour, and flavour. The most common types of onion are red, yellow and white. The taste of these vegetables can range from sweet and juicy to sharp, pungent and spicy. It is one of the most consumed and cultivated vegetable crops in the world (Brewster, 1994).

Molecular and genetic studies aimed at onion improvement have been limited. This thesis is focus on understanding the molecular genetics of adaptive traits. Onion bulb initiation is daylength-dependent, which places a significant barrier for adapting new cultivar at different latitudes. Investigating the genes responsible for the adaptation of onion under different daylength will help understand the basis of the difference, which is essential for adapting new onion cultivars at different latitudes. Premature flowering is a major problem during bulb production and results in crops loses. Developing molecular markers to bolting genes will help breeder eliminate the susceptibility to bolting in new cultivars.

1.1 Classification, Origin and Distribution of Onion

The botanical classification of the genus *Allium* has been revised using molecular techniques (Friesen *et al*., 2006) and the genus occupies the following taxonomic classification shown in Table 1.1. The Friesen *et al.*, (2006) classification is built on differences in the nucleic acid base sequence from the intergenic transcribed spacer (ITS) region of the ribosome DNA from the nucleus of 195 *Allium* species and five species from closely related genera.

Table 1.1 Classification of *Allium cepa*

Class	Monocotyledones
Order	Asparagales
Family	Alliaceae
Genus	*Allium*
Species	*A. cepa* L.

Asparagales is the second most economically important order of monocot, which comes after Poales (which includes cereal crops; Brewster, 2008). Economically important families in the order Asparagales includes the Alliaceae (chive, garlic, shallots, leek, and onion), Amaryllidaceae (snowdrop, snowflake, daffodil, and yucca), and Asparagaceae (asparagus) with *Allium cepa* being the most important species. The genus *Allium* consists of about 780 species (Friesen *et al.*, 2006). Other important members of the genus A*llium* (Figure 1.1) include garlic (*A. sativum* L.), shallot (*A. cepa* L. var. aggregatum), chive (*A. schoenoprasum* L.), leek (*A. ampeloprasum* L.) and Japanese bunching onion (*A. fistulosum*).

Figure 1.1 Important species in genus *Allium*. Onion, garlic, chives, leek and Japanese bunching onion.

The onion has been cultivated for more than 4700 years. The onion was first domesticated in the mountainous regions of Turkmenistan and north Iran, so south-west Asia is the primary centre of domestication and variability (Hanelt, 1990; Fritsch and Friesen, 2002). Other centres of great diversity, like the Mediterranean, are considered secondary centres of domestication (Hanelt, 1990; Fritsch and Friesen, 2002). Onions were introduced into different countries through travel and trade and become adapted to local condition to which they were carried (Brewster, 1994).

Cultivated *A. cepa* divided into two distinct horticultural groups, the common onion group and the *Aggregatum* group (Hanelt, 1990; Brewster, 2008). The common onion group contains most of the vast bulk of the economically important varieties. These form single bulbs and are mostly grown from seed. The bulb of the *Aggregatum* group is smaller than the common onion because they rapidly divide and form laterals, hence forming a cluster of bulbs (Brewster, 1994). The *Aggregatum* group is usually vegetatively propagated. Varieties grown for salad onions and as small bulbs for pickling are mainly from this group.

Onions are distributed widely through the temperate, tropical and sub-tropical regions of the world (Brewster, 2008). A lot of onion cultivars have been developed for size, shape, colour, pungency, storability, resistance to pests and diseases, and climatic adaptation (Griffiths *et al.*, 2002).

1.2 Importance

Onion is mainly used both, in the green and mature stage for salad and spice in a variety of flavoured dishes and soups (Craig, 2005; Brewster, 2008). Some of the economic, nutritional and, health importance of onions is described in detail in section 1.2.1 and 1.2.2.

1.2.1 Economic importance

Out of 15 vegetables listed by the Food and Agricultural organization of the United Nations (FAO), onion ranks second next to tomato in terms of total annual world production (Pathak, 2000). World production of onions in 2017 was 97.86 million tonnes from 5.2 million hectares of land (FAOSTAT, 2019). China is the major producer of bulb onions followed by India, the USA, Iran, Egypt and Poland (Figure 1.2A; FAO 2019). Bulb Onion is the most important crop in New Zealand, and it is the highest export earning vegetable crops (Figure 1.2B). Major onions growing region in New Zealand are Pukekohe, Waikato, Hawkes Bay and Canterbury (Onions New Zealand, 2019). New Zealand onion is exported to the Netherlands, China, Mexico, India, the USA and Spain (Onions New Zealand, 2019). New Zealand export approximately 85% of the total onion produce. The quality of New Zealand onions allows exporters to secure good prices in the international market (Onions New Zealand, 2019).

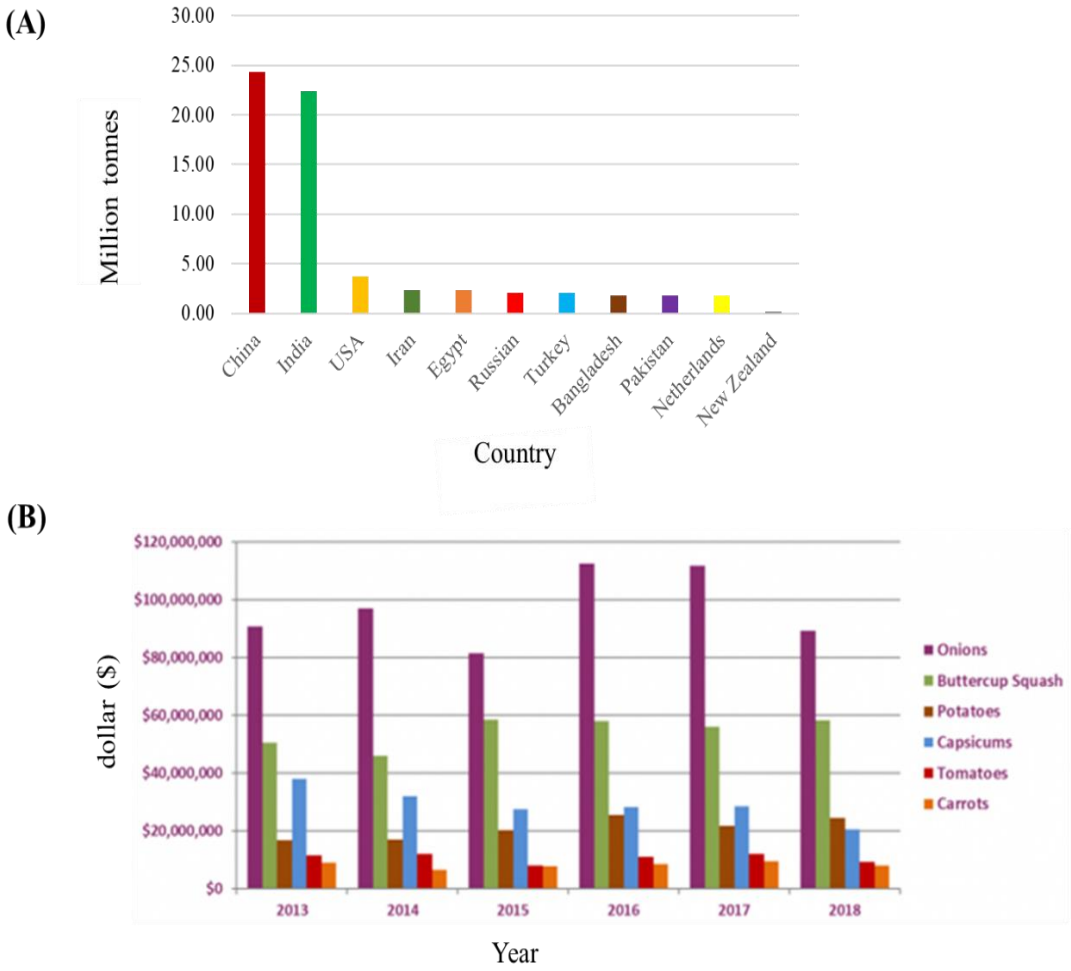

Figure 1.2 Production and export value of bulb onion. (A) Leading onion producing countries in the world in 2017(FAO, 2019) (B) New Zealand fresh vegetable export value from the year 2013 to 2018 (Onions New Zealand, 2019).

1.2.2 Nutritional and health importance

Onions are valued by people around the world for their health and nutritional values. Many health benefits of onions and other *Allium* species are due to flavonoids, fructans, and organo-sulphur compounds (Block, 1992; Koch and Lawson, 1996). The most important sulphur containing compounds are the amino acid cysteine and its derivatives, especially the S-substituted cysteine sulphoxides and glutamyl peptides (Keusgen, 2002). Various studies showed that bulb onion has antimicrobial, antioxidant, antifungal, and antidiabetic properties (Keusgen, 2002; Suleria *et al.*, 2015). Despite the benefits of onion to humans, they are toxic to cattle, cats, and dogs, and to a lesser extent, sheep and goats (Cope, 2005).

1.3 Growth and life cycle of onion

An onion plant is composed of leaves which arise alternately from a small flattened stem (bulb). The older leaves are on the outside and younger leaves on the inside of the stem (Lancaster *et al.*, 1996). Each leaf is composed of a photosynthetic leaf blade and a non-photosynthetic leaf sheath (Brewster, 1990). Onion bulb consists of a flattened stem with the disc at the base of a plant, which is known as basal plate and it acts as a shoot apical meristem (SAM). During the growth of the plant, leaf blade formation in leaves ceases and develops into swollen bladeless bulb scales to form the central storage tissues of the bulb (Garner and Allard, 1920; Heath, 1945). The outer leaf sheaths form outer dry protective skins as the bulb reach maturity and at this stage the false stem (pseudo-stem) becomes hollow and leaves start to fall, leading to the neck fall stage which is used as an indicator of maturity (Brewster, 2008). Mature onion bulbs can range in size from 5 mm to over 100 mm bulb diameter. Roots are initiated in the stem near the base of young leaves and push downwards through the outer layers of the stem disc to emerge (Brewster, 2008). The basic structure of an onion bulb is shown in Figure 1.3.

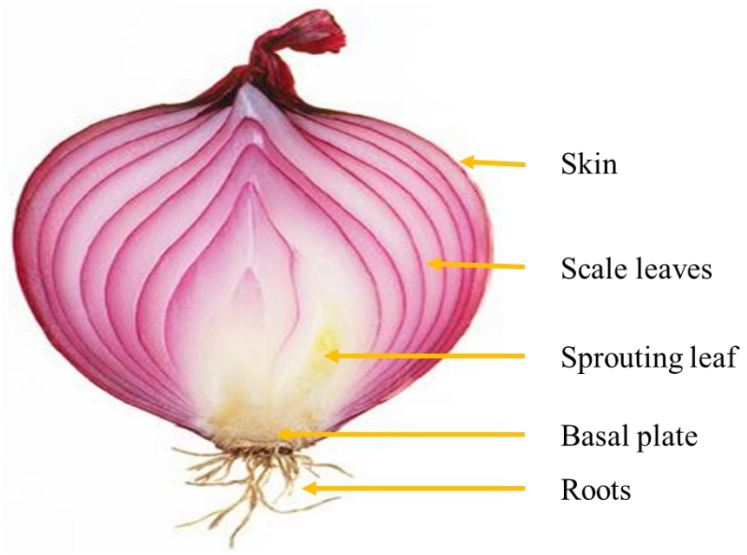

Figure 1.3 Cross section of an onion bulb. (Adapted from Rashid, 2016).

Onion has a complex life cycle compared to many other crops. Bulb onion is a biennial crop which forms a bulb during the first year and flowers in the following years (Figure 1.4; Brewster, 2008). The onion life cycle can be divided into three main stages: (1) Seedling, (2) Bulb, and (3) Flower (Bosch Serra and Casanova, 1979; Brewster, 1990).

During the first phase, onion seed will start to germinate after one or two weeks of sowing (Brewster, 1994). During the germination stage, the primary root starts to grow downwards while the cotyledon pushes upward through the soil surface as a loop or a hook and this stage is referred to as the loop stage. After few days of germination, the seedling/sets are planted in the spring and the plants undergo a juvenile phase of growth during which plants will not bulb regardless of being exposed to the inductive condition of bulbing. Onion required an inductive photoperiod to initiate and complete bulbing (Brewster, 2008). When the onion plant became mature and the daylength has reached a critical length, bulb formation, which is promoted by long-day (LD) during summer, begins (Lee *et al.*, 2013). The onion plant then overwinters as a bulb and during this time, if the environment is favourable, flowering is induced in response to prolonged cold temperature during winter, a process called vernalisation. The onion plant then flowers and sets seed during the spring/summer of its second year of growth and thus its life cycle is completed (Brewster, 1994; Lee *et al.*, 2013).

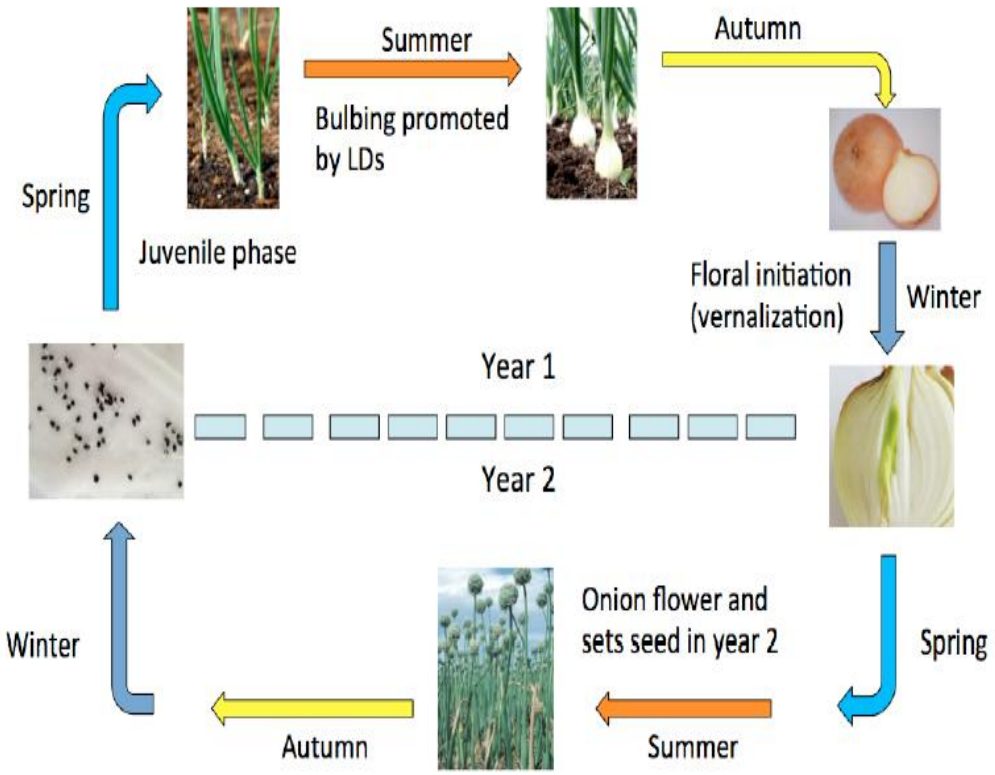

Figure 1.4 Life cycle of temperate onion. Onion produce bulb during the first year under inductive daylength. In the second year, onion bulb produces flower and seed, once the vernalization requirement fulfilled (Brewster, 1994).

1.4 Genetics of onion

Onion is a diploid plant, (2n=16) with large genome size (16,415 Mbp; Kuhl, *et al.,* 2004; McCallum, 2001), approximately equal to hexaploidy wheat and 6, 36, and 121 times larger than maize, rice, and *Arabidopsis* respectively. Genetic study of onion has been limited due to its large genome size, high inbreeding depression, cross-pollinated nature, biennial life cycle, a high percentage of repetitive DNA and small research community (Kuhl *et al.,* 2004; McCallum *et al.,* 2006; McCallum 2007; Duangjit *et al.,* 2013). Improving the onion genetic resources are important to promote germplasm conservation and to increase crop productivity, quality, resistance to disease and adaptability (McCallum, 2007). With the help of next-generation sequencing technologies, a large quantity of genomic resources have been created in bulb onion that is providing new insights into the genetic and molecular basis of important economic traits (Brewster, 2008; Baldwin *et al.,* 2012a, *b;* Duangiit, *et al.,* 2013; Lee *et al.,* 2013; Kim, *et al.,* 2014; Finkers, *et al.,* 2015).

1.5 Classification of onion

Bulb onion is classified into three categories based on critical day length (CDL) required for bulbing (Currah and Proctor, 1990; Brewster, 2008). This classification gives an indication of the latitude where a specific variety can be grown (Magruder and Allard 1937; Currah and Proctor 1990; Brewster 2008).

(1) Short-day (SD) onion: SD onions are cultivated in tropical regions and require a minimum of 10 hours of daylength for bulb formation and are well adapted to low latitudes between 30^0 north and south from the equator.

(2) Intermediate-day onion (ID): ID onions require at least 12 to 14 hours of the daylength and are cultivated in the area between 30^0 to 45^0 latitudes.

(3) Long-day (LD) onion: LD onions are cultivated in temperate regions where bulb formation occurs when the daylength reaches at least sixteen hours per day and is well adapted to higher latitudes between 45^0 to 60^0 latitude.

1.6 Bulbing in onion

Daylength, light quality and quantity, and temperature influence bulb formation in onions. Onion has to pass the juvenile phase before the plant can respond to daylength to initiate bulbing. The physiology of bulb initiation has been studied extensively. It is a process which is photoperiodically driven in temperate onions, drawing parallels with the photoperiodic control of flowering in *Arabidopsis* and other plant species (Mettananda and Fordham 1997; Thomas *et al.,* 2006; Taylor *et al.,* 2010). Both these processes are stimulated by LD, signal perception is in the leaf, signal transported via phloem and response is at the shoot apex. *Arabidopsis* flowering and onion bulb formation can be compared in terms of the involvement of phytochrome, and both processes are promoted by far-red light (Brewster, 1977). Flowering and bulbing can be compared in terms of the existence of a juvenile phase, and during both processes, initiation will not occur irrespective of being exposed to favourable environmental conditions till this phase has been passed (Massiah, 2007; Rashid, 2016).

1.7 Factors involved in the bulbing process of onion

1.7.1 Daylength

Daylength is defined as the daily duration (number of hours) of light, a plant is exposed to (Denisen, 1979). Garner and Allard (1920) first time showed that onions develop bulbs in response to long photoperiods. Onion leaves have to be exposed continuously to bulb-inductive photoperiods in order to complete bulbing, if bulbing plants are transferred to short, non-inductive photoperiods then leaf production can start again and bulbing is arrested, even in plants at an advanced stage of bulb development (Brewster, 2008). Bulb onion is originated as a long-day plant, but it shows extensive variation from 10 to 16 hours for the critical day length required to induce bulbing in different onion cultivars (Magruder and Allard, 1937; Currah and Proctor, 1990; Brewster, 2008). Cultivars adapted to lower latitudes require shorter photoperiod to initiate bulbing than those adapted to higher latitudes. Cultivars from higher latitudes do not form bulb under lower latitude and move on producing leaf blades (Magruder and Allard, 1937). In opposition, cultivars from lower latitudes when sown in spring at high latitudes start bulbing when the plants are small due to the long photoperiods, and plant produce small bulbs (Magruder and Allard, 1937).

1.7.2 Light quality and quantity

Light quality is an important factor for bulb development in onion (Austin, 1972). Onions require far-red light to initiate bulbing under inductive daylength (Austin, 1972; Lercari, 1982a, b; Bertaud, 1986; Mondal *et al.,* 1986). When far-red light is low or absent during bulb development, bulbing is arrested even under inductive daylength (Mondal *et al.,* 1986). In addition to quality, light quantity also affects bulb formation and high irradiance accelerates bulbing in LD onion (Sobeih and Wright, 1986). Bulb initiation in daylength varies with the quality of light received by the plants and high levels of far-red light accelerate bulbing. Bulb maturity is also associated with leaf area index (LAI). Light interception is affected by higher density plantation. Study shows that any agronomic practices, like high plant density, early sowing and the use of starter fertilizer that tends to increase LAI will also speedup bulb maturity date (Brewster *et al.,* 1992). At the physiological level, bulb initiation in LD onion is regulated in a similar way to the photoperiodic regulation of flowering in *Arabidopsis* (Taylor *et al.,* 2010). Various pathways controlling flowering time in *Arabidopsis* is described in the next section.

1.8 Pathways controlling flowering time in model plant *Arabidopsis*

The transition from vegetative growth (producing stems and leaves) to reproductive (producing flowers) state is the most crucial and complex developmental process in the life cycle of flowering plants. The correct timing of this transition is crucial to maximizing the reproductive success of plants. The timing of flowering is widely regulated by endogenous (plant age, sugar metabolism, and hormones level) and exogenous (daylength, temperature, cold and light intensity) signals through complex regulatory networks (Murfet, 1977; Higgins *et al.,* 2010; Abou-Elwafa *et al.,* 2011; Romera-Branchat *et al.,* 2014).

Arabidopsis thaliana has been used as a model plant over the years and there is extensive progress in the physiology, genetics and molecular mechanism of flowering regulation (Koornneef and Meinke, 2010; Zhang *et al.,* 2014; Blumel *et al.,* 2015). The different molecular and genetic studies of *Arabidopsis* have identified six major regulatory pathways viz., vernalization, photoperiod, gibberellin (GA), temperature, age and autonomous pathways to regulate flowering (Figure 1.5; He and Amasion, 2005;

Fornara *et al.*, 2010; Zhang *et al.*, 2014). However, this research project focuses on the photoperiodic pathway. Our understanding of photoperiodic flowering mechanisms in *Arabidopsis* has greatly assisted our understanding of these mechanisms in major crops (wheat, barley, and rice; Bevan *et al.*, 2017).

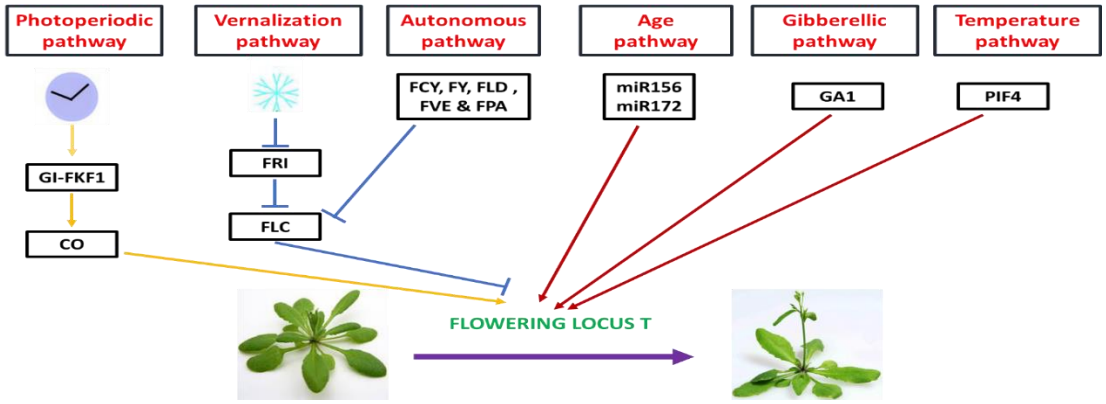

Figure 1.5 Pathways controlling flowering time in *Arabidopsis*. The several genes play a role in the different pathways and measure change in plant internal factors (age and hormones) and environmental cues to control flowering. Lines with arrows denote up-regulation of gene expression and lines with bars denote repression of gene expression.

1.9 The Photoperiod Response Pathway

Photoperiodism is a physiological process which allows plants to detect seasonal changes by measuring fluctuations in daylength over the seasons to regulate different development processes (Garner and Allard, 1920). The photoperiod is the amount of light and darkness in a daily cycle of 24 hours. Day length and night length (12 hours light and 12 hours dark) are equal throughout the year at the equator (zero latitudes). As we move away from the equator toward either side of the poles, the days become longer in summer and shorter during winter. Seasonal changes in daylength are consistent from year to year thus, many plant species have evolved the ability to detect these seasonal changes in daylength, and their specific photoperiodic responses are strongly influenced by the latitude in which they originated (Taiz and Zeiger, 2010; Thomas and Vince-Prue, 1996). Photoperiod is a reliable indicator of the time of year, much more reliable than the temperature which also shows seasonal variation but is far less predictable due to climate change (Jackson, 2009).

German plant physiologist Erwin Bunning (1936), proposed an internal timekeeping mechanism separated each day into 12-hours periods. He suggested that circadian rhythm consists of two phases, first 12-hour period, beginning at dawn, was called the photophile ("light requiring") phase and the second 12-hour period was called skotophile ("dark-requiring") phase. Flowering is induced in a long-day plant (LDP), when the light period is longer than 12 hours, such that light is still present at the begging of the skotophile phase. In the short-day plant (SDP), flowering induced if the light remains in the photophile phase (Figure 1.6A). This model is known as Bunning's hypothesis. Later, Colin Pittendrigh and Dorothea Minis (1964) proposed a model known as the external coincidence model, that was based on Bunning's model but modified in two keyways (Figure 1.6B). They proposed the occurrence of two factors: substrate and enzyme. Substrate levels oscillate throughout the day that induces a photoperiodic response when it is processed and an enzyme, which is active only under the light. When the peak of the substrate coincides with the presence of the active enzyme, the photoperiodic response is triggered. Second, the circadian clock regulates the timing of the substrate peak, the timing of this peak changes depending on daylength throughout the year. Further, the molecular mechanism of photoperiodism in different plants strongly supports the external coincidence model to regulate photoperiodic flowering, bud cessation and tuberization (Song *et al.,* 2015; Blumel *et al.,* 2015).

Garner and Allard (1920) clearly demonstrated that daylength plays a vital role in flowering regulation and classified various plants into three categories viz, long-day plant (LDP), short-day plant (SDP) and day-neutral plant (DNP). LDP flower when the day length exceeds the critical daylength (CDL); SDP flower when the daylength is shorter than the CDL and, DNP does not respond to daylength. Thus, CDL is the point at which the daylength switches from being noninductive to inductive and the value of the CDL varies significantly among species and among plants within the same species (Jackson, 2009). Plants can measure time by means of an endogenous time-keeping mechanism called circadian clock, which is described in more detail in section 1.9.1.

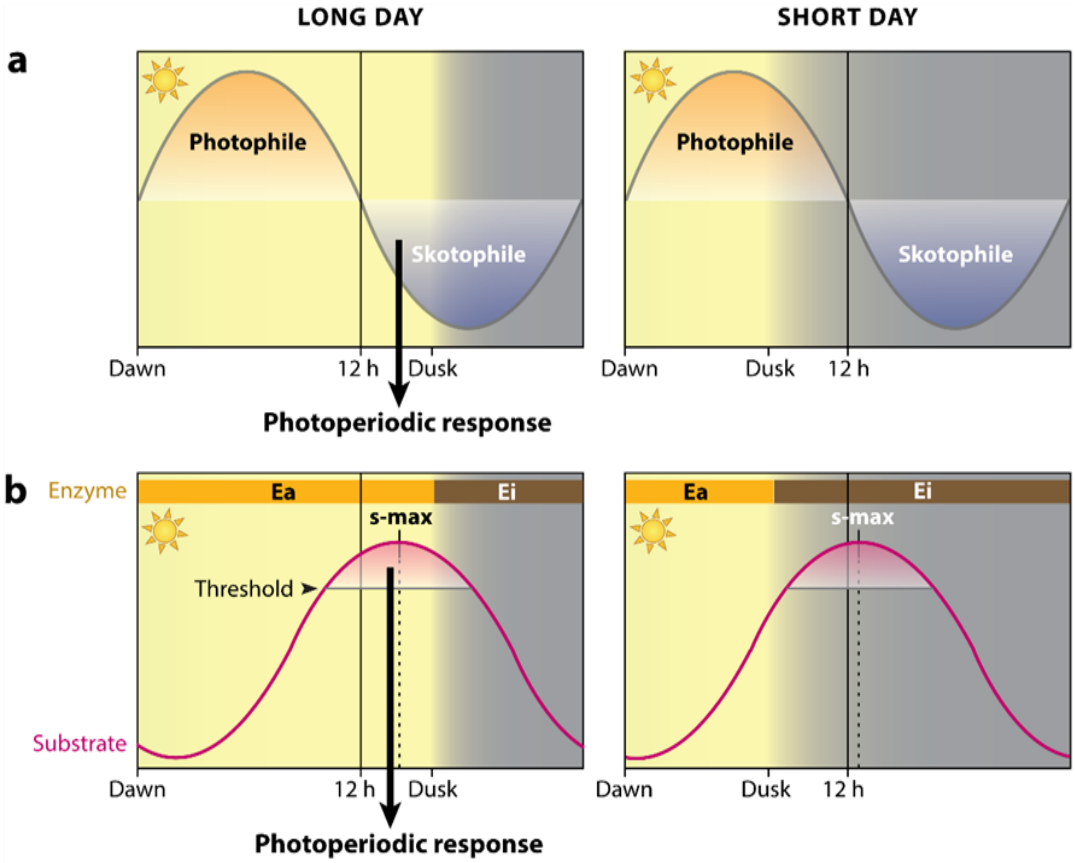

Figure 1.6 Models of photoperiodic response. (a) Bunning's hypothesis. In this model, organism possesses 12 hours photophile and 12 hours skotophile phase, which is characterized by an internal oscillator. When daylight lengthens into the skotophile phase, the photoperiodic response occurs in LD plants but repress in SD plants. (b) External coincidence model. According to this model, the enzyme is present throughout the day and the light triggers enzyme to convert from inactive form (Ei) to active form (Ea) via the circadian clock. The photoperiodic responses only occurred when the peak of enzyme activity coincides with a maximum activity of the substrate (red dotted line; Taken from Song *et al.*, 2015).

1.9.1 Circadian Clock

Photoperiodic response model in previous section evaluates plant needs to determine the time of the day to regulate various biological process. In a plant, the time of the day (the daily light and dark period) is measured by the circadian clock. The circadian clock is an endogenous timekeeping mechanism that allows organisms to anticipate and prepare for daily and seasonal changes in the surrounding environment. The clock uses environmental signals, such as temperature and light to entrain the clock (Green *et al.*, 2002; Inoue *et al.*, 2018). In plants, the clock regulates a wide variety of biological processes (Figure 1.7), including hypocotyl and root growth (Yazdanbakhsh *et*

al., 2011), flowering time (Imaizumi *et al.*, 2005; Johansson and Staiger, 2015), sugar metabolism (Dodd *et al.*, 2015), photosynthesis (Webb and Satake, 2015), nutrient homeostasis (Haydon *et al.*, 2015), hormonal signaling and immunity (Bolouri Moghaddam and Van den Ende, 2013).

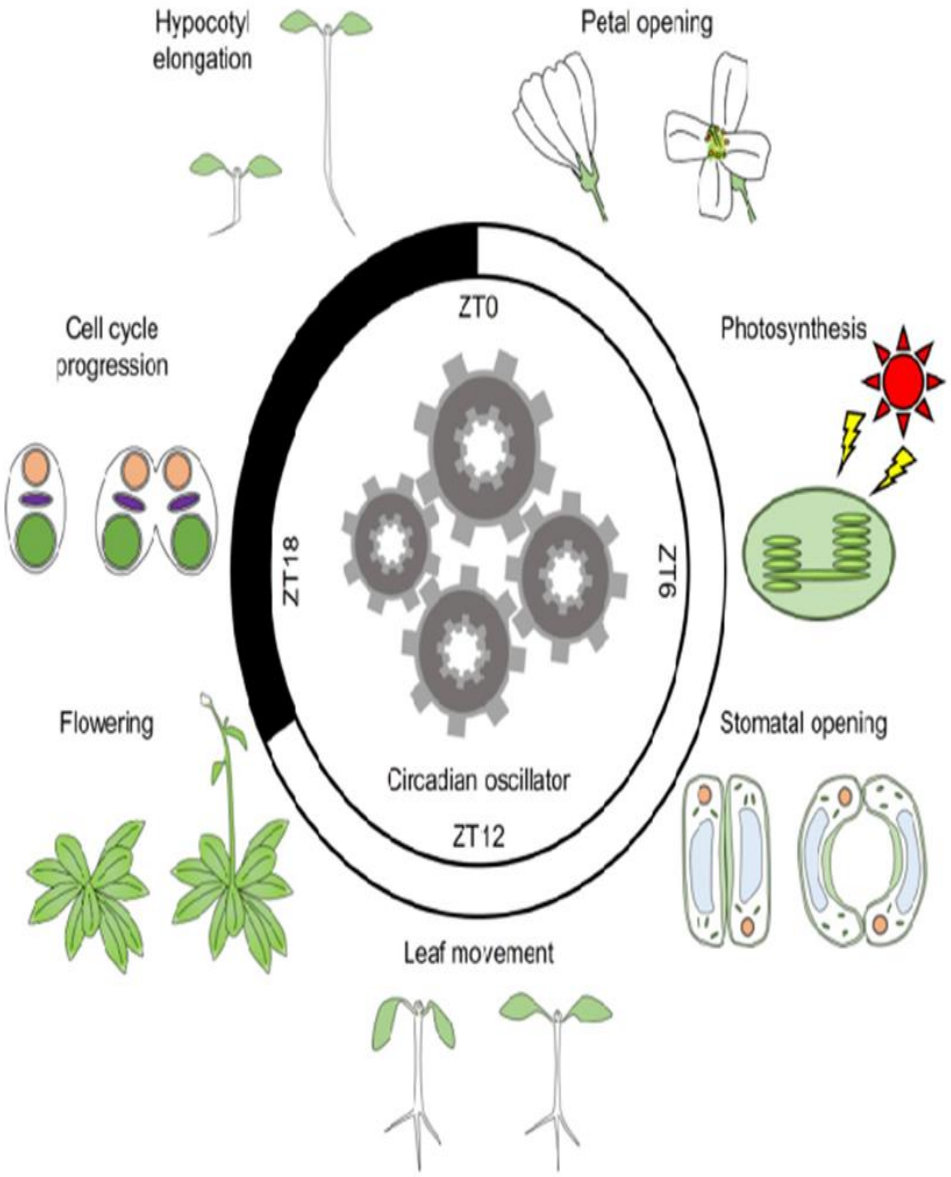

Figure 1.7 The circadian clock in plants. The clock regulates various biological processes. The circadian clock adjusts the timing of output responses, such as petal opening, photosynthesis, stomatal opening, leaf movement, flowering, cell cycle progression, and hypocotyl elongation to an appropriate time of day (taken from Inoue *et al.*, 2018).

1.9.2 Light input to the circadian clock via photoreceptors

Plants use sunlight not only for photosynthesis but also as a signal to regulate a broad range of developmental and physiological responses throughout their life cycle (Franklin *et al.,* 2005; Kami *et al.,* 2010). Light plays an important role in resetting the circadian clock and is also needed to convert an inactive form of enzyme into an active form to regulate the various biological process. Plants have evolved multiple photoreceptors to sense the surrounding environment precisely (Kong and Okajima 2016). Light input to the circadian clock in plants is mainly through phytochromes, cryptochromes, and ZEITLUPE (ZTL) family proteins.

Phytochromes are one of the most important photoreceptors that perceive and respond to the red and far-red light spectrum (Bae and Choi, 2008). Red light activates phytochromes by converting the inactive Pr (phytochrome red) form to the active Pfr (phytochrome far-red) form, whereas far-red light inactivates phytochromes by converting the Pfr form back to the Pr form. The inactive Pr form resides in cytosol, whereas the active Pfr form of all phytochromes translocated into the nucleus (Van Buskirk *et al.,* 2012). Phytochrome has N terminal domains act as a photosensory region and C terminal domains involved in dimerization and signal transduction.

In *Arabidopsis*, there are five phytochromes designated phyA to phyE. (Quail *et al.,* 1995). These phytochromes are encoded by five members of the phytochrome gene family and they are classified into two groups according to their stability in light (Sharrock and Quail, 1989). phyA is light liable phytochrome (type I) and phyB to phy E is light stable phytochrome (type II). Contrasting to the dicots, monocots contain three different phytochromes designated as phyA, phyB, and phyC (Mathews and Sharrock, 1997).

In *Arabidopsis*, photomorphogenesis responses are categorized into three types: Very Low Fluence Response (VLFR) and Far-Red High-Irradiance Response (FR-HIR; Schafer and Bowler, 2002), that is mainly mediated by phyA and Low Fluence Response (LFR) that is mainly ensured by phyB (Casal *et al.,* 2014; Possart *et al.,* 2014).

Cryptochromes absorb a blue and ultraviolet spectrum of light to regulate seedling, de-etiolation, cotyledon opening and expansion, anthocyanin accumulation, circadian clock and photoperiodic flowering (Cashmore *et al.,* 1999). *Arabidopsis* has two

cryptochromes; cry1 and cry2. The light stable cry 1 regulates germination, seedling de-etiolation, and seed dormancy, whereas cry 2, a light liable cry2 is involved in leaf senescence and photoperiodic flowering (Cashmore *et al.,* 1999; Lin and Shalitin, 2003; Yu *et al.,* 2010). The structure of the cryptochromes are related to DNA photolyases, but they do not possess DNA photolyase activity (Sancar, 2003). Cryptochromes have an N-terminal photolyase homology region (PHR) domain and C-terminal domain (Lin and Shalitin, 2003).

1.9.3 Central oscillator

Central oscillator incorporates different environmental signal through different auto-regulatory transcriptional and translational feedback loops (McClung, 2014). Genes involved in the central oscillator expressed at a different time of the day and night to regulate output pathway gene (Sanchez and Kay, 2016; Figure 1.8). In *Arabidopsis*, the central oscillator consists of CCA (CIRCADIAN CLOCK ASSOCIATED1) and LHY (LATE ELONGATED HYPOCOTYL), a MYB transcription factors, which directly bind to the promoters and repress the transcription of the evening phased genes (Wang *et al.,* 1997; Kamioka *et al.,* 2016).

The CCA/LHY dependent repression is partly mediated by the co-repressor complex CONSTITUTIVE PHOTOMORPHOGENIC10(COP10), DE-ETIOLATED1(DET1) and DAMAGED DNA BINDING1(DDB1; Endo *et a*l., 2014). During mid-day, PRR9, PRR7, and PRR5 directly repress the expression of *CCA1* and *LHY* (Matsushika *et al.,* 2000). In the evening, *EARLY FLOWERING3 (ELF3*), *EARLY FLOWERING4* (*ELF4*), and *LUX ARRYTHMO* (*LUX*) genes are upregulated and form a complex known as the evening complex and repress the *PRRs* (Nusinow, *et al.,* 2011; Chow, *et al.,* 2012). During the night, *TOC1* is expressed and directly represses expression of *CCA1* and *LHY* or indirectly promotes the expression of *CCA1* and *LHY* by inhibiting the repressive *PSEUDO-RESPONSE REGULATOR* (*PRR*) genes by binding their promoters (Gendron *et al.,* 2012; Huang *et al.,* 2012). FKF1, ZTL, and LKP2 degrade the TOC protein that leads to the upregulation of *CCA1*/*LHY* the next morning (Baudry *et al.,* 2010). These feedback loops create repeated outputs to regulate the activity of the different output genes.

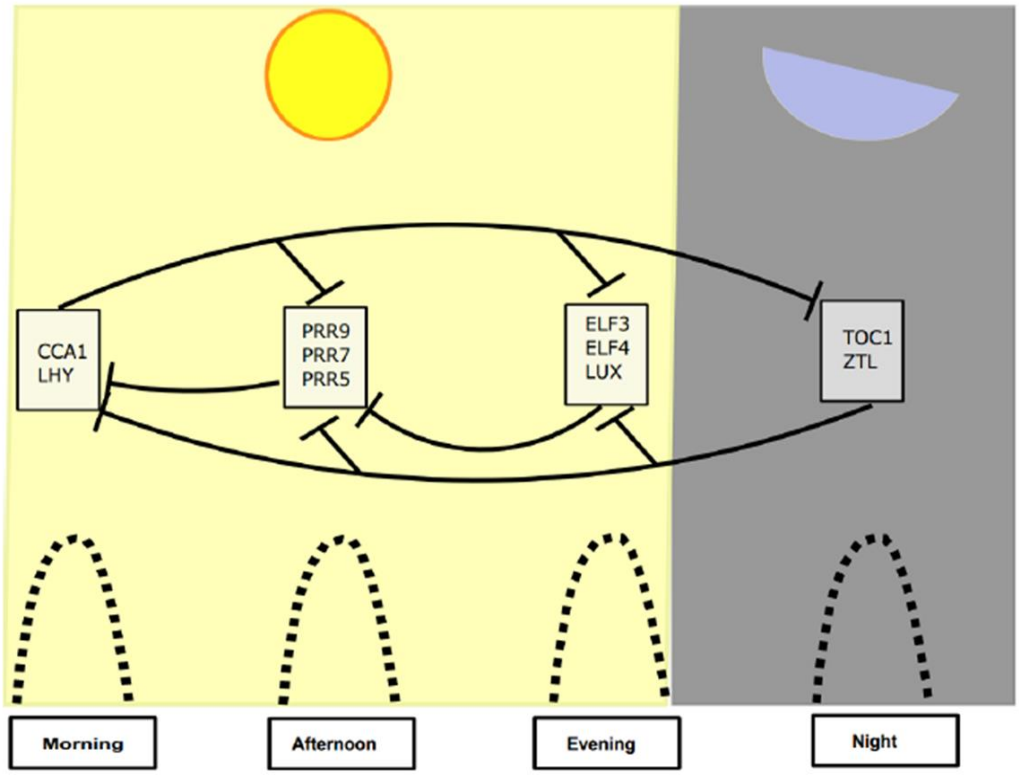

Figure 1.8 Regulation of the circadian clock central oscillator in *Arabidopsis* thaliana under LD condition. The different circadian clock genes are expressed during the specific time of the day and interact with each other to form interconnected loops and regulate the activity of different output genes (Taken from Khosa, 2018).

1.10 *FT* and its activation via the photoperiodic pathways

The photoperiodic flowering mechanism induced by *FT* expression is most well characterized in the model plant, *Arabidopsis thaliana*. In *Arabidopsis*, high levels of *FT* expression induce under long-day conditions that consequently accelerate flowering, whereas short-day conditions lead to very low levels of *FT* expression (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). The daylength dependent induction of *FT* is regulated by the B-box transcriptional regulator *CONSTANS* (*CO*; Samach *et al.*, 2000; Valverde *et al.*, 2004; Song *et al.*, 2012). In SD, CO mRNA accumulates only during the night time, but in LD, CO mRNA accumulates during the late afternoon to early evening via the circadian clock, thus coinciding with light mainly in the summer, when the CO protein is stabilized, and *FT* expression occurs. This LD specific peak of CO mRNA is depending on the activity of *GIGANTEA* (*GI*) as well as *FLAVIN KELCH F BOX* (*FKF1*) gene.

Transcription of *FKF1* and *GI* is regulated by the circadian clock and FKF1-GI interact when plants are exposed to light, and thus this complex only form and stabilizes FKF1 under LD (Fowler *et al.,* 1999; Park, *et al.,* 1999; Nelson *et al.,* 2000). Daylength dependent differences in CO transcript abundance under the light are tightly associated with the amount of the FKF1-GI complex. This FKF-GI complex is responsible for the degradation of a *CYCLINGDOF FACTOR* (*CDF*), repressors of CO transcript. (Imaizumi *et al.,* 2005; Sawa *et al.,* 2007; Fornara *et al.,* 2009).

1.11 *FT* signalling

FT is the major component of the floral signal molecule, florigen (Andres and Coupland, 2012). Under the inductive condition, FT mRNA is expressed in the companion cells of the phloem and its protein transported to the phloem sieve elements from where it is transported to the shoot apical meristem (Figure 1.9; Mathieu, *et al.,* 2007; Tamaki, *et al.,* 2007). FT protein is unloaded into the shoot meristem and interact with 14-3-3 proteins and a bZIP transcription factor called FD (Wigge *et al.,* 2005). The complex referred to as florigen activation complex (FAC), translocated to the nucleus and induces the expression of *APETALA1* (*AP1*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) to initiate flowering (Abe *et al.,* 2005; Wigge *et al.,* 2005).

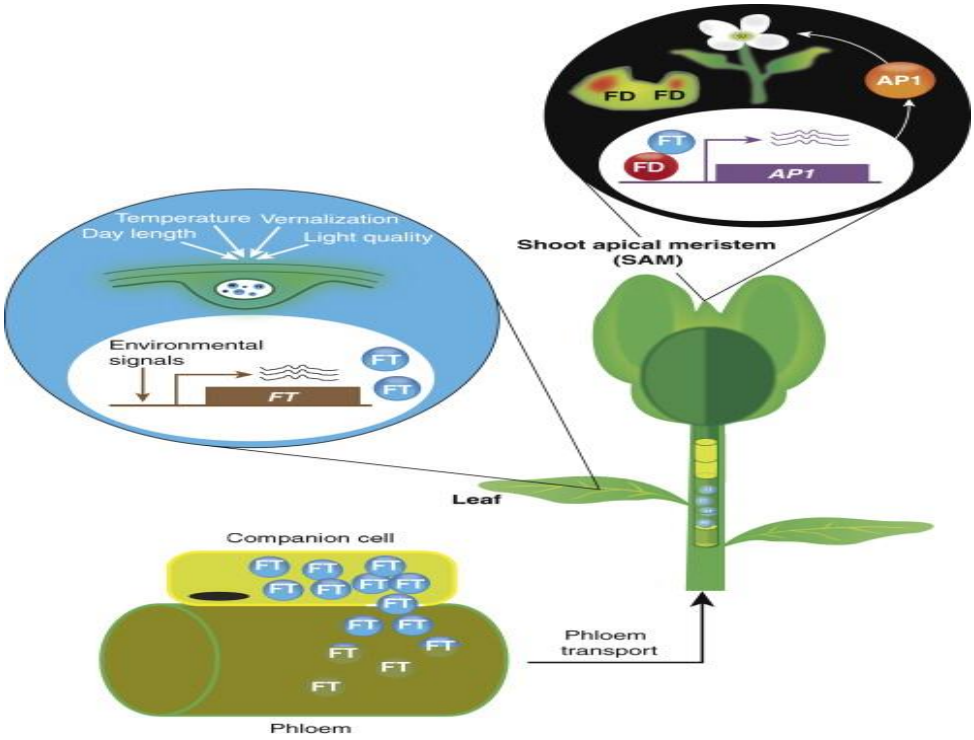

Figure 1.9 *FT* signalling system in plants. *FT* is expressed in the leaves and FT protein transported to SAM through the phloem. At the SAM, FT interacts with the FD, bZIP transcription factor and activate floral meristem identity genes such as *APETALA1* (*AP1;* Taken from Wigge, 2011).

1.12 Molecular basis of bulbing and flowering in onions

In recent time, the availability of transcriptomic resources in onion allows the researcher to identify genes involved in different pathways (Taylor *et al.,* 2010; Lee *et al.,* 2013; Khosa *et al.,* 2016). Several studies have revealed that FTs perform multiple roles in plant development (Pin and Nilsson, 2012; Navarro *et al.,* 2015). Bulbing and flowering in onion is regulated by the *FLOWERING LOCUS T* (*FT*) genes. Our laboratory identified six *FT-like* genes (*AcFT1* to *AcFT6*) in a DH2150 and found that two *FTs* (*AcFT1* and *AcFT4*) regulate bulbing; *AcFT4* suppress bulb formation whereas *AcFT1* promotes bulbing (Lee *et al.*, 2013; Figure 1.10). Our laboratory further revealed that the *AcFT2* gene responded to the cold and was upregulated by vernalization. Tagashira and Kaneta (2015) opined that the expression levels of *AcFT4*, *AcFT5* and *AcFT6* goes up as it got closer to a condition in long days in association with the onion bulbing. Manoharan *et al.,* (2016) identified eight *FT-like* genes, which were identical reported by our laboratory (Lee *et al.*, 2013). Dalvi *et al.*, (2016) stated that the *AcFT6* expression level in SD onion was high during the bulb initiation stage and indicated that it might be

involved in bulb initiation. Taylor *et al.*, (2010) explored the genetics underlying onion flower and bulb development and identified *GI* and *FKF1*, key circadian clock genes, which exhibit diurnal expression patterns similar to other plants. Khosa (2018) showed that photoreceptor and circadian clock genes are conserved in bulb onion and other *Allium* species. It is important to investigate whether these photoreceptors and circadian clock genes play any functional role in photoperiodic bulbing in onion (Khosa, 2018).

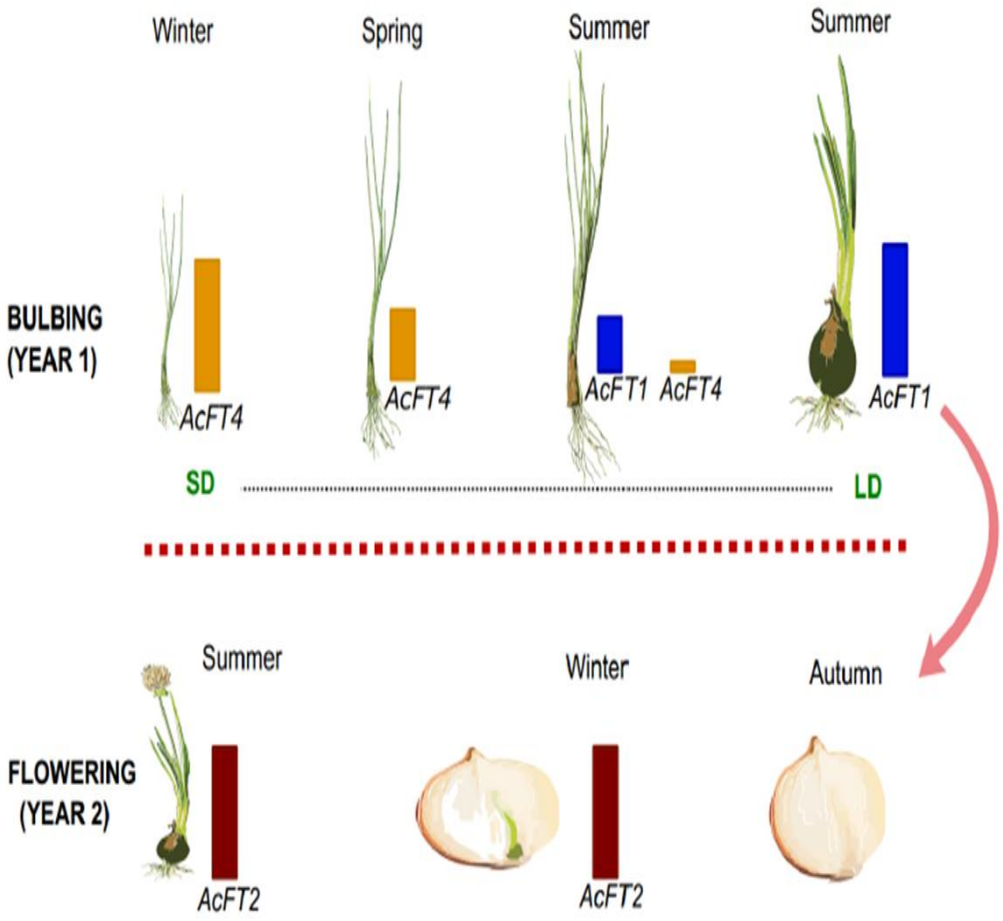

Figure 1.10 Role of *FT* genes in bulb onion life cycle. *AcFT4* prevents bulbing in young seedlings. Once plant pass the juvenile phase, under inductive daylength *AcFT4* is downregulated, and this leads to upregulation of *AcFT1* that promotes bulbing in onions during the first year. The bulb is an overwinter stage which is exposed to low temperatures during autumn and winter that leads to upregulation of *AcFT2* and promote flowering in the second year (Taken from Lee *et al.*, 2013, Khosa, 2018).

1.13 How onion adapted to different latitudes?

Adaptation is the special features which allow a plant to live in a particular place or locality. The onion was first domesticated in the south-west Asia and adapted to diverse geographic region (Hanelt, 1990; Fritsch and Friesen, 2002; Figure 1.11). Onions were introduced into different countries through travel and trade and become adapted to local conditions to which they were carried (Brewster, 1994). Photoperiodic pathway genes are conserved in a broad range of LD and SD plants, but few genes evolve opposite functions as *CO* which acts as an activator of flowering in LD plants but act as a repressor of flowering and tuberization in SD plants. Thus, circadian clock genes regulating *CO* stabilization also act differently in SD and LD plants (Romera-Branchat *et al.*, 2014; Blümel *et al.*, 2015; Song *et al.*, 2015).

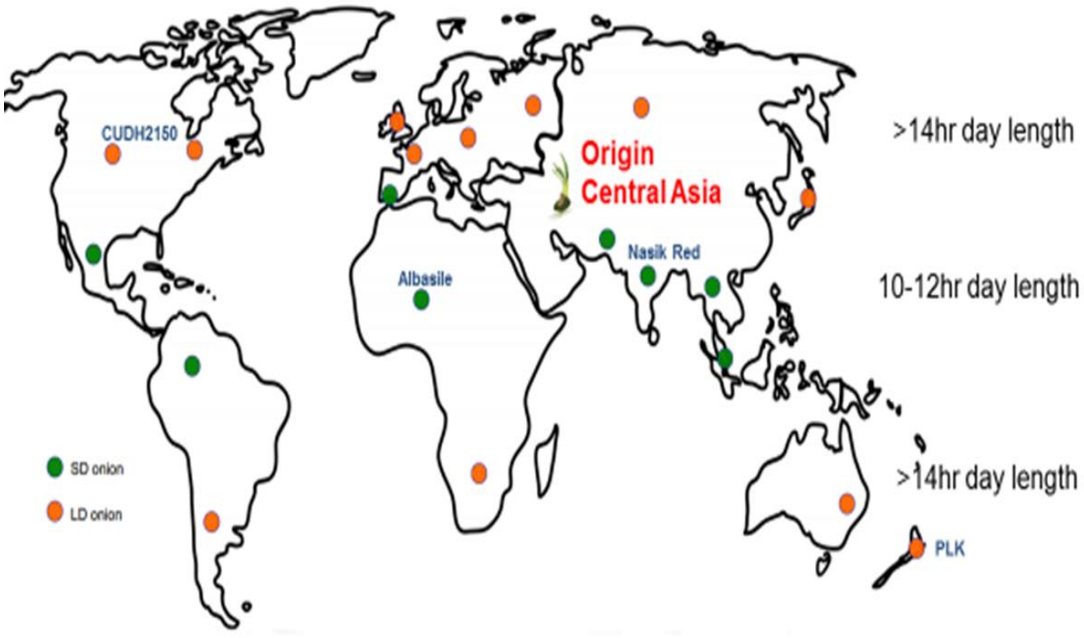

Figure 1.11 Adaption of onion cultivars to different latitudes. Onion originated in south-west Asia (shown by onion plant) and spread to different region. SD onions are grown at lower latitudes (indicated by green dots), whereas LD onions are grown at higher latitudes (indicated by orange dots; Taken from Khosa, 2018).

Greenham *et al.*, (2017) stated that *Arabidopsis* plants subjected to natural or artificial selection would exhibit variation in photoreceptor and circadian clock period at different latitudes. Photoreceptors sense the daylength and light quality and give input signals to the circadian clock to regulate output protein which active *FT1* gene. We hypothesized that photoreceptor and circadian clock genes are altered between LD and SD onion and

involved for the adaptation of onion to different latitudes. The proposed photoperiodic bulbing pathway model shown in Figure 1.12.

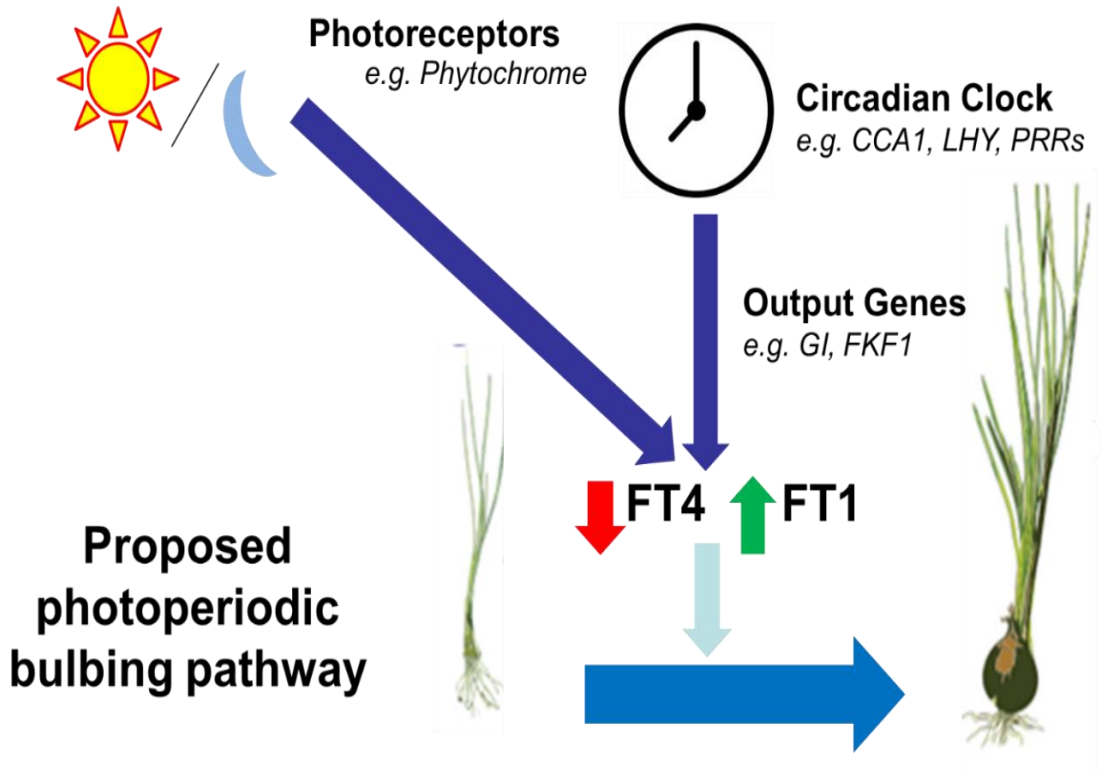

Figure 1.12 Proposed photoperiodic bulbing pathway. Plant sense the light through PHYTOCHROME (PHY) and interacts with the circadian clock and output genes to regulated *FT* expression.

1.14 Onion flowering

In onion, flowering is referred as bolting and inflorescence is known as umbel. Flowering is an essential prerequisite for seed production in onion. Flowering in onions occur in the second year once onion bulb meets its vernalization (exposure to low temperature) requirement.

1.14.1 Vernalization pathway in *Arabidopsis*

In many plant species (winter annuals and biennials), flowering is promoted by a period of exposure to low temperature through a process known as vernalization. Vernalization requirement prevents flowering in the fall season prior to winter but allows flowering the following spring. *A. thaliana* is a much-studied model plant for vernalization. Some

ecotypes (varieties) called ‘winter annuals’ have delayed flowering without vernalization (Figure 1.13), whereas others ‘summer annual’ do not (Sung *et al.*, 2006). In winter-annual *Arabidopsis*, the vernalization requirement is largely due to allelic variation at *FRIGIDA* (*FRI*) and its downstream target *FLOWERING LOCUS C* (*FLC*); winter accessions have dominant alleles of both genes (Michaels and Amasino, 1999; Sheldon *et al.*, 1999; Johanson *et al.*, 2000). In the absence of active *FRI* allele, *FLC* expression level is reduced and plants do not require vernalization for accelerated flowering. *FRI* is involved in activating *FLC*, and FLC represses flowering by preventing the transcription of *FT* in leaves and *SOC1* in the SAM (Michaels and Amasino, 1999; Sheldon *et al.*, 1999; Searle *et al.*, 2006). The expression of *FLC* is repressed by exposure to cold (Amasino, 2010).

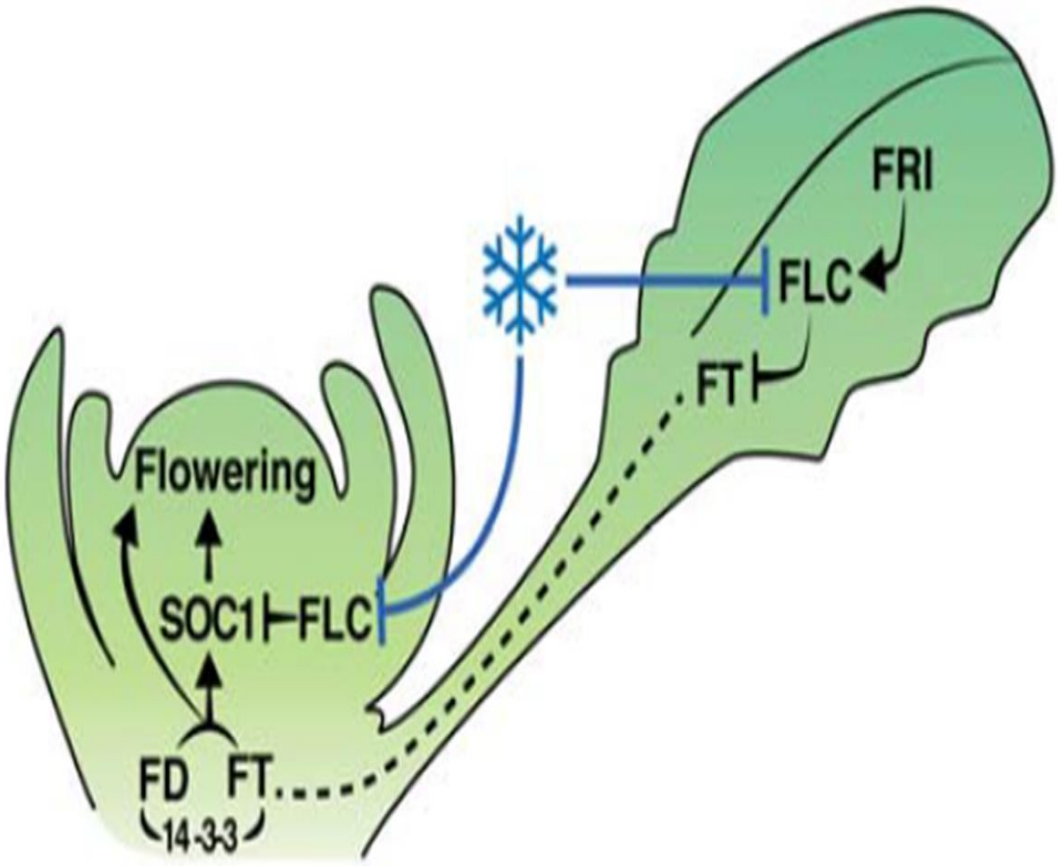

Figure 1.13 Schematic representation of the mechanisms governing vernalization in *A. thaliana*. *FRI* acts as a positive regulator of *FLC* and FLC act as a floral repression. *FLC* is expressed before cold exposure and its expression is repressed by vernalization. Pointed-headed arrows indicate activation, whereas horizontal headed arrows indicate repression. (Taken from Jansen *et al.*, 2007).

1.14.2 Physiology and molecular basis of vernalization in onion

Premature flowering or bolting before bulb development is an undesirable trait and strongly selected against by breeders during the adaption of onion germplasm. All the onions produce flowers in the spring. Physiology of flowering in onion is studied extensively and reviewed by Brewster (2008). Onion plant can start to initiate flowering once it passes the juvenile phase by exposure to low temperatures. There may be competition between the bulb and flower development depending upon the different onion cultivar, such that flower development may be suppressed in the first year (Van Kampen, 1970). Vernalization period is all that is needed to initiate flowering from onion bulbs. The optimum temperature for onion vernalization varies with cultivar; onion cultivars grown in the temperature climate require 3 to 4 °C, whereas cultivars grown in tropical climate require 10-21 °C (Brewster, 2008). Time (number of days) required for vernalization also varies with the cultivar and it is in the range of approximately 20-40 days. The vernalization pathway is less conserved and evolved separately in grasses (cereal crops) and dicots (Greenup *et al.*, 2009). Though the extensive genetic studies of grasses are often assumed to generalise to all monocots, studies of adaptive trait genetics in other monocot clades are very limited (Baldwin *et al.*, 2014). Onion, which is in order Aspargales, diverged 122 million years ago from the monocot grasses (Janssen and Bremer 2004), thus the genetic mechanisms controlling cereal flowering may differ in the Aspargales.

The physiological basis of flowering and vernalization is well studied in bulb onion, but not much work has been done at the molecular level (Macknight *et al.*, 2016a). Our laboratory has identified that the *AcFT2* gene is involved in the vernalization responsive initiation of flowering (Lee *et al.*, 2013). The optimum temperature for onion vernalization varies with cultivars; onion cultivars grown in the temperature climate require 3 to 4 °C, whereas cultivars grown in tropical climate require 10-21 °C (Brewster, 2008).

Baldwin *et al.*, (2014) mapped a *Bolt1* locus responsible for premature bolting in onion on chromosome 1. *AcVRN1* gene turns on by vernalization but optimum temperature for vernalization varies; temperate cultivar requires 4 °C, whereas tropical cultivar requires 10-21 °C. In this research, one of aims is to investigate whether *Bolt1* locus and *AcVRN1*

gene are the same or not. If both are the same, then to develop markers to identify which onion cultivar bolt at 4° C and which one at 10-21 °C. For this, various LD, SD and F1 population (cross between SD x LD) will be used in this study.

1.15 Onion transformation

The genetic transformation method has become an important tool for modern crop improvement. *Agrobacterium-mediated* transformation of dicot plants is well established and widely used but monocot plants, on the other hand, are not the natural hosts of the A. *tumerfaciens* (Zheng *et al.,* 2001). But recently *Agrobacterium*-mediated transformation has been done in monocot plant using a specific strain of *Agrobacterium* (Hiei *et al.,* 1997; Li *et al.,* 1996; Park *et al.,* 1996). Onion is a biennial crop and it takes more time to improve by conventional breeding methods. Daylength sensitivity places a major barrier to onion breeding programmes as a trait in a particular daylength cultivar (LD or SD onion) cannot be transferred to another daylength cultivars by cross-breeding as the specific daylength response of the progeny will be unknown (Rashid, 2016). It is quite challenging to establish the functional role of genes in bulb onion due to a lack of efficient transformation protocols (Eady, *et al.*, 2000; McCallum, 2007). *In vitro* culture and plant regeneration from *Allium* species have been reported and reviewed but it is not suitable for onion (Eady, 1995). Onion transformation from the mature zygotic embryo (Zheng *et al.,* 2001), immature zygotic embryo (Marinangeli *et al.,* 2005), immature flower bud (Pike and Yoo, 1990), and mature flower bud (Luthar and Bohanec, 1999) have been reported. Hence one of the aims of this research is to develop an efficient and reliable protocol for generating transformed onion callus cell from shoot tip tissue of germinating seed.

1.16 Research aims

This research project was carried out with the following three aims:

1. To identify the photoreceptor and circadian clock genes with altered expression which might be responsible for altered bulbing in LD and SD onion. To identify the photoreceptor and circadian clock genes with altered expression in LD and SD cultivars, the expression of photoreceptor and the circadian clock gene was compared in plants that bulb and those they did not bulb when grown under SDs (Chapter 3).

2. Develop protocol for generating transformed onion callus cells. As no reliable method of generating transgenic onions exist, we set out to establish a method of generating transgenic onion cells so that the function of onion genes such as *AcAP1* involved in bulbing, could be analysed (Chapter 4).

3. To develop molecular markers to the *AcVRN1* genes and determine if this gene is responsible for premature bolting. *Bolt1* locus a major determinant of premature bolting. To determine if the *VRN1* gene and *Bolt1* are the same gene, we set out to develop markers and map the *AcVRN1* gene (Chapter 5)

Chapter 2 Materials and Methods

The experiments were carried out at the Department of Biochemistry, the University of Otago, Dunedin, New Zealand, during the period from February 2019 to January 2020. The methods and experimental protocols presented in this chapter were used throughout the experiment.

2.1 General Buffers, Solutions and Media

All buffers, solutions and media were made with milliQ H_2O (ddH_2O), sterilised via autoclaved (121 ℃ for 20 minutes at 15 psi), and stored at room temperature, unless stated otherwise.

Bacterial media

LB liquid media (1 L)	10 g NaCl
	10 g bacto-tryptone
	5 g yeast extract
	pH 7.0
LB agar	LB liquid media
	1.5% (w/v) bacto-agar

Buffers and solutions

50x TAE buffer (1 L)	242 g Tris
	100 mL 0.5 M EDTA, pH 8.0
	57.1 mL Glacial acetic acid
	842.9 mL ddH_2O
	Used 1x TAE
TE Buffer	10 mM Tris, pH 8.0
	1 mM EDTA, pH 8.0

1% Agarose gel	1% (w/v) agarose
	1x TAE Buffer
	RedSafe™ Nucleic acid staining solution
	1.5 µL per 30 mL Agarose gel
10x Loading dye	50 % glycerol
	10 mM EDTA, pH 8.0
	0.25 % (w/v) bromophenol blue
	0.25 % (w/v) xylene cyanol FF

2.2 Plant material and growth condition

The various LD (CUDH2150, CUDH2107, Pukekohe Long Keeper), SD (Albasile, Nasik Red) and F1 generation (cross between 'CUDH2150 x Nasik Red' and 'CUDH2107 x Albasile') onion cultivars were grown under either SD conditions (12 hours light/ 12 hours dark), or LD conditions (16 hours light/ 8 hours dark) at 20° C in a controlled growth room. Onion seeds were sown in plastic trays containing 3-parts Yates Professional Potting Mix and 1-part vermiculite.

2.3 Plant tissue harvest and storage

Onion leaf tissue was harvested (~100 mg) in 1.5 mL microcentrifuge tubes, immediately frozen in liquid nitrogen and stored at -80℃.

2.4 RNA Manipulations

To avoid contamination of samples with ribonuclease (RNases) latex gloves, barrier filter tips and UltraPure™ Distilled Water (Invitrogen™) were used.

2.5 Total RNA extraction from onion leaf tissue

RNA was isolated from ≤100 mg of frozen, onion leaf tissues. Onion tissues were placed in a plastic zip-lock bag, then 500 µL of Plant RNA Extraction Reagent (Invitrogen™, stored at 4 °C) added. The sample was immediately crushed with a corex glass tube and

the resulting homogenised mixture transferred to a 1.5 ml microcentrifuge tube. Samples were vortexed and incubated horizontally (to maximize surface area) for 30 minutes at room temperature. Samples were centrifuged at 12,000 rpm at 20 °C for 3 minutes, supernatants were transferred to a new clean 1.5 ml microfuge tube without disturbing debris. 100 µL 5 M NaCl was added to tube followed by vortexing. After that 300 µL chloroform was added and vortexed thoroughly then, samples were centrifuged at 12,000 rpm at 4 °C for 10 minutes and the upper aqueous phase was removed into a new clean 1.5 ml microfuge tube. 300 µL chloroform was added and vortexed thoroughly then, samples were centrifuged at 12,000 rpm at 4 °C for 10 minutes and the upper aqueous phase was removed into a new clean 1.5 ml microfuge tube. An equal volume of isopropanol was added, mixed thoroughly by inverting the tube 5-6 times and incubated at room temperature for 10 minutes. Centrifuge the sample at 12,000 rpm at 4 °C for 10 minutes then, decant the supernatant, taking care not to lose the pellet. Pellet was washed twice with 1 ml of 75% ethanol and centrifuge at 12,000 rpm at 4 °C for 10 minutes. Discard the supernatant with a micro pipettor then, samples were dried for 10 minutes and the pellet was dissolved in 20 µL RNase-free water by pipet the liquid up and down over the pellet to resuspend the RNA. RNA was stored at –80 °C.

2.5.1 RNA purification

To purify the total RNA isolated using Plant RNA Extraction Reagent (Invitrogen^{TM}), the total volume of 400 µL was made by adding RNase-free water into 20 µL RNA. 100 µL 5 M NaCl was added to the tube followed by vortexing. After that 300 µL chloroform was added and vortexed thoroughly then, samples were centrifuged at 12,000 rpm at 4 °C for 10 minutes and the upper aqueous phase was removed into a new clean 1.5 ml microfuge tube (this step done twice). An equal volume of isopropanol was added, mixed thoroughly by inverting the tube 5-6 times and incubated at room temperature for 10 minutes. Centrifuge the sample at 12,000 rpm at 4 °C for 10 minutes then, decant the supernatant, taking care not to lose the pellet. Pellet was washed twice with 1 ml of 75% ethanol and centrifuge at 12,000 rpm at 4 °C for 10 minutes. Discard the supernatant with a micro pipettor then, samples were dried for 10 minutes and the pellet was dissolved in 20 µL RNase-free water by pipet the liquid up and down over the pellet to resuspend the RNA. RNA was stored at –80 °C.

2.5.2 RNA quality assessment

To ensure quality and quantity of extracted RNA, concentration and absorbance level at 230 nm, 260 nm and 280 nm of RNA sample was measured using a Nanodrop Spectrophotometer (Thermo Scientific). The absorbance ratios of $A_{260:280}$ and $A_{260:230}$ were used to determine the purity of RNA. The RNA quality was considered high if the $A_{260:280}$ and $A_{260:230}$ rations is near 2 and 2.2 respectively.

2.5.3 DNAse I treatment

To remove gDNA contamination from total RNA, DNase I treatment was given. 500 ng of isolated RNA was mixed with 1 µL 10x DNase I reaction buffer (Invitrogen™), 1 µL DNase I (Amp Grade, 1U/ µL, Invitrogen™) and nuclease-free water to make the final volume 10 µL. Tubes were incubated at room temperature for 15 minutes. After 15 minutes of incubation, 1 µL EDTA (Invitrogen™) was added and tubes heated to 65 ºC for 10 minutes to inactivate the DNase I. The RNA is ready to use in reverse transcription.

2.5.4 cDNA synthesis

Genomic-DNA free of RNA (section 2.5.3) was then used to synthesize the first-strand cDNA. 1 µL 50 µM oligo$(dT)_{20}$ (Invitrogen™) primers and 1 µL 10 mM (Invitrogen™) of dNTPs were added in RNA, heated to 65 ºC for 5 minutes and then incubated on ice for 2 minutes. Samples were briefly centrifuged to collect contents to the bottom of the tube and added 4 µL 5x First-Strand Buffer (Invitrogen™), 1 µL 0.1 M DTT (Invitrogen™), 1 µL RNaseOUT (40U/µL, Invitrogen™) and 1 µL Superscript III Reverse Transcriptase (200U/µL, Invitrogen™) were added and mixed by pipetting up and down. Tubes were incubated at 50ºC for 60 minutes followed by 70 ºC for 15 minutes. cDNA was stored at -20 ºC or -70 ºC for long term storage. The resulting cDNA was diluted in ddH_2O (1 in 30) before use in qRT-PCR.

2.6 Polymerase chain reaction (PCR)

2.6.1 General PCR protocol:

General PCR protocol using Invitrogen™ Taq DNA polymerase was as follows unless stated. Each 20 µL PCR reaction contained 1 µL of template DNA (~20 ng), 2 µL 10x PCR reaction buffer (-MgCl2), 0.6 µL MgCl2, 0.4 µL dNTP (10 µM), 0.4 µL each of forward and reverse primer (10 µM) and 0.1 µL of Platinum Taq Polymerase (Invitrogen™); ddH_2O was used to make the volume up to 20 µL. Reactions were run in the PCR machine (Mastercycler® Nexus, Eppendorf) with the following thermocycling conditions (Table 2.1) for a general PCR.

Table 2.1 The thermal cycling condition for general PCR

PCR Steps		Temperature	Time
1. Initial denatureation		94 ˚C	2 minutes
2. Denaturation		94 ˚C	20 seconds
3. Primer annealing	30 cycles	58 ˚C	20 seconds
4. Extension		72 ˚C	1 minutes per kb
5. Final Extension		72 ˚C	5 minutes

2.6.2 Colony PCR protocol

In order to confirm plasmid into vectors, PCR was carried out on bacterial colony cells suspended in water. Sterile toothpick tip was used to collect a single bacterial colony and resuspend in 20 µL water. 1 µL of the cell suspension would serve as a template in a general PCR reaction, using a forward primer binding to the promoter of CaMV, and a reverse primer that would bind the *AcAP1* gene (see appendix 1&2 for primer sequence). Reactions were run in the PCR machine (Mastercycler® Nexus, Eppendorf) with the following thermocycling conditions provided in Table 2.2 for a colony PCR. PCR products were run on agarose gels and those of the correct size would result in the corresponding bacterial colonies being selected for further processing.

Table 2.2 The thermal cycling condition for colony PCR

PCR Steps		Temperature	Time
1. Initial denatureation		94 ˚C	2 minutes
2. Denaturation	30 cycles	94 ˚C	20 seconds
3. Primer annealing		58 ˚C	10 seconds
4. Extension		72 ˚C	30 seconds per kb
5. Final Extension		72 ˚C	5 minutes

2.6.3 Quantitative real-time PCR (qRT-PCR)

To determine relative gene expression, qRT-PCR was performed using three biological replicates. Reactions took place in 10 µL volumes using 3 µL diluted cDNA (1:30) in 96 multi-well LightCycler 480 plates (Roche), using the LightCycler 480 machine (Roche). A reaction would comprise 5 µL SYBR FAST qPCR master mix (Kapa Biosystems), 1 µL nuclease-free H_2O and 0.5 µL each of forward and reverse primers (10 µM stock) and 3 µL dilute cDNA. Negative controls for each primer pair were included by replacing the template with nuclease-free ddH_2O. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method using Roche LC480 software. Data were analysed in Microsoft Excel. Tubulin was used as the reference gene. Primers used for qRT-PCR experiments are listed in Appendix 1. The thermocycling conditions are provided in Table *2.3* for qRT-PCR.

Table 2.3 The thermal cycling condition for qRT-PCR

PCR Steps		Temperature	Time	Ramp rate °C/second
1. Initial denatureation		95 ˚C	5 minutes	4.4
2. Denaturation	50 cycles	95 ˚C	10 seconds	4.4
3.Primer annealing		58 ˚C	10 seconds	2.2
4. Extension		72 ˚C	08 seconds	4.4
5. Melt curve acquisions		95 ˚C	5 seconds	4.4
		65˚C	1 minutes	2.2
		97˚C		0.11

2.6.4 TAIL-PCR (Thermal Asymmetric Interlaced PCR)

TAIL-PCR was used to amplify the unknown sequence of the promoter and first intron region of *AcVRN1* gene of onion. TAIL-PCR was performed as described by Singer (2003) with some modifications. Two reverse specific primers were designed from exon1 sequence of AcVRN1 gene; AcVRN1TAIL2 and AcVRN1TAIL3 to amplify promoter region of *AcVRN1* gene. Also, two reverse specific primers were designed from the exon2 sequence of *AcVRN1* gene; AcVRN1TAIL9 and AcVRN1TAIL10 to amplify the first intron. The forward primers were the arbitrary degenerate (AD) primers AD1, AD5 and AD6. All the primers sequence used in TAIL-PCR is listed in appendix 2. Primary TAIL-PCR was performed using 10 µL genomic DNA as a template in 20 µL reactions containing 2 µL 10x Taq polymerase buffer, 0.6 µL MgCl2, 0.4 µl dNTPs (10 µM), 1 µl 10 µM specific primer, 3 µl 20 µM AD primer, 0.2 µl Taq polymerase (Invitrogen™); and 2.8 µl ddH20. Reverse primers for the primary PCR reaction was AcVRN1TAIL2 for promoter and AcVRN1TAIL10 for intron1 region amplification. For the secondary reaction, the primary PCR reaction was diluted 1:100 and used as a template. The secondary reaction solution was the same as that for the primary reaction except for 2 µl template and 12.4 µl ddH20 was used with AcVRN1TAIL3 and AcVRN1TAIL9 as a reverse primer of the promoter and intron1 region respectively. The PCR products were run on 1% agarose gels and sequenced via

Sanger sequencing (see section 2.11.3). Cycling condition used for primary and secondary TAIL-PCR in as below:

Primary TAIL-PCR

One cycle of 1 minute at 93 ˚C, 1 minute at 95 ˚C; five cycles of 30 second at 94 ˚C, 1 minute at 62 ˚C, 2.5 minute at 72 ˚C; one cycle of 30 second at 94 ˚C, 3 minute at 25 ˚C ramping to 72 ˚C at 0.3˚C/second, 3 minute at 72 ˚C; fifteen cycles of 30 second at 94 ˚C, 1 minute at 68 ˚C, 2.5 minute at 72 ˚C, 30 second at 94 ˚C, 1 minute at 68 ˚C, 2.5 minute at 72 ˚C, 30 second at 94 ˚C, 1 minute at 44 ˚C, 2.5 minute at 72 ˚C; 5 minute at 72 ˚C; one cycle of 5 minute at 72 ˚C then a hold at 4 ˚C.

Secondary TAIL-PCR

Twelve cycle of 30 second at 94 ˚C, 1 minute at 64 ˚C, 2.5 minute at 72 ˚C; 30 second at 94 ˚C, 1 minute at 64 ˚C, 2.5 minute at 72 ˚C; 30 second at 94 ˚C, 1 minute at 64 ˚C, 2.5 minute at 72 ˚C; one cycle of 5 minute at 72 ˚C then a hold at 4 ˚C.

2.7 Agarose gel electrophoresis

To analyse PCR products, agarose gel electrophoresis was used to separate DNA products. Normally, 1% (w/v) agarose gels were used, however higher, or lower percentage gels were used to improve the resolution of smaller, or larger DNA fragments, respectively. To prepare the gel, agarose powder melted in 1x TAE buffer in a microwave oven for 1-2 minutes until agarose powder completely dissolves in the buffer. Agarose solution allowed to cool down to 40 ˚C (about 5 minutes). RedSafe™ (iNtRON Biotechnology) 20,000x nucleic acid stain solution added (2-3µL of laboratory stock solution per 100 mL gel), and then cast in a gel tray with the well comb and gate in place. Once the gel set, 1x TAE buffer was added to the tank. To prepare nucleic acid samples for electrophoresis, approximately 0.25 volumes of loading buffer were added to each sample; 10 µL of the sample was typically loaded onto the gel. As a molecular marker, ~5 µL of the DNA ladder (HyperLadder™, Bioline; Appendix 3) was used to identify the size of sample bands. Electrophoresis was performed at 120 V for 25-30 minutes. A Gel Doc™ XR+ gel documentation system (BioRad) was used to view and photograph the nucleic acids in the agarose gel under UV light.

2.8 Purification of PCR product from agarose gels

DNA fragments of interest were excised from agarose gels with sterile razor blades under UV light and purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research) in accordance with the manufacturer's protocol. 10 µL sample was run on an agarose gel to ensure the integrity of the isolated DNA was maintained. Furthermore, a volume of 1 µl purified DNA was quantified using a Nanodrop Spectrophotometer (Thermo Scientific).

2.9 Preparation of primer

Primers were synthesised by Integrated DNA Technologies (IDT®). List of primers used in PCR, sequencing, restriction digest and qRT-PCR is listed in Appendix 1 and 2. Primers were resuspended in ddH_2O to make the final concentration 100 µM. Working stock solutions of 10 µM were made by diluting in ddH_2O and stored at -20 ˚C. Primers used for Sanger sequencing were further diluted to 3.2 µM in ddH_2O.

2.10 Primer efficiency

Primer efficiency of each primer used in RT-qPCR was calculated and provided in Appendix 6. The acceptable PCR primer efficiency (standard curve, slopes on log amplification curves) is between 90-110%, R2 higher than 0.985 were appropriate and the melt curve should how a unique peak to indicate that only a single type of expected amplicon is present.

2.11 DNA Manipulations

To maintain DNA integrity and purity, samples were kept on ice while aliquots were taken for use in various reactions, or stored at -20 ˚C.

2.11.1 Extraction of genomic DNA from onion leaf

Onion genomic DNA (gDNA) was extracted using GeneJET Plant genomic DNA Purification Mini Kit (Thermo Scientific). Around 100 mg of onion leaf tissues, which were stored at -80 ℃ used for gDNA extraction.

2.11.2 Purification of DNA

Prior to Sanger sequencing, PCR products were purified using DNA Clean and Concentrator™ (Zymo Research) purification kit. DNA Clean and Concentrator™ purifications were carried out using the manufacturer's instructions. The kit aims to remove unwanted DNA or RNA polymerases, free dNTPs, modifying enzymes nucleases, phosphatases and restriction endonucleases, which may interfere with downstream experiments.

2.11.3 Sanger DNA Sequencing

Isolated PCR products or plasmids were sequenced via Sanger sequencing using a capillary ABI 3730xl DNA Analyser (Applied Biosystems) at Genetic Analysis Services (GAS), Department of Anatomy, University of Otago, Dunedin. DNA samples were delivered to GAS containing 1 ng/100bp (for PCR products) or ~150 ng of DNA (for plasmids), and either a forward or reverse primer (3.2 μM) and made up to 5 μL ddH_2O.

2.11.4 Geneious for bioinformatic analyses

Geneious® version 10.0.9 was used to analyse sequencing data and carry out the bioinformatic analysis.

2.12 Vector construction and cloning

2.12.1 pCR™8/GW/TOPO® TA cloning®

DNA sequences (*AcAP1* gene) for cloning purposes utilised gDNA template, extracted with the protocol described in section 2.11.1 and amplified by PCR using Platinum High Fidelity *Taq* DNA polymerase (Invitrogen™) as described in section 2.6.1. 10 μL PCR products were run on agarose gels to confirm the product size. PCR products amplified with Platinum High Fidelity *Taq* DNA polymerase has 5' A-tails, required for cloning into the pCR™8/GW/TOPO vector (entry vector; Appendix 4). A pCR™8/GW/TOPO® TA Cloning® kit (Invitrogen™) was used to clone the gene of interest into the pCR™8/GW/TOPO® vector. Cloning was carried out in a 6 μL reaction containing: 2 μL linear DNA construct (gene of interest), 1 μL salt solution, 0.5 μL pCR™8/GW/TOPO® vector and 2.5 μL ddH_2O. This reaction was mixed gently and incubated for 5 minutes at room temperature.

2.12.2 Restriction enzyme digest

Restriction enzyme digests were used to isolate specific DNA fragments. Restriction enzymes XhoI used in this study were sourced from New England Biolabs® (NEB®), and the digestion was carried out in 50 µL reaction containing: 1 µL XhoI restriction enzyme, 5 µL CutSmart buffer, 5 µL DNA (TOPO-*AcAP1*) and 39 µL ddH_2O. Incubations were normally carried over an hour to ensure complete digestion of products. Prior to gateway cloning, the reaction was purified using DNA Clean and Concentrator™ (Zymo Research) purification kit to remove enzyme and buffer.

2.12.3 Gateway cloning

Gateway cloning was utilised to introduce specific DNA fragments within a pCR™8/GW/TOPO® vector (entry vector) into the pARTB-GW-egfpER vector (destination vector; Appendix 5). This reaction uses recombination sites that are complimentary between plasmids. A typical gateway reaction was made up as follows (unless otherwise stated): 1 µL destination vector, 1 µL of the entry vector containing gene of interest, 1 µL of Gateway™ LR clonase™ II (Invitrogen™) and 2 µL TE buffer. The reaction was incubated at room temperature for 1 hour.

2.12.4 Transformation of *E. coli* via heat shock

Plasmid DNA (from 2.12.3) and chemically competent *E. Coli* DH5α (~50 µL) cells were thawed slowly on ice for 10-15 minutes. 2 µL of the thawed plasmid were added into chemically competent *E. coli* cells and then incubated on ice for 15 minutes. *E. coli* DH5α cells were heat shocked at 42 ˚C for 45 seconds and returned immediately to the ice for 2 minutes. 500 µL of room temperature LB liquid media was added into *E. coli* DH5α cells and incubated with shaking for 1 hour at 37 ˚C. After this, ~300 µL *E.* coli DH5α cells were plated on LB solidified medium containing the appropriate antibiotics via a sterile spreader, dried, and incubated overnight at 37 ˚C. Colony PCR followed by gel electrophoresis was carried out to confirm the presence of insert DNA in plasmid constructs. Colonies testing positive by colony PCR were picked for growth on 3mL LB solidified medium containing the appropriate antibiotics and incubated with shaking for 16 hours at 37 ˚C.

2.12.5 Storage of bacteria

For long term storage of *E.* coli DH5α, *A. tumefaciens* strain GV3101 and EHA105, 100 µL of 80% glycerol added into 400 µL bacterial culture and stored at -80 ˚C.

2.12.6 Isolation of Plasmid DNA from bacteria

ZR Plasmid Miniprep™ Classic Kit (Zymo Research) was used to extract plasmid from *E.* coli DH5α and *A. tumefaciens.* The concentration and the quality of the plasmid DNA extraction were measured via the Nanodrop Spectrophotometer (Thermo Scientific). The extracted plasmid was sequenced to further confirm correct insert orientation and sequence reliability. These checks took place prior to the transformation of plasmids into *Agrobacterium tumefacien*.

2.12.7 Transformation of Agrobacterium tumefaciens via heat shock

Chemically competent *A. tumefaciens* cell stocks were all created in the laboratory and stored at -80 ˚C. The *A. tumefaciens* strain, i.e. GV3101 and EHA105, were used in the experiment. Plasmid DNA and chemically competent *A. tumefaciens* cells were thawed slowly on ice for 10-15 minutes. 2 µL of the of thawed plasmid were added into frozen *A. tumefaciens* cells immediately and then heat shocked at 37 ˚C for 5 minutes and placed back on ice. After that, 500 µL of LB liquid media was added to the cells, which were then incubated at 28 ˚C for 2 hours with agitation. *A. tumefaciens* cells were plated on LB solidified medium containing the appropriate antibiotics and incubated at 28 ˚C for 2-3 days. Colony PCR followed and gel electrophoresis was carried out to confirm the presence of insert DNA in plasmid constructs. Colonies testing positive by colony PCR were picked for growth on 5 mL LB agar containing the appropriate antibiotics and incubated with shaking for 2 days at 28 ˚C.

2.13 Onion transformation

2.13.1 Antibiotics

Antibiotic powders were obtained from Sigma-Aldrich® and stored at 4 ºC. All antibiotics were made into a filter sterilised stock solution of 1000x working concentration and stored at -20 ºC. working and stock concentrations of antibiotics used for bacterial and plant transformation in this study listed in Table 2.4. L-phosphinothricin

Table 2.4 List of antibiotics used for bacterial and plant transformation

Antibiotic	**Working Concentration**	**Stock concentration**
Rifampicin	50 μg.mL^{-1}	50 mg.mL^{-1} in DMSO
Gentamycin	25 μg.mL^{-1}	25 mg.mL^{-1} in water
Spectinomycin	100 μg.mL^{-1}	100 mg.mL^{-1} in water
Timentin	500 μg.mL^{-1}	500 mg.mL^{-1} in water
Nystain	25 μg.mL^{-1}	25 mg.mL^{-1} in DMSO
BASTA (L-Phosphinothricin)	5 μg.mL^{-1}	10 mg.mL^{-1} in water

2.13.2 Plant material

The tissue used in this onion transformation experiment was shoot tip tissue of germinating seed. Two onion cultivar, Pukekohe long keeper and double haploid line CUDH2107 were used.

2.13.3 Seed sterilization, germination and explant preparation

Seeds were surface sterilized with 70% ethanol for 30 seconds, rinsed in ddH_2O for 1 minute. After that seed were soaked in detergent (1 uL Silwett in 10 ml ddH_2O) for 5 minutes, rinsed in ddH_2O. Then seeds were immersed in 2% sodium hypochlorite (50 bleach:50 Water) for 12-20 minutes with agitation, rinsed with ddH_2O five times and store the seeds in ddH_2O at 4 ˚C over- night. The next day, seeds were immersed in 2% sodium hypochlorite for 10 minutes with agitation and washed with ddH_2O five times. Seeds were placed on filter paper for 5 minutes to dry. The sterilized seeds were then

placed in callus induction media (CIM; Table 2.5) at 22 °C and allowed to germinate in the dark. Shoot tips, not more than 5mm were excised as soon possible once seed germinate and transferred on callus induction media. Calluses were transferred on to fresh CIM every 10 days and cultured on CIM for five weeks.

2.13.4 Co-cultivation with Agrobacterium

Two strains of *A. tumefaciens*, i.e. GV3101 and EHA105 were streaked out on LB solidified medium with appropriate antibiotics and grown at 28 ˚C for three days for colonies to appear. A single colony was selected from plate and suspended for further cultured in 5 uL LB liquid medium with appropriate antibiotics at 28 ˚C for two days. 500 uL of this culture were taken and inoculated in 50ml LB liquid medium with appropriate antibiotics and incubated on shaker for 4 hours at 28 ˚C. After that, 100 uM acetosyringone were added and incubated a further 3 hours on the shaker at 28 ˚C. Suspensions were centrifuged at 4000 rpm for 10 minutes, the supernatant was removed and the *Agrobacterium* pellet was resuspended into the co-cultivation liquid medium at an optical density (OD) of 0.4 to 0.6 (OD_{600}). The resuspended *Agrobacterium* cultures were transferred to the petri dish. 40-50 calli were immersed in the *Agrobacterium* suspension to soak for at least an hour, swirling occasionally to mix. After an hour, excess bacterial suspension was removed from the callus by placing them on a sterilized filter paper in a petri dish. After, the callus was put on solidified co-cultivation medium (*Table 2.1*) and incubated in the dark at 22 °C for 3 days. The empty petri dish was placed on top of the stack of callus containing plates to prevent the accumulation of condensation in the uppermost plate.

2.13.5 Selection of transform cells and tissues

After 3 days in co-cultivation media, calli were transferred on a pre-selection media containing 500 mg/L timentin (to kill *Agrobacterium*) and incubated in the dark at 22 °C for one week (*Table 2.5*). Calluses were examined after the co-cultivation period of 3 days for GFP expression under the fluorescent microscope.

After one week on pre-selection media, callus was transferred on a selection media (Table 2.5) containing BASTA, selective agent to kill untransformed plant tissue and incubated in the dark at 22 °C for three weeks. Callus was transferred on to fresh selection media every week. After three weeks, callus was transferred to the light.

Table 2.5 Composition of various media used in onion transformation

Callus induction media	1X MS with vitamin 3% Sucrose 250 mg/L casein hydrolysate 1 mg/L 2,4-D 0.7 bacto-agar
Co-cultivation media	1X MS with vitamin 3% sucrose 1% glucose 250 mg/L casein hydrolysate 1 mg/L 2,4-D 100 μM acetosyringon 0.3% gelrite
Pre-selection media	1X MS with vitamin 3% sucrose 0.3% gelrite 1 mg/L 2,4-D 500 mg/L timentin
Selection media	1X MS with vitamin 3% sucrose 0.3% gelrite 1 mg/L 2,4-D 500 mg/L timentin 25 mg/L nystatin 5-10 mg/L BASTA

Chapter 3 Investigating genes responsible for adaptation of onion under different daylength

3.1 Introduction

Onion is a biennial plant and completes its life cycle in two growing seasons: forming bulbs in the first year, and flowering and producing seed in the second year. The physiology of bulb initiation has been studied extensively. It is a process that is driven by photoperiod and light (Garner and Allard, 1920; Brewster, 2008).

Onion bulb formation and *Arabidopsis* flowering are both daylength dependent processes (Thomas *et al.,* 2006). At the physiological level, bulb initiation in temperate regions is regulated in a similar manner to the photoperiodic flowering of *Arabidopsis* (Mettananda and Fordham, 1997; Taylor, 2010; Lee *et al.,* 2013). Both processes are induced by long-day (LD), with signal perception in the leaves, the response at the shoot apical meristem (SAM; Okporie and Ekpe, 2008). Both *Arabidopsis* flowering and onion bulbing are promoted by far-red light (Sobeih and Wright, 1987). Flowering and bulbing also can be compared in terms of the existence of a juvenile phase, and during both processes, induction will not occur regardless of being exposed to favourable environmental conditions until they passed juvenile phase (Brewster, 2008).

Onion is grown in diverse geographical regions and has adapted to different environmental conditions. As described in section 1.5 of chapter 1, onion cultivars are classified based on their day length requirement for bulbing and are known as a short-day (SD), long-day (LD) and intermediate-day (ID) types. SD onion requires 10-12 hours of day length, ID onion require 12-14 hours of day length, and LD onion require 16 or more hours of daylength. This critical day length (CDL) requirement for bulb initiation indicates the adaptability of different onions cultivars in different latitudes in the world (Thomas *et al.,* 2006).

Genetic study of onion aimed at onion improvement has been limited due to its large genome size, high inbreeding depression, cross-pollinated nature, biennial life cycle, high percentage of repetitive DNA (Kuhl *et al.,* 2004; McCallum *et al.,* 2006; McCallum 2007; Duangjit *et al.,* 2013). Onion bulb initiation is daylength dependent, which places a significant barrier to adapting onion cultivars at different latitudes.

Various SD and LD plants adapted to different latitudes by altering genes involved in photoperiodic pathway (Nakamichi, 2015). In *Arabidopsis*, allelic variation in these

genes are associated with variation in flowering time and has been shown to be responsible for adaptation of plants to diverse geographic regions (Andres and Coupland, 2012; Olsen and Wendel, 2013; Nakamichi, 2015). Studies showed that different components of the photoperiodic pathway (photoreceptor, circadian clock, and output genes) have changed during crop domestication and this allowed crop to adapt to different latitudes (Olsen and Wendel 2013; Shrestha *et al.,* 2014; Blumel *et al.,* 2015; Nakamichi, 2015; Blackman, 2017). In onion, working in our laboratory by Khosa (2018) indicated that the shift in photoreceptor and circadian clock genes might be responsible for differences in daylength sensitivity and bulbing in SD and LD onion. The aim of this chapter is to identify the photoreceptor, circadian clock and output genes with altered expression in LD and SD onion and to correlate which of these genes might be responsible for altered bulbing in LD and SD onion.

3.2 Molecular basis of *FT* dependent bulbing in onion:

The *FLOWERING LOCUS T* gene (*FT*), first identified in *Arabidopsis (*Kardailsky *et al.,* 1999; Kobayashi *et al.,* 1999), has been shown to be the key component of the floral signal molecule, florigen (Andres and Coupland, 2012). For bulbing, daylength perception occurs in the leaves, while the response is in the shoot apical meristem (located in basal plate). These suggest that a mobile signal with properties to *FT* might be involved (Lee *et al.,* 2013). As mention in section 1.12 of chapter 1, bulbing in onion is regulated by the *FLOWERING LOCUS T (FT)* genes. *AcFT4* suppress bulbing whereas *AcFT1* promotes bulb and, *AcFT2* regulates flowering (Lee *et al.,* 2013). Khosa (2018) also found that *AcFT1* expression was high in SD onion under 12 hours of light but at a low level in LD onion.

Daylength and light (quality and quantity) are major environmental parameters that control bulbing. Onions form bulbs when grown under inductive daylength and a sufficient amount of far-red light. But when the far-red light is low or absent, even for a short duration even under inductive daylength onions never forms bulbs (Lercari, 1982a, b; Brewster, 2008; Khosa, 2018). LD onions form bulb only when the day length reaches around 16 or more hours, whereas SD onions form bulb when the daylength around 12 (Brewster 2008). During bulbing, *AcFT1* level goes up and *AcFT4* goes down. To determine if the expression of *AcFT1* and *AcFT4* changes over 24 hours period under LD and SD conditions was examined in this chapter.

3.3 Results

3.3.1 *AcFT1* expression in SD and LD onion

We set out to examine the expression of *AcFT1* in LD (DH2150) and SD (Albasile) onion varieties over a 24 hours period of either LD condition where both LD and SD varieties should form bulbs and, SD condition where only SD variety (Albasile) form bulb. To do this LD onion and SD onion plants were grown in a controlled environment under LD conditions (16 hours light/ 8 hours dark) at 20° C with ~40% relative humidity. After 8 weeks from sowing, onion plants were moved to far-red light for 5 days. For SD condition, plants were given 12 hours white light supplement with far-red light (12 hours white light + FR light/ 12 hours dark). For LD condition, plants were given 16 hours white light supplement with far-red light (16 hours white light + FR light/ 8 hours dark). On the sixth day, onion leaves were harvested at 4 hours intervals over 24 hours period into liquid nitrogen and stored at -80℃. Total RNA was isolated, cDNA was made, and RT-qPCR was done.

In LD condition, *AcFT1* follows diurnal expression patterns, expressed at a low level in the morning, peaking up in mid-afternoon to dusk (Figure 3.1). Expression of *AcFT1* was seen higher in SD onion (Albasile) compared to LD onion (DH2150). The expression of *AcFT1* was seen to peak around ZT 17 in LD onion compared with ZT 13 in SD onion. Under LD condition, *AcFT1* expression was seen 4 hours early (ZT 13) in SD onion than the LD onion (ZT 17; Figure 3.1).

Expression of *AcFT1* was next examined under the SD light condition in the LD and SD onion. SD onion varieties shows diurnal expression patterns, expressed in low level in the morning, peaking up at dusk (ZT 12; Figure 3.2). In LD onion cultivar, *AcFT1* was not induced by 12 hours of daylength. This suggests that 12 hours daylength was sufficient to activate high levels of *AcFT1* to initiate bulbing in SD onions but not in LD onion varieties. These results demonstrate that the difference between these cultivars is that different CDL are required to regulate *AcFT1* expression.

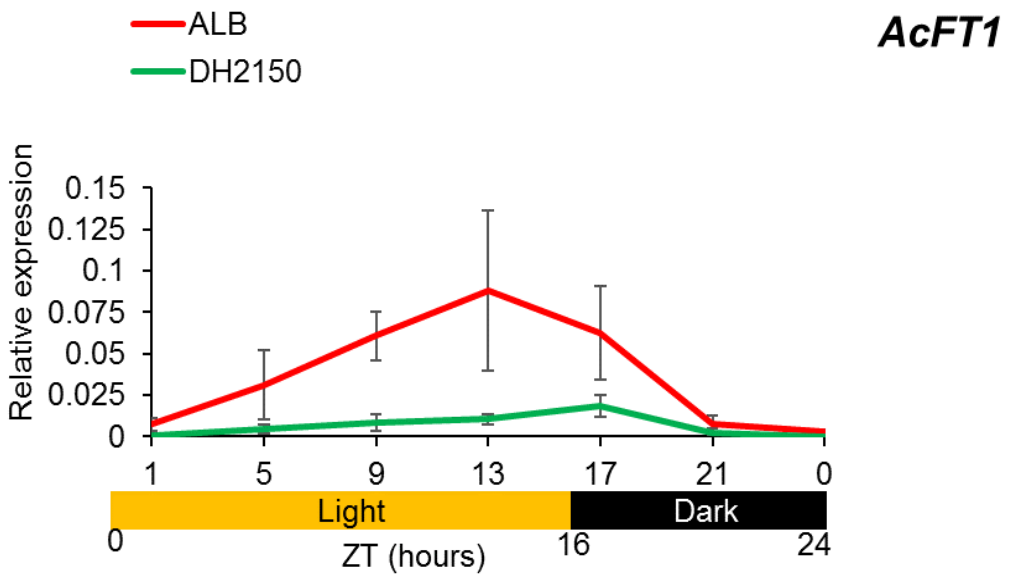

Figure 3.1 Diurnal expression of *AcFT1* in SD and LD onions under LD condition. *AcFT1* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring the plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

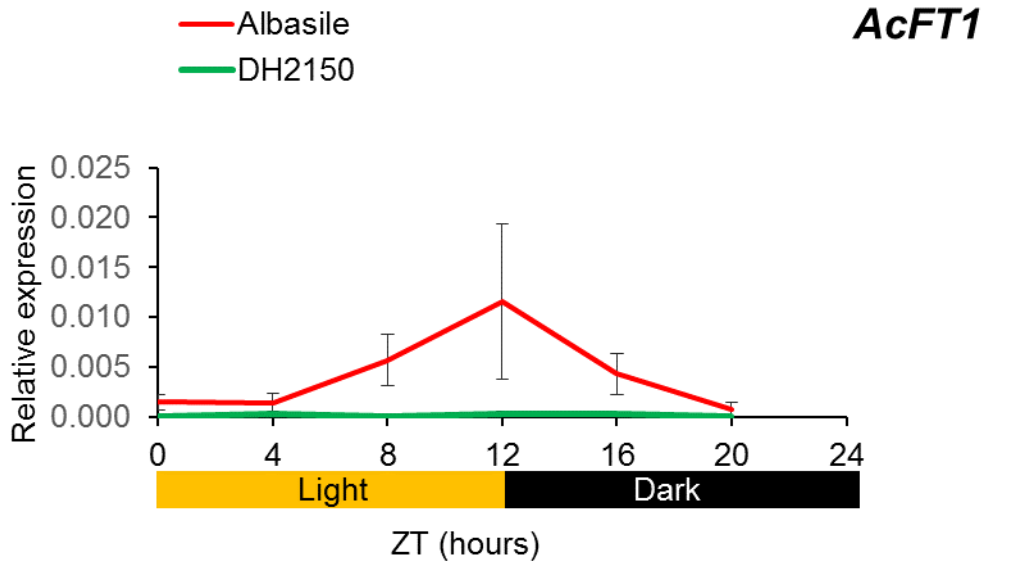

Figure 3.2 Diurnal expression of *AcFT1* in SD and LD onions under SD condition. *AcFT1* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring the plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

3.3.2 *AcFT4* expression in SD and LD onion

Next the expression level of *AcFT4* in SD and LD onion varieties under both conditions was measured. In the LD condition, *AcFT4* showed limited expression with no obvious trend in LD onion (Figure 3.3). In SD onion, *AcFT4* showed limited expression during the daytime, peaking up after dusk at ZT 21. However, this peak was due to the presence of an outlier in the sample (0.81 relative expression compared with 0.05 and 0.04; see the error bar in Figure 3.3), otherwise it expressed at the low level at ZT 21.

In the SD condition, for the LD onion variety *AcFT4* was expressed throughout the day and early part of the night, going down after mid-night. For SD variety, *AcFT4* is expressed at a low level. However, peak around ZT 8 was due to sample variation present within replicates (0.005, 0.170, and 0.035; see the error bar in Figure 3.4). 12 hours photoperiod is not sufficient to induce bulbing in LD onion. As seen the higher expression of *AcFT4* to repress the bulbing whereas, in SD onion, 12 hours photoperiod is sufficient to initiate the bulbing, so *AcFT4* expressed at a low level. The result is consistent with the previous study done by Lee *et al.*, (2013), where they establish that *AcFT4* prevents bulbing by repressing *AcFT1*.

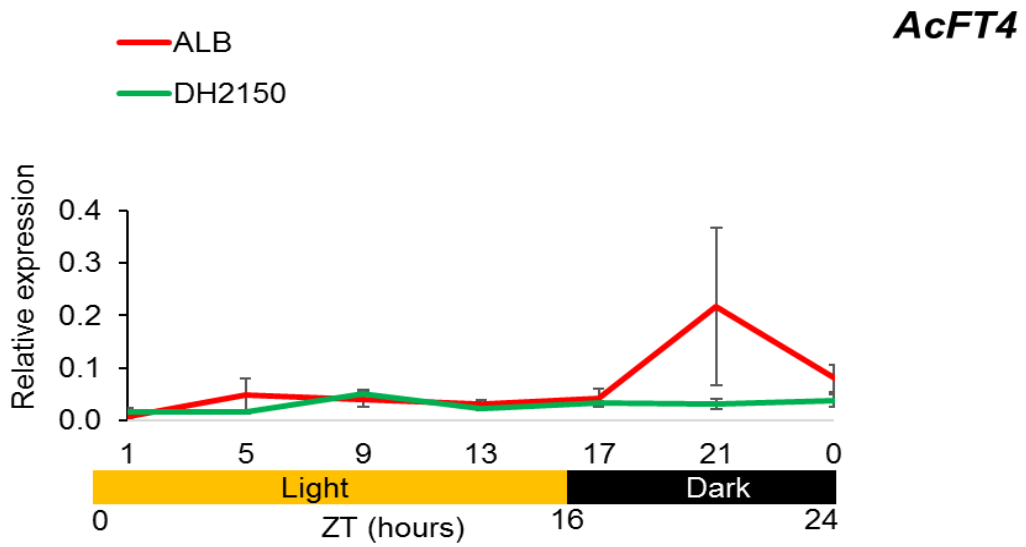

Figure 3.3 Diurnal expression of *AcFT4* in SD and LD onions under LD condition. *AcFT4* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring the plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

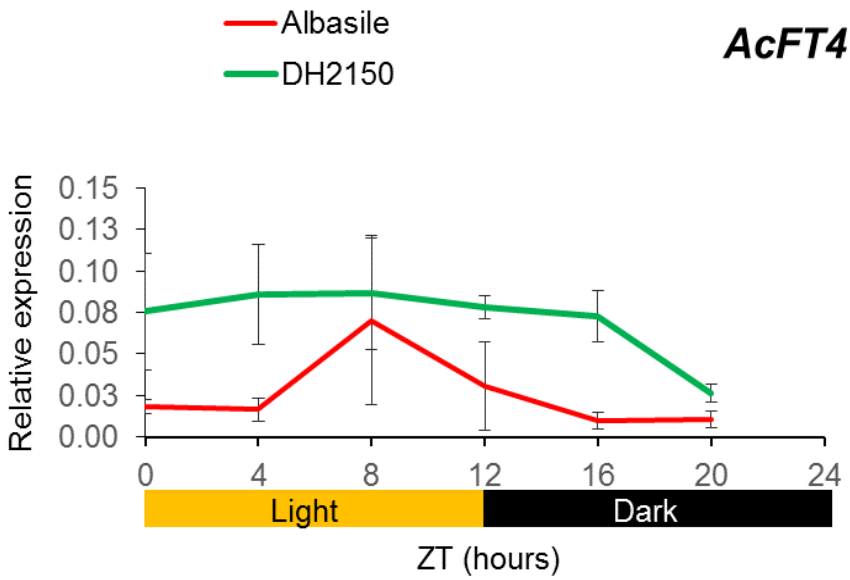

Figure 3.4 Diurnal expression of *AcFT4* in SD and LD onions under SD condition. *AcFT4* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring the plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

3.3.3 *AcAP1* expression in SD and LD onion

AP1 (APETALA1*)* is a MADS-box transcriptional regulator play important roles during plant development (Irish and Sussex, 1990). In *Arabidopsis* and other plants, *FT* activates *AP1* gene at SEM and show a positive expression correlation with each other to impart floral identity (Wigge *et al.,* 2005; Taoka *et al.,* 2011). *FT* normally promotes flowering in *Arabidopsis* and the temperate grasses under long days (Amasino, 2010) while the equivalent gene in rice (*Hd3a;* monocot plant), promotes flowering in short days but represses flowering in long days (Sun *et al.,* 2014).

In onion, working in our laboratory by Khosa (2018) identified two distinct related *AP1-like* genes; *AcAP1* and *AcVRN1*. Based on expression patterns, Khosa (2018) suggested that *AcAP1* is involved in bulbing, whereas *AcVRN1* is involved in flowering. *AcAP1* promotes the bulbing. *AcFT1* expressed differently in SD and LD onion cultivars under SD condition (*AcFT1* was not induced in LD onion under SD light condition). To know whether *AcAP1* expressed differently in SD and LD onion cultivars, the relative expression of the *AcAP1* gene was measured under the SD condition.

In SD onion (Albasile), *AcAP1* follows diurnal expression patterns, expressed at a low level in the morning, peaking up in mid-afternoon and going down at dusk (Figure 3.5). *AcAP1* was seen to peak around ZT 8 in SD onion. In LD onion (DH2150), *AcAP1* was not induced by 12 hours of photoperiod (Figure 3.5). 12 hours of daylength (SD condition) is not enough to trigger *AcAP1* expression. Compared to *AcFT1*, *AcAP1* peak was seen 4 hours earlier (ZT 8; Figure 3.2 and 3.5), which suggest that *AcAP1* might work upstream of *AcFT1*.

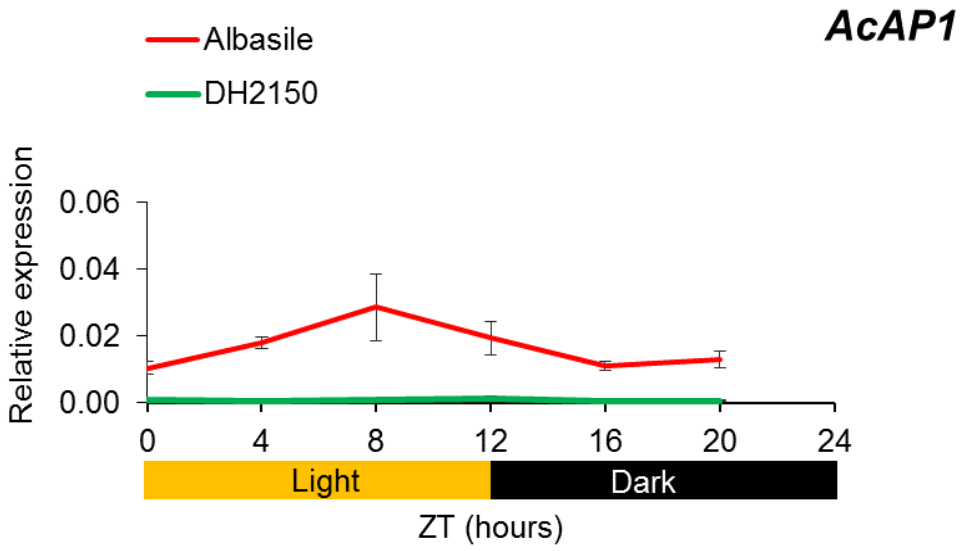

Figure 3.5 Diurnal expression of *AcAP1* in SD and LD onions under SD condition. *AcAP1* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring the plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

3.3.4 Photoperiodic pathway genes

Next to investigate onions adapted at different latitudes have altered photoperiodic pathway genes (photoreceptor, circadian clock, and output genes), expression of photoperiodic pathway genes were measured in LD and SD onion. *AcFT1*, *AcFT4*, and *AcAP1* express differently in SD and LD onions in response to daylength required for bulbing initiation. It might be possible that photoreceptors involved in daylength sensing get altered and as a result, circadian clock genes act differently to regulate *AcFT1* expression in SD and LD onion cultivars for better adaptation at different latitudes. Thus, relative expression of photoreceptors and circadian clock genes was measured over 24 hours period in LD and SD onion cultivars grown under LD and SD condition to

investigate whether bulb onions adapted at different latitudes have altered photoperiodic genes.

3.3.5 Expression of photoreceptor genes in SD and LD onion

Light is perceived by plants at different wavelengths by photoreceptors. Light input to the circadian clock in plants is mainly through phytochromes, cryptochromes and *ZEITLUPE* (*ZTL*) family proteins. Phytochromes are one of the most important photoreceptors that perceive and respond to the red and far-red light spectrum (Bae and Choi, 2008). In laboratory conditions, onion only forms bulb when white light is supplemented with far-red light (Khosa, 2018). Thus, the level of expression of the phytochrome genes were measured in LD and SD onion under both conditions.

AcPHYC followed diurnal expression patterns in LD (DH2150) and SD (Albasile) onions under SD condition, expressed at a high level at dawn, going down in mid-afternoon (Figure 3.6). *AcPHYC* was expressed to peak at ZT 0 in both onion cultivars under the SD condition. There was no difference or shift in the expression pattern of *AcPHYC* in LD and SD onion varieties under the SD condition. In LD condition, *AcPHYC* expression was seen almost similar throughout the day in LD onion but in SD onion, expression showed peak at ZT 1 and ZT 21 (Appendix 7).

The relative expression of *AcPHYA* and *AcPHYB* also measured over 24 hours period under the SD condition. Both genes showed a clear diurnal expression pattern. Expression pattern of the *AcPHYA* was seen almost similar throughout 24 hours period in both, LD and SD onion (Figure 3.7). Further *AcPHYB* also showed diurnal expression pattern, expressed at a high level during the early part of the day (Figure 3.8). These results indicate that under the SD condition, the expression pattern of all the phytochrome genes are almost similar in LD and SD onions. This suggests that phytochrome gene expression is not altered under SD light conditions and thus not involved in the different bulbing behaviour of two LD and SD cultivars examined here.

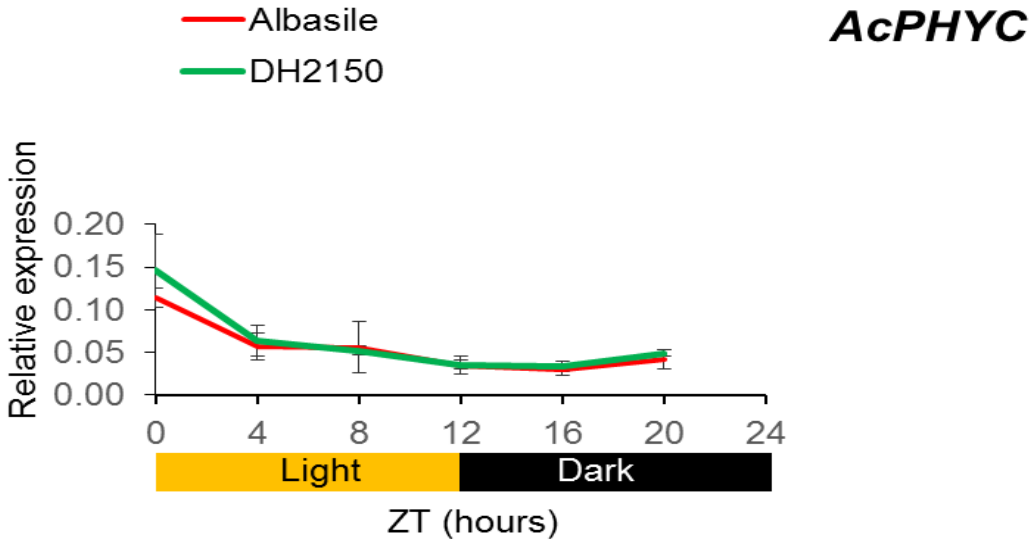

Figure 3.6 Diurnal expression of *AcPHYC* in SD and LD onions under SD condition. *AcPHYC* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring the plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

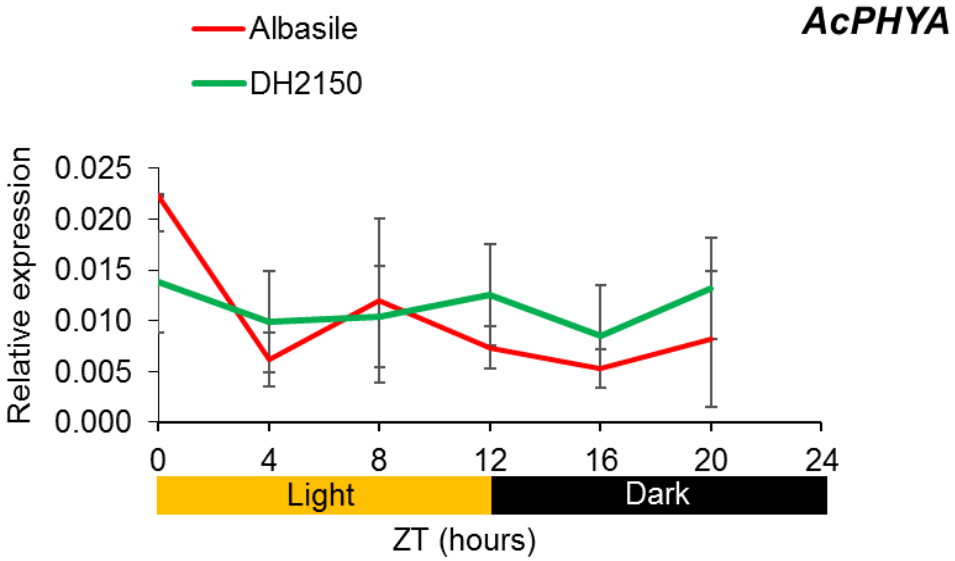

Figure 3.7 Diurnal expression of *AcPHYA* in SD and LD onions under SD condition. *AcPHYA* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

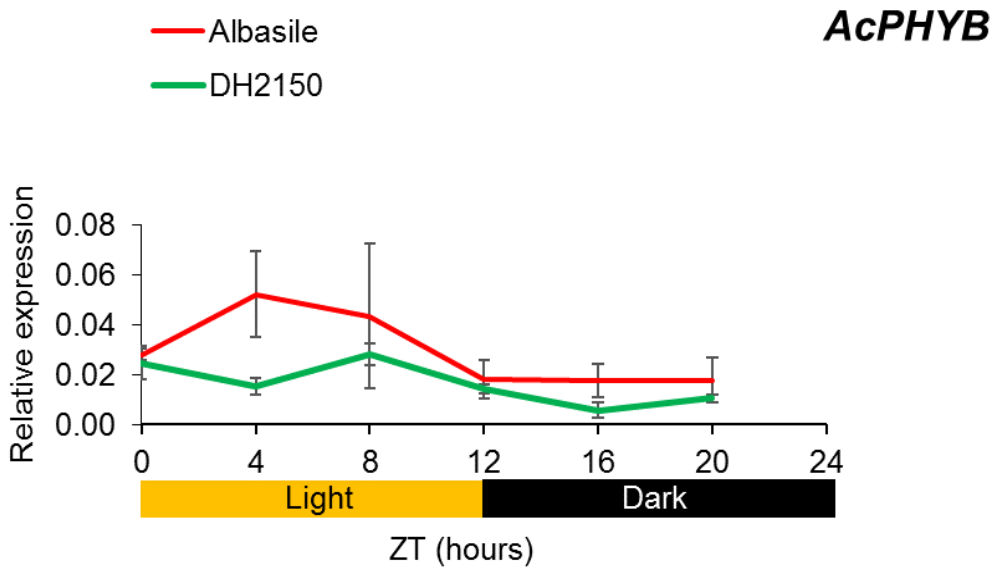

Figure 3.8 Diurnal expression of *AcPHYB* in SD and LD onions under SD condition. *AcPHYB* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

3.3.6 Expression of circadian clock genes in SD and LD onion

Photoreceptor genes are not altered in LD (DH2150) and SD (Albasile) onion under the SD condition. So next objective was to find out whether the circadian clock genes are altered and act differently to regulate *AcFT1* expression in SD and LD onion cultivars for better adaptation at different latitudes. Central oscillator integrates different environmental signal through different auto-regulatory transcriptional and translational feedback loops (McClung 2014). Genes involved in the central oscillator expressed at a different time of the day and night to regulate the output pathway gene (*GI*, *FKF1*, and *CO*; Sanchez and Kay 2016; Figure 1.8 of chapter 1). Thus, the relative expression of the circadian clock genes *AcCCA1* (*CIRCADIAN CLOCK ASSOCIATED1*), *AcPRR9* (*PSEUDO-RESPONSE REGULATOR9*), and *AcPRR7* (*PSEUDO-RESPONSE REGULATOR7*) was measured over 24 hours period using RT-qPCR.

In the SD condition, *AcCCA1* follows diurnal expression patterns, expressed at a high level at dawn, going down in early-afternoon and low expression in afternoon onward (Figure 3.9). No difference in the expression pattern of *AcCCA1* in LD and SD onion cultivars under the SD condition. Similar expression pattern of *AcCCA1* also observed

in LD and SD onion varieties under LD condition but showed higher expression in the Albasile than the DH2150 (Appendix 8). *CCA1* is the morning phased genes that function mainly as the repressors of the afternoon, evening and night phased genes (Figure 1.6 of chapter 1; Wang *et al.*, 1997; Kamioka *et al.*, 2016). The result suggests that *AcCCA1* is not altered in both cultivars under the SD condition and thus not involved in the different bulbing behaviour of these two onion cultivars studied here.

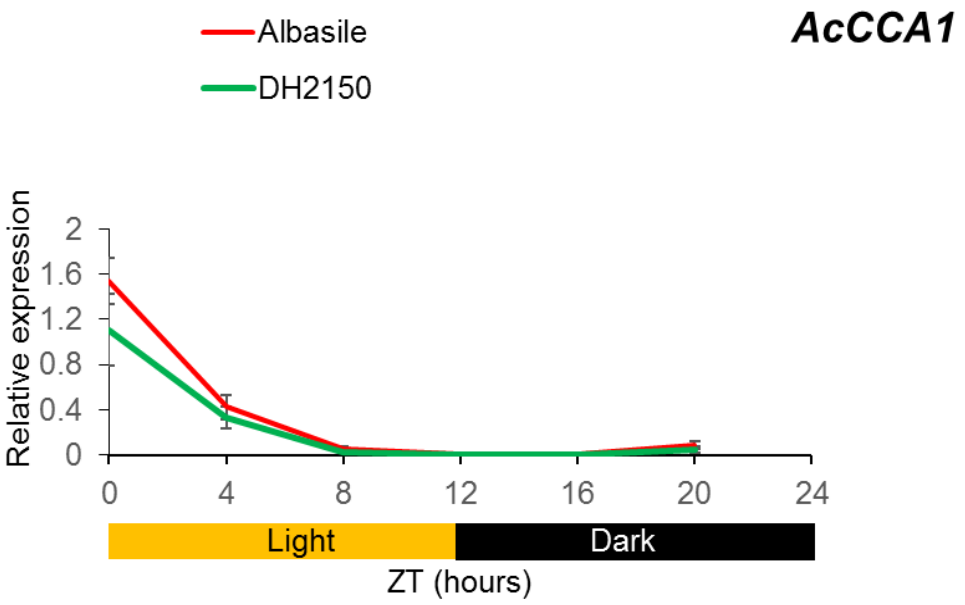

Figure 3.9 Diurnal expression of *AcCCA1* in SD and LD onions under SD condition. *AcCCA1* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

Next, the relative expression of two more circadian clock genes *AcPRR9* (in SD and LD condition) and *AcPRR7* (only in SD condition) were also measured over 24 hours period. *AcPRR9* and *AcPRR7* act in the central oscillator to regulate output genes (*FKF1*, *GI*, and *CO*) of the circadian clock and these output genes regulate *FTs* to promote flowering in different plant species (Hayama *et al.*, 2017).

In the SD condition, *AcPRR9* showed diurnal expression patterns, expressed at a low level in the morning, peaking up in the afternoon, and going down at dusk in LD and SD onion (Figure 3.10). In both cultivars, expression showed a peak at ZT 8. There was no

difference in the expression pattern of *AcPRR9* in LD and SD onion cultivars under the SD condition.

In the LD condition, *AcPRR9* followed diurnal expression patterns, expressed at low level in the morning, peaking up in the afternoon in SD and LD onion (Appendix 9). Expression of *AcPRR9* is higher in the SD onions than the LD onions at different times under LD condition. *AcPRR9* expression showed a peak at ZT 5 in SD onions, whereas in LD onions peak at ZT 9.

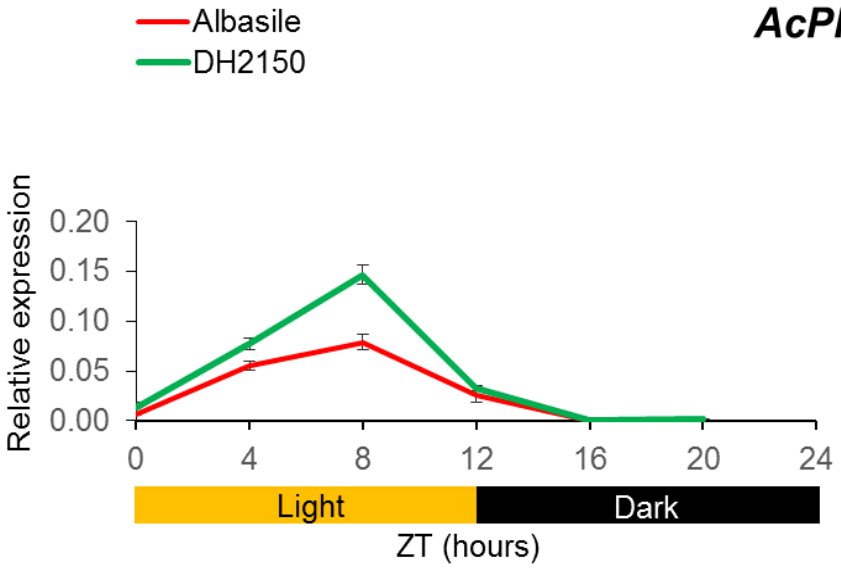

Figure 3.10 Diurnal expression of *AcPRR9* in SD and LD onions under SD condition. *AcPRR9* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring the plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

Next, I measured expression level of *AcPRR7* under SD condition. Expression of *AcPRR7* showed diurnal expression patterns, expressed at low level in the morning, peaking up in the afternoon, and going down at dusk in both onion cultivars (Figure 3.11). Maximum expression was seen at ZT 8 in SD and LD onions. There was no difference or shift in the expression pattern of *AcPRR7* in SD and LD onion. *PRR9* and *PRR7* is the afternoon phased genes that function mainly as the repressors of the morning phased genes (*CCA1* and *LHY*; Figure 1.8 of chapter 1; Matsushika *et al.*, 2000). These results suggest that the expression of *AcPRR9* and *AcPRR7* are not altered in both cultivars under

the SD condition and therefore, not involved in the different bulbing behaviour of two LD and SD onion cultivars examined here.

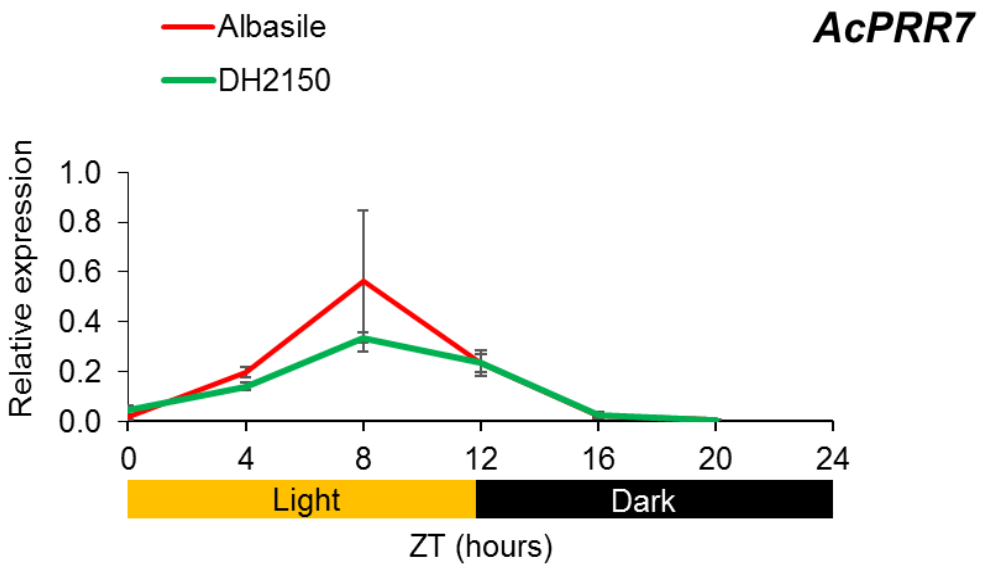

Figure 3.11 Diurnal expression of *AcPRR7* in SD and LD onions under SD condition. *AcPRR7* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring the plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

3.3.7 Expression of output pathway genes

CCA1, *PRR9*, and *PRR7* genes involved in the central oscillator to regulate the output pathway gene (*GI*, *FKF1*, and *CO*; Sanchez and Kay 2016) in *Arabidopsis* and other plants. The results of the circadian clock genes (*AcCCA1*, *AcPRR9*, and *AcPRR7*) showed that they are not altered in LD and SD onion under the SD condition. This suggests that they are not involved in the different bulbing behaviour of LD (DH2150) and SD (Albasile) onions studied here. *FKF1* and *GI*, an output pathway gene interacts with each other only when plants are exposed to light and this happens in late evening (around dusk) to stabilize *CO* by degrading *CDF1* (*CYCLING DOF FACTOR1*). To know whether the expression pattern of output pathway genes is altered between two cultivars under SD and LD condition. Thus, the relative expression of *AcGI* and *AcFKF1* was measure over 24 hours period under both SD and LD condition whereas expression of *AcCO* was measure in the SD condition.

In SD condition, *AcGI* showed diurnal expression, expressed at a low level at dawn, peaking up in the late afternoon, and going down in the late afternoon to dusk in SD onions, whereas in LD onions, expressed at a low level at dawn, peaking up in the evening, and going down after dusk. Relative expression of *AcGI* was seen to peak at ZT 12 in DH2150 compared with ZT 8 in Albasile (Figure 3.12). The peak at ZT 8 in Albasile was due to the presence of the outlier in the samples (0.47 relative expression compared with 0.15 and 0.12; see the error bar in Figure 3.12) otherwise expressed at low level (similar to DH2150).

In LD condition, *AcGI* follows diurnal expression, expressed at a low level at dawn, peaking up in afternoon in SD onion, whereas in LD onion expressed at a low level at dawn and peaking up in evening. The relative expression of *AcGI* was seen to peak at ZT 9 in DH2150 compared with ZT 5 in Albasile (Appendix 10).

There is no phase shift in expression pattern of *AcGI* in both, SD and LD onions under SD condition. This result suggests that the *AcGI* are not altered in SD and LD onions under the SD condition and thus not involved in the different bulbing behaviour of two LD and SD cultivars examined here.

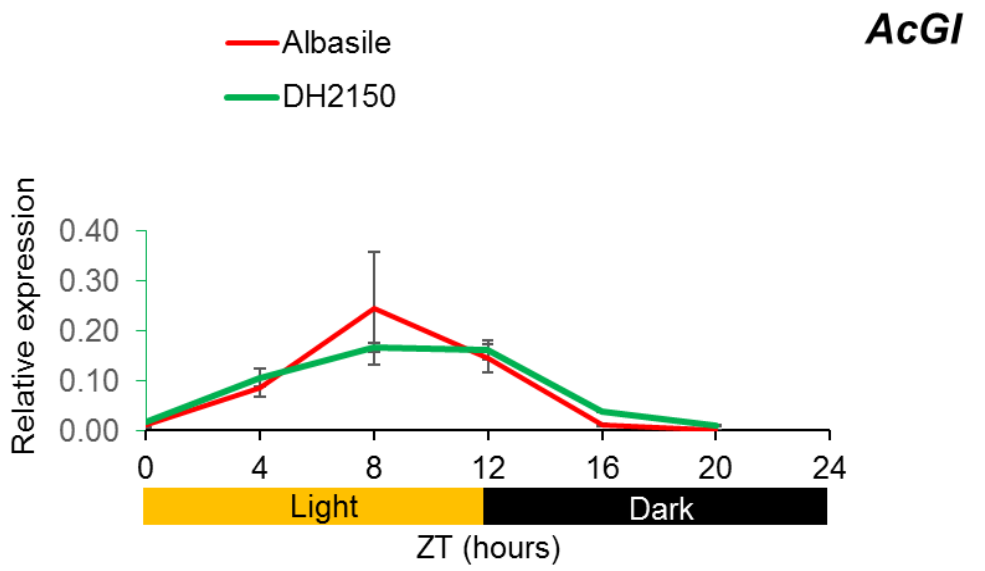

Figure 3.12 Diurnal expression of *AcGI* in SD and LD onions under SD condition. *AcGI* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring the plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

Expression of *AcFKF1* was next examined under the LD and SD condition in the LD and SD onion varieties. In the SD condition, *AcFKF1* showed diurnal expression patterns, expressed at a low level in the morning, peaking up in the afternoon, going down at dusk in SD and LD onions. The peak of *AcFKF1* was seen at ZT 8 in both onion cultivars.

In LD condition, expression of *AcFKF1* was almost identical during the daytime in LD and SD onions (Appendix 11). In SD onion, expression was seen to peak at ZT 21.

No phase shift in the expression pattern of *AcFKF1* was seen in LD and SD onions under the SD condition. This result suggests that the *AcFKF1* are not involved in the different bulbing behaviour of two onion cultivars studied here.

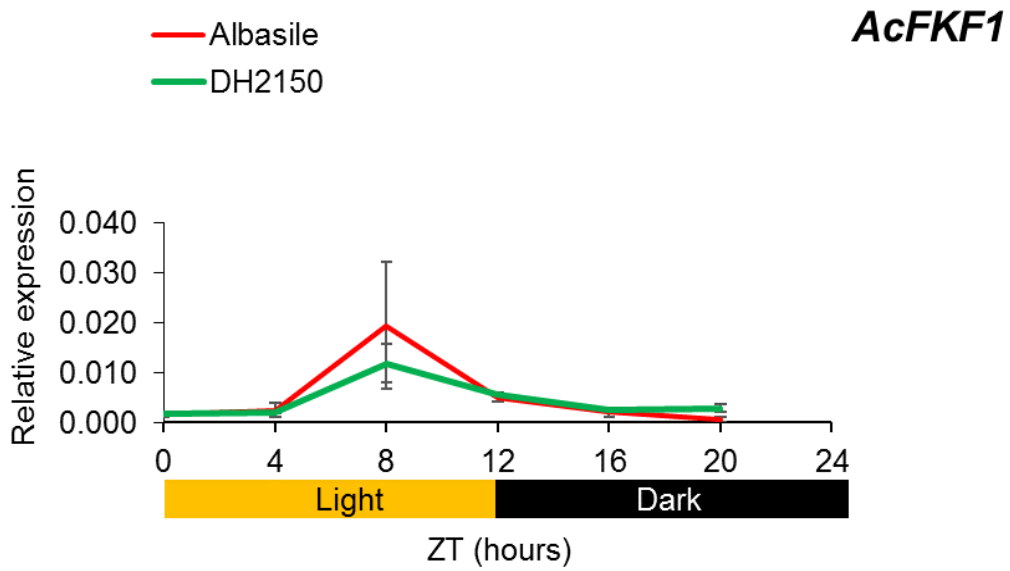

Figure 3.13 Diurnal expression of *AcFKF1* in SD and LD onions under SD condition. *AcFKF1* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring the plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

The *CO* acts upstream of the *FT*, which promote flowering in response to LD in *Arabidopsis* and accumulation of CO mRNA occurs at the end of the light period in LD, whereas in SD, CO mRNA level peaks during the night (Samach *et al.*, 2000; Valverde *et al.*, 2004; Song *et al.*, 2012). Thus, expression of *AcCO* was next examined under SD light condition in the LD and SD onion cultivars.

The *AcCO* showed no distinct peak or apparent diurnal expression pattern and expressed constitutively in both LD and SD onions (Figure 3.14). This result suggest that the *AcCO* is not regulated by the circadian clock or diurnal regulation.

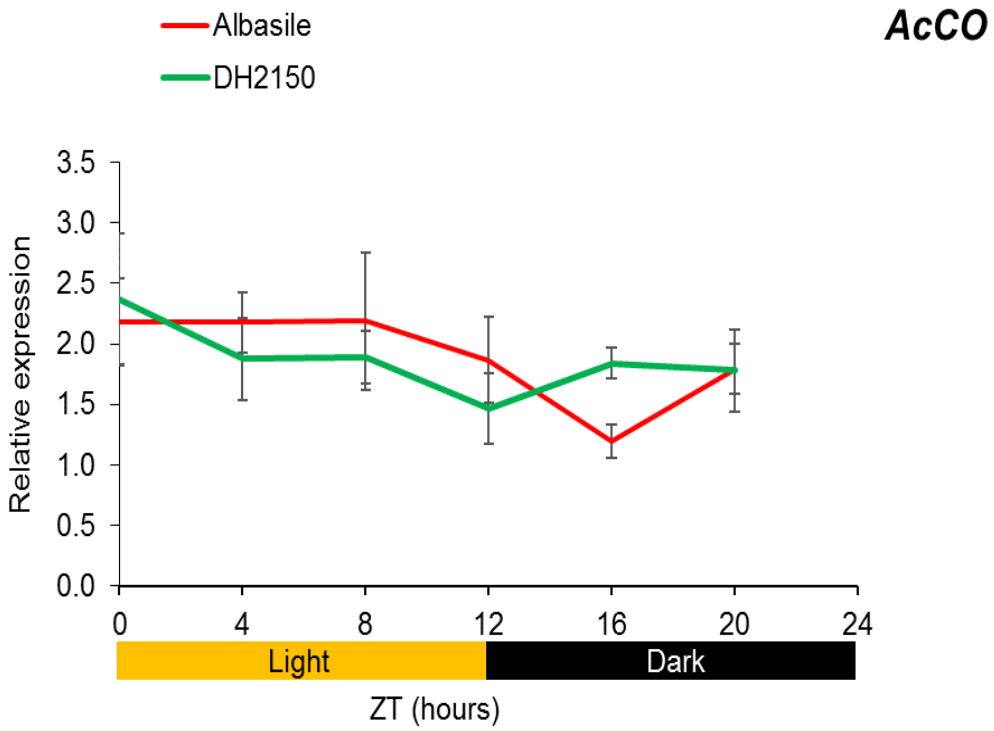

Figure 3.14 Diurnal expression of *AcCO* in SD and LD onions under SD condition. *AcCO* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring the plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

3.4 Discussion

The aim of this chapter was to characterize photoperiodic pathway genes responsible for the adaptation of onion under different daylength. In order to achieve this, the expression of photoreceptor and circadian clock and output genes were measured using RT-qPCR over a 24 hours period under the LD and SD light condition in LD (2150) and SD (Albasile) onions cultivars.

In the present investigation, *AcFTs* results indicate that expression of these genes (*AcFT1* and *AcFT4*) is altered in LD and SD onion under different daylength. Diurnal experiment

(Figure 3.1 to 3.4) shows that *AcFTs* in LD and SD onions respond differently under different conditions. *AcFT4* was not expressed in both, LD and SD onion varieties under the LD condition, but in the SD condition, it was expressed throughout 24 hours period in LD onions and low level of expression in SD onions. This consistent with that *AcFT4* role in repressing bulbing under non-inductive daylength. *AcFT1* was not expressed in LD onion under SD light condition (Figure 3.2) but expressed in the LD condition. Also, under the LD condition, *AcFT1* was expressed in both cultivars (Figure 3.2). This suggest that *AcFT1* is promoting bulbing under inductive daylength. Overall results of *AcFTs* suggest that onion cultivars adapted at different latitudes required different CDL to regulate *AcFT1* to form bulbs. Also, under LD condition, *AcFT1* expression was seen 4 hours early (ZT 13) in SD onion than the LD onion (ZT 17; Figure 3.1). So, there is a shift in the expression of *AcFT1* in the LD light condition, and this could be due to alter expression of upstream photoperiodic pathway genes. These results indicate that *FTs* regulated by upstream photoperiodic pathway genes and these genes might act differently in SD and LD onion.

Alongside *AcFTs*, onion has two distantly related *AP1-like* genes (*AcAP1* and *AcVRN1*) that evolve in bulb onion through gene duplication and are also involved in bulbing and flowering. *AcAP1* and *AcVRN1* are thought to be involved in bulbing and flowering respectively. *AcAP1* was not expressed in LD onion under the SD condition but was expressed in SD onion under the SD condition. This result suggests that *AcAP1* is also involved in bulb initiation under inductive daylength. Expression of *AcAP1* was seen 4 hours earlier than the *AcFT1,* which indicate that *AcAP1* might regulate *AcFT1*. In future, more experiments need to be done to confirm this. The experiment could be overexpression *AcAP1* gene in transgenic onion and see which other genes are activated or RNA-Seq experiment, to identify which genes are expressed in the bulbing and non-bulbing population. At this point, we do not know whether *AcAP1* is regulating *AcFT1* or vice versa.

The different plants adapted to different latitudes by altering genes involved in day length sensing (Nakamichi, 2015). In *Arabidopsis* and other plants, light signals perceived by phytochrome and cryptochrome stabilize CO protein in long days and this CO protein plays a crucial role in translating photoreceptor-perceived light signals into the expression of the *FT* (Valverde *et al.*, 2004). In this study, the expression of *PHYA*,

PHYB, and *PHYC* are not altered in both onion cultivars under SD condition (Figure 3.6 - 3.8). This indicates that the photoreceptor genes are not altered in LD and SD onion under SD condition and hence, not involved in the different bulbing behaviour of these two onion cultivars examined here.

Light-signalling pathways from photoreceptors regulate the clock components (central oscillator and output pathway genes) to entrain the clock. Results of circadian clock genes (*AcCCA1*, *AcPRR9*, and *AcPRR7*; Figure 3.9 – 3.11) and output pathway genes (*AcFKF1*, *AcGI*, and *AcCO*; Figure 3.12 – 3.14) reveals that there is no phase shift in these genes under SD condition in both LD and SD onion cultivars and might be not involved in the different bulbing behaviour of these two onion cultivars examined here under different daylength.

CO is known to promote flowering in long days by activating the expression of *FT*. In this study, the expression of *AcCO* showed no distinct peak or apparent diurnal expression pattern. This suggests that the *AcCO* might be not regulated by the circadian clock. 'Khosa (2018) identifies six transcripts encoding *CO-like* genes in RNA-seq datasets of bulb onion. Bulb onion CO-like proteins grouped into three groups (group 1-3; Appendix 12). Four bulb onion CO-like proteins cluster in group two and one bulb onion CO-like (*AcCOL9*) cluster in group three. One bulb onion CO-like (*AcCOL*) cluster in group one and displayed a close relationship with *Arabidopsis AtCOL3* to *AtCOL5* in clade C. However *CO-like* genes involved in photoperiodic flowering and tuberization in other plants grouped in clade A and B'. The *AcCO* looked in this study might be not involved in bulbing.

Working in our laboratory by Khosa (2018) identified a mutation in the AcFKF1 gene in SD onions. He found missense mutations (Appendix 13) in SD onion (Pusa Madhavi, H-6, and Albasile) but not in LD onion (CUDH2150 and CUDH2107). This mutation could be responsible for the adaption of onion to different daylength. But to confirm this, more experiment needs to be done in future.

This study will help to understand the difference between LD and SD onion adaptation, which is important for adapting new onion cultivars at different latitudes.

Chapter 4 Genetic transformation of onion

4.1 Introduction

The genetic transformation method has become an important tool for modern crop improvement as well as for studying gene function in plants (Nyaboga *et al.,* 2015). *Agrobacterium tumefaciens*-mediated transformation is a preferred method in many plants. *A. tumefaciens* is normally used in gene transfer to dicot plants. While monocot plants were thought to be recalcitrant to this technology as they were outside the host range of the bacterium (Zheng *et al.,* 2001). However genetic transformation has been done in monocot plant using specific strains of *Agrobacterium* (Hiei *et al.,* 1997; Li *et al.,* 1996; Park *et al.,* 1996).

It is challenging to establish the functional role of genes in bulb onion due to a lack of efficient transformation protocols (Eady, *et al.,* 2000; McCallum, 2007). Thus, it is important to develop efficient plant transformation protocol in bulb onion to study various genes responsible for bulbing and flowering in onion. *In vitro* culture and plant regeneration from *Allium* explant has been widely reported and reviewed but only a few contain regeneration protocols suitable for use in onion transformation (Eady, 1995). Eady *et al.,* (2000) developed *Agrobacterium tumefaciens* mediated transformation protocols, but unfortunately, these have very low transformation efficiency and are highly dependent upon a genotype. Several other studies also reported plant transformation from the mature zygotic embryo (Zheng *et al.,* 2001), immature zygotic embryo (Marinangeli *et al.,* 2005), immature flower bud (Pike and Yoo, 1990), and mature flower bud (Luthar and Bohanec, 1999). But plant transformation is highly dependent upon the cultivars (Tanikawa *et al.,* 1998). While on the other hand, somatic embryogenesis (SE) is considered a useful tool for genetic study and large-scale propagation of the plant Sivanesan *et al.,* 2015). SE is a developmental process in which a plant somatic cell can dedifferentiate to a totipotent embryonic cell that has the capability to give rise to an embryo under appropriate conditions and this new embryo can further than develop into a whole new plant (Nyaboga *et al.,* 2015). SE is most suitable for physiological and morphological studies in the plant during morphological development. In onion, SE has been reported in the past from mature zygotic embryos and regeneration occurred via callus (van der Valk *et al.,* 1992; Zheng *et al.,* 2001).

The lack of an efficient and reliable genetic transformation protocol has significantly hampered the functional studies of genes in the bulb onion. As described in chapter 1,

AcAP1 and *AcFT1* promotes bulbing and result suggests that *AcAP1* might work upstream of *AcFT1*. But to confirm this in the future, *AcAP1* needs to be overexpressed in the plant. It is better to generate the whole plant from the callus to study gene function but even overexpressing genes in callus one can study the gene function. Thus, the aim of this chapter was to develop an efficient and reliable *Agrobacterium tumefaciens*-mediated protocol for generating transgenic onion callus cells from shoot tip tissue of germinating seed.

4.2 Results

The purpose of this study was to develop an efficient protocol for generating transgenic onion callus cells to study gene function. The tissue used in this onion transformation experiment was shoot tip tissues of germinating seed. Shoot tips, not more than 5 mm were excised once seed germinated and used to produce callus. The *A. tumefaciens* strain GV3101 was used for the transformation of the PLK, whereas *A. tumefaciens* strain EHA105 was used for the DH2107 transformation. Gateway cloning was used to introduce DNA sequence of *AcAP1* gene within a TOPO vector (entry vector) into the pARTB-GW-egfpER plasmid (destination vector; section 2.12.1 – 2.12.3 of chapter 2). The construct was transferred to *A. tumerfaciens* GV3101 and EHA105 via electroporation. *eGFP* (*enhanced green fluorescent protein*) and *Bar* (*bialaphos acetyltransferase*) gene was used as a selectable marker for the selection of transformed cells and tissues.

4.2.1 Effect of bleach sterilization on germination

Seeds collected from an open field and greenhouse are very often contaminated by fungi and bacteria. Therefore, germinating seeds on sterile media can be particularly challenging due to the contamination of the plates. Bleach is toxic to the plant tissues, and hence optimum concentrations, duration of exposure of explants to bleach need to be determined to minimize explant injury and to achieve a better germination rate. *In vitro* seed germination is negatively affected by bleach at high concentration, whereas bleach is ineffective for sterilization at low concentration (Hsiao and Hanes, 1981). In this study 50% (v/v) commercial bleach was used to sterilize the onion seeds. When seeds were soaked in 50% commercial bleach for 20 minutes, the germination rate was very low (approximately less than 50%; data not provided). Treatment with 50%

commercial bleach and soaking time of 12 min was selected as the best sterilization treatment (germination rate >80%; Figure 4.1B). The optimum concentration of bleach and soaking time of seeds into bleach is crucial otherwise seeds are killed in the process.

4.2.2 Callus induction

Callus was induced on two onion cultivars (PLK and DH2107) from shoot tip tissue of germinating seed. Composition of callus induction media (CIM) is mention in Table 2.5 of chapter 2. Previously, the induction of embryogenic callus from several explants of onion has been achieved by supplementing growth hormone Auxin such as 2,4-D and picloram into CIM (van der Valk *et al.,* 1992; Eady *et al.,* 1998; Zhang *et al.,* 2004). In the present investigation 1 mg/L 2,4-D was used as the source of Auxin. Callus formation from excised shoot tips (Figure 4.1C) became visible after two to three weeks on the CIM containing 1 mg/L 2,4-D (Figure 4.1D). Two types of callus were form from embryos: a compact type and a friable type (Figure 4.1E). Callus growth on CIM was very slow. Mikami and Kinoshita (1988) also observed the slow growth of callus of *Allium* compared with other monocots such as rice. After five weeks, both callus types were selected for transformation. In this study, callus was effectively and efficiently induced from PLK and DH2107 (Figure 4.1D).

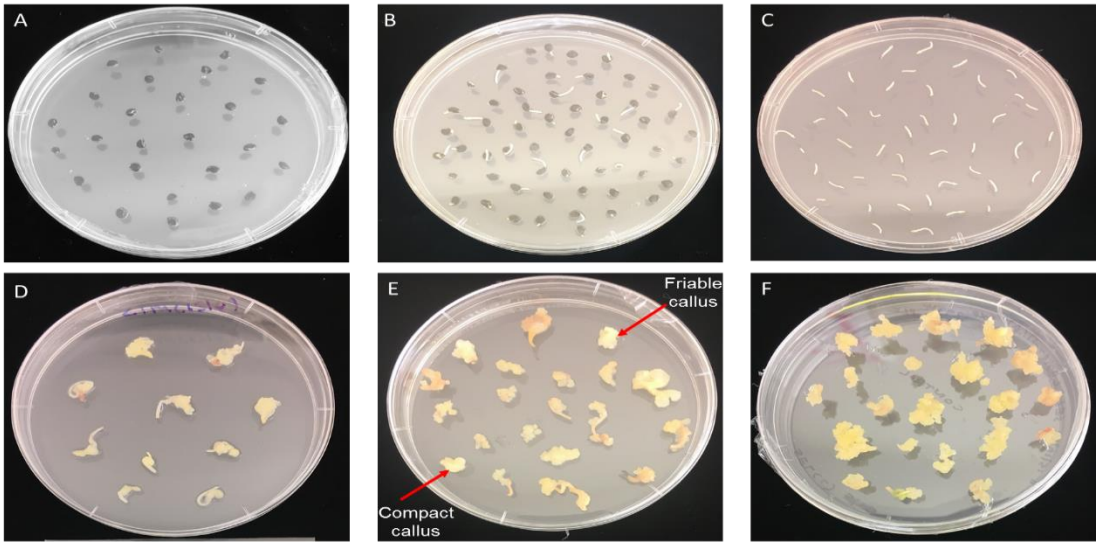

Figure 4.1 Steps for *Agrobacterium* mediated transformation of *Allium cepa*. (A) Seeds on CIM (B) Seed germination on CIM after 5-7 days (C) 4-5 mm shoot tips excised from germinated seed (D) Callus initiation on callus induction media after 3 weeks (E) Callus after co-cultivation with *Agrobacterium* after 3 days (F) Callus on selection media after 5 weeks.

4.2.3 Preparation of *Agrobacterium* for transformation

The DNA sequence of the *AcAP1* gene was cloned into the pCR™8/GW/TOPO vector (section 2.12.1 of chapter 2). The plasmid pARTB-GW-egfpER-*AcAP1* was obtained by gateway cloning the DNA sequence of *AcAP1* gene from the pCR™8/GW/TOPO-*AcAP1* plasmid via restriction digest with enzymes XhoI into the plasmid pARTB-GW-egfpER (section 2.12.1 – 2.12.3 of chapter 2). Calli of PLK and DH2107 were transformed with *Agrobacterium tumefaciens* strain GV3101 and EHA105 respectively, harbouring the binary vector pARTB-GW-egfpER containing onion *AcAP1* gene. The schematic map of T-DNA region is shown in Figure 4.2. *AcAP1*, *eGFP*, and *Bar* genes were under the control of the CaMV35S, pMAS (promoter of *mannopine synthase* gene), and pNOS (promoter of *nopaline synthase* gene) promoter respectively. Both *bar* and *enhanced green fluorescent protein* (*eGFP*) genes were used as a selectable marker for selection of transgenic calli based on BASTA resistance and eGFP fluorescence. BASTA contains L-phosphinothricin (PPT), which inhibits the action of enzyme glutamine synthetase. Resistance to this agent is conferred by the *bar* gene.

AcAP1 and *AcFT1* gene are involved in the bulbing process. Whether the *AcAP1* gene works upstream of *AcFT1* is not clear. By overexpressing the *AcAP1* gene under 35S promoter, one can establish the role of the gene in bulb induction.

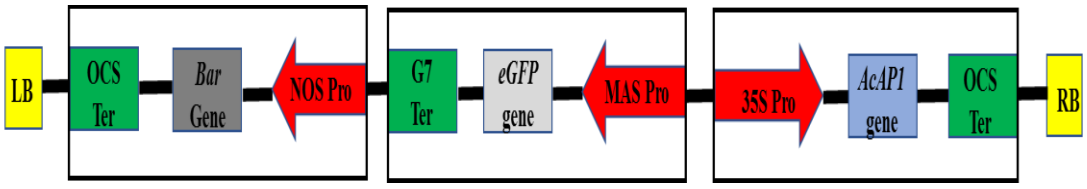

Figure 4.2 Schematic map of T-DNA region of plasmid pARTB-GW-egfpER. LB, left border; OCS Ter, octopine synthase terminator; *Bar* gene, herbicide resistance gene; NOS Pro, promoter of *nopaline synthase* gene; *eGFP* gene, *enhanced green fluorescent protein* gene; MAS pro, promoter of *mannopine synthase* gene; 35S pro, CaMV 35S promoter; *AcAP1*, *Allium cepa AP1* gene; RB, right border.

4.2.4 Transformation and Selection

Five weeks old callus was subjected to Agro-infection using *Agrobacterium* carrying the gene construct (Figure 4.2) and co-cultivated for 3 days on co-cultivation medium in the dark. After 3 days on co-cultivation media, callus was transferred to the pre-selection media for 1 week in dark. After 1 week, callus was transferred to the selection media for

3 weeks in dark then shifted to light. After 3 weeks on the selection medium, some of the callus turned brownish (very few) while the other remained creamish (Appendix 21). Creamish colour callus was shifted to fresh selection medium. Brown colour callus was not looking healthy, this could be due to dying cells and thus, it was discarded.

One of the important factors for the genetic transformation of any plant is the selectable marker system that allows the identification of transformed cells and tissue (Wilmink and Dons, 1993). Hence, an efficient selection protocol is essential. In this study, eGFP and BASTA were used for selecting transformed cells and tissues. Two concentrations of BASTA (5 and 10mg/L) were used in this study.

For identification of transformed tissues, the callus was examined for GFP expression using a fluorescent microscope after the co-cultivation period of 3 days. It was hard to differentiate transformed and non-transformed callus with GFP expression. This could be due to the big and thick size of callus. To see transform cell easily, callus was divided into small pieces, but still was not able to differentiate the transformed cells.

After one week on pre-selection media, the callus was transferred on a selection media containing BASTA, selective agent to kill untransformed plant tissue. The first four weeks 5 mg/L BASTA concentration was used on plates to kill the untransformed plant tissue. After four weeks, all the callus looks the same. This indicated that the 5 mg/L concentration of BASTA might be not effective to kill the untransformed plant tissue. So, half of the callus was subsequently transformed to selection media containing 10 mg/L BASTA. The same results were observed. This result indicates that BASTA might be not working effectively to kill the untransformed tissues. In future, higher concentration of BASTA could be used (10-30 mg/L) to see the effectiveness as a selective agent. Due to time limit this was beyond scope of this project.

4.3 Discussion

In this study, callus was developed efficiently from the shoot tip tissue of germinating seed from both the onion cultivars (DH2107 and PLK) using *A. tumefaciens* strain GV3101 and EHA105. The choice of the explant for genetic transformation is very crucial. In onion, plant transformation from mature zygotic embryo (Zheng *et al.*, 2001), immature zygotic embryo (Marinangeli *et al.*, 2005), immature flower bud (Pike and Yoo, 1990), and mature flower buds (Luthar and Bohanec, 1999) were reported but

transformation efficiency was very low. Immature embryos are the preferred explants due to actively dividing embryonic cells (Dong *et al.,* 1996; Eady *et al.,* 2000) but the biennial life cycle of onions makes difficult to get immature embryos all year round. Callus induction from the mature seeds is a major step ahead because it is possible to carry out genetic transformation of onions all year round.

Surface sterilization of the explant is the most important step prior to culture initiation. In this study, more than 80% seed germination rate was observed when onion seeds were soaked in 50% commercial bleach for 12 minutes and were selected as the best sterilization treatment. Optimum sterilization time is very important, leaving too long will kill the seed and taking out seed early will not kill the microorganism present on the seed surface.

Reliable callus induction and regeneration of viable plants are crucial for the genetic improvement of the major crops (Murphy, 2003). It is better to develop the whole plant from the callus to study gene function. But the rate of plant regeneration via somatic embryogenesis of onion has been limited to specific cultivars and short-lived (Eady 1995; Eady *et al.,* 1998). Further due to the biennial life cycle of onion, it is difficult to obtain immature embryos any time of the years. Also, it is hard to dissect embryo from the immature seed. Due to time constrain, it was beyond my scope to generate the whole plant from the callus. In this study, callus was induced efficiently from the shoot tip tissue of germinating seed of both onion cultivars using two different strains of *Agrobacterium*.

BASTA and GFP was used as selectable markers for onion transformation. The size of callus during co-cultivation was thick and big which could be hindering the visualization of the GFP. Even after chopping the callus into small pieces unable to see any GFP expression. Both the concentration (5 and 10mg/L) of BASTA were not able to kill the untransformed cells and tissues. Few calli were showing browning otherwise calli looked in healthy condition. This suggests that the BASTA concentration used in this study was not able to kill the untransformed cells. These results suggest that cells were not transformed.

Overall, callus was induced efficiently from shoot tip tissue of PLK and DH2107 using *Agrobacterium* strains GV3101 and EHA105 respectively within three weeks times.

4.4 Future direction

Following point needs to be consider in future for improvement of genetic transformation in onion:

- Additional strains of *A. tumefaciens* could be compared to determine if another would result in higher transformation efficiency.

- Successful genetic transformation required effective selection systems. BASTA was not effective as a selective agent in these two onion cultivars. In future, Hygromycin could be used as selective agents for selection of transform cells and tissues.

- In this study five weeks old callus was transformed. In future, transforming callus early could increase transformation rate.

- Dividing callus before co-cultivation could also help to transform cells and tissue efficiently.

Chapter 5 Development of molecular marker for *AcVRN1* gene

5.1 Introduction

Over the years, several types of molecular markers have been developed and used in plant breeding. Onion is one of the most important vegetable crops cultivated throughout the world, ranks second after tomato in terms of total world production annually (Pathak, 2000). Despite its economic significance as one of the major vegetable crops worldwide, genetic study of onion is limited. This is due to its large genome size, cross-pollinated nature, biennial life cycle, high percentage of repetitive DNA, and high inbreeding depression (Kuhl *et al.,* 2004; McCallum *et al.,* 2006; McCallum 2007; Duangjit *et al.,* 2013). Various conventional plant breeding methods have been used till date to improve yield, quality, and resistance to pest and diseases to allow onion to be grown around the world but to meet the demand of fast growing population of the world, there is an urgent need to enhance onion breeding through the integrated use of genomics tools (McCallum, 2007). The development of genomic resources such as genetic markers, mapping populations and saturated linkage maps are providing new insights into the genetic and molecular basis of important economic traits (Brewster, 2008; Baldwin *et al.,* 2012a, *b;* Duangiit, *et al.,* 2013; Lee *et al.,* 2013; Kim, *et al.,* 2014; Finkers, *et al.,* 2015; Macknight *et al.,* 2016a). In onion, biennial life cycle and high inbreeding depression make the use of molecular markers extremely attractive for breeding programmes (McCallum, 2007).

Molecular markers are the most abundant and most widely used types of marker in crop improvement. Several classes of molecular markers have been developed and mapped in onion. Gel electrophoresis and recombinant DNA technologies permitted for the identification of numerous molecular markers and for the development of the first genetic maps (Havey, 2019). At first, polymorphisms were discovered by isozymes, restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs), and single nucleotide polymorphisms (SNPs; King *et al.,* 1998; Rouamba *et al.,* 2001 Martin *et al.,* 2005; Baldwin *et al.,* 2012a, b; Macknight *et al.,* 2016a). Among these markers, SNPs are the most widely and preferred genetic markers used in breeding programme due to their high abundance, even distribution, and strong association with the traits of interest (Hayward *et al.,* 2012). With the arrival of next generation sequencing (NGS) technologies, SNP markers have been developed from the expressed regions of the genome of an inbred onion line and this SNP used to construct a genetic map (Duangjit *et al.,* 2013). Onion genome has been not

been sequence so far, however RNA-sequencing has been utilized to develop genomic resources and these genomic resources facilitate the researcher to identify the genes and molecular markers associated with important traits in onion (Khosa *et al.*, 2016).

Premature flowering (bolting) during bulb production is an undesirable trait and serious losses for growers. Bolting resistance is particularly important for bulb onions that are sown in the fall, overwinter in the field, and produce bulbs for harvest in the spring (Cramer, 2003). After setting true leaves (passing juvenile phase), onion plants can be induced to flower by exposure to low temperatures (Brewster, 2008). Basal plate (shoot apical meristem) is the most sensitive region to vernalization (Brewster, 2008; Khokhar, 2014). Vernalization period is all that is needed to initiate flowering from onion bulbs, but temperature and photoperiod influence the time taken for the inflorescence to appear (Khokhar *et al.*, 2007; Lee *et al.*, 2013). There are clear phenotypic differences in the ability of specific germplasms to bolt; onion cultivar grown in the temperature climate require 3 to 4 °C, whereas cultivar grown in tropical climate require 10-21 °C (Brewster, 2008; Khokhar, 2014). The growing of un-adapted onions and early sowing cause premature bolting during bulb production (Brewster, 2008).

Over the year's physiological basis of vernalization in onion has been studied, but not much emphasis has been given on the genetic and molecular basis (Brewster, 2008; Lee *et al.*, 2013; Baldwin *et al.*, 2014; Khosa *et al.*, 2016; Macknight *et al.*, 2016a). Vernalization has been studied at molecular level in model plant, *Arabidopsis*. In *Arabidopsis*, vernalization results in the repression of the floral repressor *FLC* and this allows the expression of *FT* gene, which triggers flowering (Song *et al.*, 2012). Our laboratory identified six *FT-like* genes (*AcFT1* to *AcFT6*) in double haploid line DH2150, and found that *AcFT1* and *AcFT4* regulate bulbing, whereas *AcFT2* regulates flowering (Lee *et al.*, 2013). Khosa (2018) found that *AcRVR1* and *AcVRN1* genes respond to cold and play an important role in the process of vernalization. In temperate grasses, coding and intron regions of *VRN1* display polymorphism in spring and winter cultivars and this polymorphism are linked with vernalization requirement and flowering time (Fjellheim *et al.*, 2014). But in bulb onion, no polymorphisms were found in the coding region of *VRN1* gene (Khosa, 2018).

Studies showed that vernalization pathway has evolved separately in monocots and dicots (Greenup *et al.*, 2009). Onion diverged 122 million years ago from the monocot

grasses (Janssen and Bremer 2004), therefore the genetic mechanisms controlling cereal flowering may differ in the Aspargales.

Hyun *et al.,* (2009) reported first genetic analysis of onion bolting, who examined F_2 family segregating for time to flowering using proteomic methods. They revealed that late flowering segregated as a dominant character and identified proteomic difference among parents, but they did not examine segregation of these with bolting traits. Baldwin *et al.,* (2014) studied variation at 10 candidate genes involved in flowering response across eight diverse populations and identified a major quantitative trait locus (QTL), *AcBlt1* on chromosome 1 affecting pre-mature bolting. *AcVRN1* gene turns on by vernalization but optimum temperature for vernalization varies; temperate cultivar requires 4 °C, whereas tropical cultivar requires 10-21 °C.

Aims of this chapter is to investigate whether *AcVRN1* gene and *AcBlt1* locus are the similar or not. If so, then plans to develop markers to identify which onion cultivar bolt at 4 °C and which one at 10-21 °C. For this, we use various LD (DH2107, DH2150, Pukekohe long keeper), SD (Albasile, Nasik red), and F1 population (cross between 'DH2107 x Albasile', 'DH2150 x Nasik Red'). Developing molecular markers will be useful for breeders to identify cultivars which are susceptible to bolting at early stage of onion development.

5.2 Results

The goal of this chapter was to identify polymorphism to the *AcVRN1* gene. The onion genome is yet to be fully sequenced and very little genomic sequence is available. Therefore, cDNA sequences had only been used identify polymorphism in promoter and intron 1 region of *AcVRN1* gene. Unfortunately, working in our laboratory, Khosa (2018) found no polymorphisms in the coding region of the *AcVRN1* gene. Also, Robyn Lee working in our laboratory did not find any polymorphisms in the intron 2 (unpublish data). So, in this study, promoter and intron 1 region targeted to find out polymorphism in the *AcVRN1* gene.

5.2.1 Isolation of unknown *AcVRN1* promoter sequence using TAIL-PCR

Previously in our laboratory with a specific primer on both sides of the exon boundary did not amplify the intron region. Therefore, genome walking was used for determining the DNA sequence of the unknown promoter and intron 1 region flanking a region of known DNA sequence (Exon 1 and 2). The flanking sequences adjacent to the known sequences cannot be amplified by conventional PCR because the sequences are unknown. Therefore, specific primers pairs cannot be designed to amplify such region. The most used methods for flanking sequence amplification are the random primed methods and ligation-mediated methods (Uchiyama and Watanabe 2006). In this study, random primed PCR called thermal asymmetric interlaced PCR (TAIL-PCR; develop by Liu and Whittier, 1995) is used for isolation of unknown promoter and intron 1 region from known sequence of exon 1 and exon 2 respectively (Figure 5.1 and Figure 5.5). Two types of primers; specific primers (SP) that have a high T_m which bind to the known sequences and arbitrary degenerate (AD) primers that have a low T_m which bind to the unknown sequences were used in TAIL-PCR (see Appendix 2 for primer sequence).

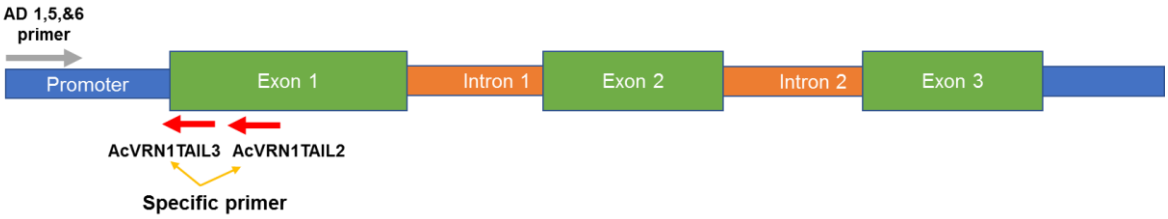

Figure 5.1 Schematic diagram of TAIL-PCR showing specific and AD primers binding sites of promoter region of *AcVRN1* gene. Specific primer AcVRN1TAIL2 and AcVRN1TAIL3 bind to known sequence of exon 1. AD primers (1,5, and 6) bind to unknown region. gDNA from DH2150 used as a template for TAIL-PCR.

The first task was to isolate the *AcVRN1* promoter region from one of onion cultivar DH2150. In order to isolate the promoter sequence of the *AcVRN1* gene using the TAIL-PCR, specific primer AcVRN1TAIL2, AcVRN1TAIL3 and three AD (1,5,6) primer were used. During primary TAIL-PCR all the three AD primers along AcVRN1TAIL2

primer were found to produce different size band (Figure 5.2; Lane 4,5,6). For negative control (water instead of gDNA) no bands were detected (Lane 7).

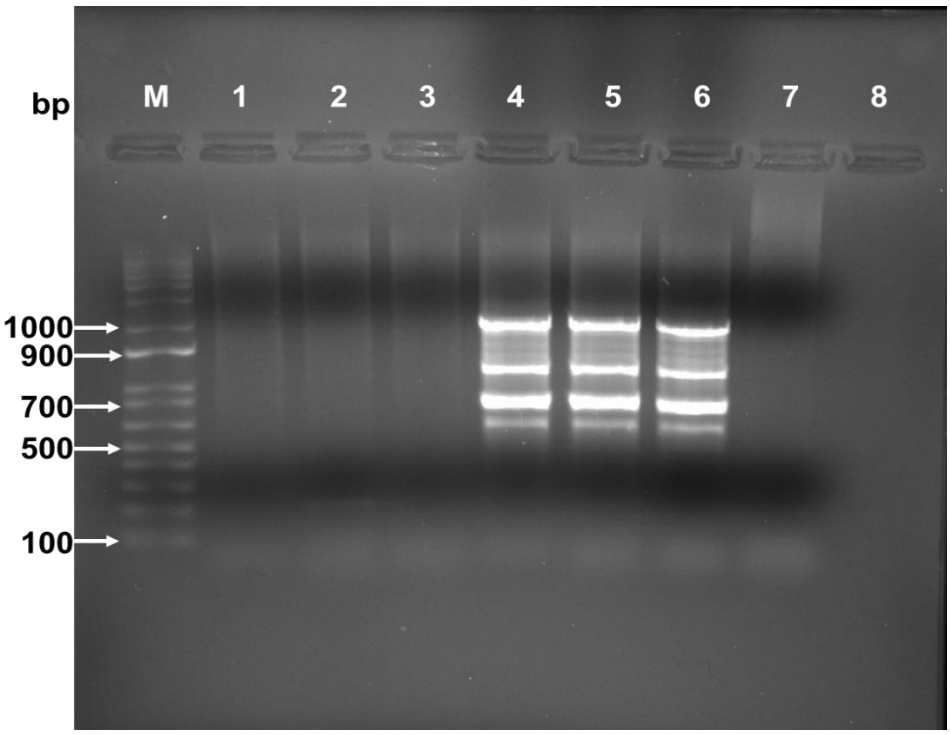

Figure 5.2 Gel electrophoresis of the primary TAIL-PCR products. Lane M – ladder, Lane 4,5,6 – products of the TAIL-PCR using AD1, AD5, and AD6 primers respectively, with AcVRN1TAIL2 as a specific primer, Lane 7 – negative control (water), and Lane 1,2,3,8 – empty lane.

The primary PCR reaction was diluted 1:100 and used as a template in secondary TAIL-PCR. During secondary TAIL-PCR only AD1 primers along with AcVRN1TAIL3 primer were found to produce three different size products (Figure 5.3; Lane 7). one of these three products, (approximately 550 bp) was present at higher amounts. To purify this, the PCR product (Lane 7) were run on lower percentage agarose gels at lower voltage (0.7%; Appendix 14) and purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research; Section 2.8 of Chapter 2). The purified PCR product was then sequence using sanger sequencing. To find polymorphism in the *AcVRN1* promoter region, several onion cultivars (LD, SD, and F1 population; Total 7 cultivars were used) *AcVRN1* promoter sequence needs to be amplified. To do that specific primer were design from the *AcVRN1* promoter sequence of one of onion cultivar sequence earlier.

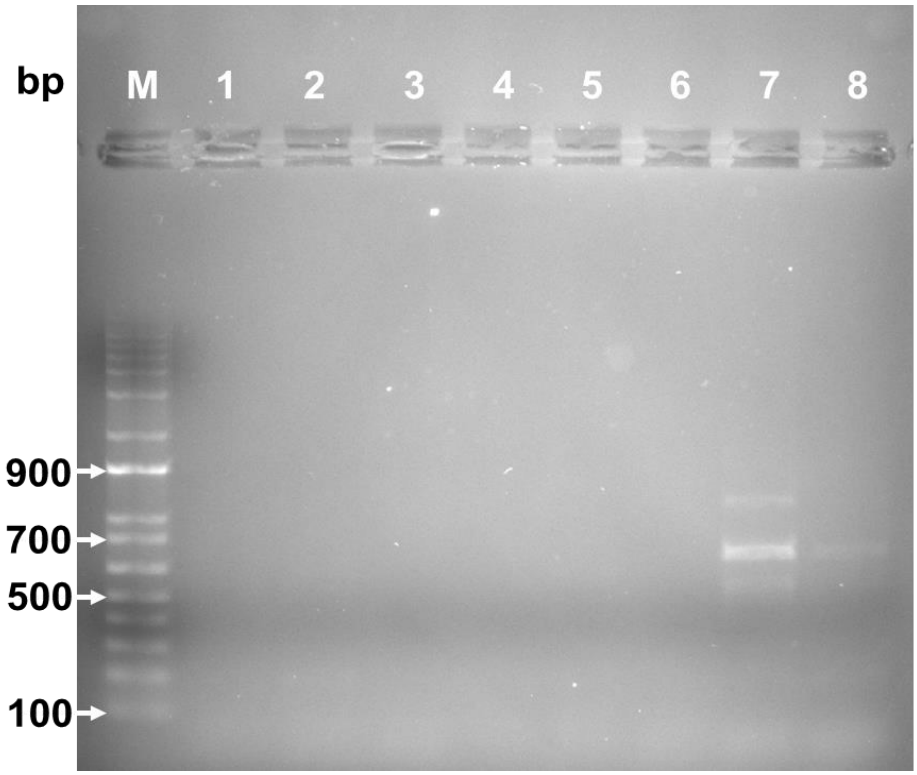

Figure 5.3 Gel electrophoresis of the secondary TAIL-PCR products. Lane M – Ladder, Lane 5 – negative control (water), Lane 6,7,8 – products of the TAIL-PCR using AD6, AD1, and AD5 primer respectively, with AcVRN1TAIL3 as a specific primer, Lane 1,2,3,4 – empty lane.

After designing specific primer, promoter region of *AcVRN1* were amplified using normal PCR. A total of seven onion cultivars were sequenced using sanger sequencing to analyse sequence variation and find SNPs. After eliminating the unreadable sequences, a fragment of 447 bp was obtained. Nucleotides alignment of promoter sequence of *AcVRN1* gene of all seven onion cultivars were done in Geneious software (Figure 5.4 – 1 to 100bp; Appendix 19 – 101 to 447bp).

Nucleotides alignment of *AcVRN1* promoter region of different onion cultivar (Figure 5.4, Appendix 19) showed that no polymorphism was detected in all of onion cultivars. Across all onion cultivars, heterozygous genotypes occurred at certain location but heterozygous were present in most all of onion cultivars.

Figure 5.4 Nucleotides alignment of *AcVRN1* promoter region of different onion cultivar. DH2107A – cross between 'DH2107 x Albasile', DH2150NR – cross between 'DH2150 x Nasik Red', ALB – Albasile, NR – Nasik Red, and PLK – Pukekohe long keeper.

5.2.2 Isolation of unknown *AcVRN1* intron 1 sequence using TAIL-PCR

The *AcVRN1* promoter region did not show polymorphism. So next task was to amplify the intron 1 region of *AcVRN1* gene using TAIL-PCR to find out polymorphism. To isolate the unknown intron region of *AcVRN1* gene, specific primer AcVRN1TAIL9, AcVRN1TAIL10 and three AD (1,5,6) primer were used (Figure 5.5). During primary TAIL-PCR all the three AD primers along AcVRN1TAIL10 primer were found to produce four different size PCR products (Appendix 17; Lane 1,2,3). No PCR products were detected for negative control (Lane 4,5,6).

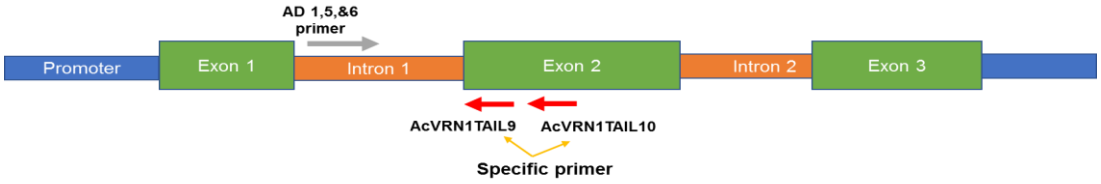

Figure 5.5 Schematic diagram of TAIL-PCR showing specific and AD primers binding sites of intron 1 region of *AcVRN1* gene. Specific primer AcVRN1TAIL9 and AcVRN1TAIL10 bind to known sequence of exon 2, AD primers (1,5, and 6) bind to unknown region. gDNA from DH2150 used as a template for TAIL-PCR.

During the secondary TAIL-PCR, only AD1 and AD6 primers along with AcVRN1TAIL9 primer were found to produce one size PCR products (Appendix 18; Lane 6, 4). Out of AD1 and AD6, AD1 produced PCR products (approximately 450 bp) in high amount. PCR products were purified and sequence using sanger sequencing. To find polymorphism in the intron 1 region, several (LD, SD, and F1 population) onion cultivars *AcVRN1* intron 1 sequence needs to be amplified. To do that specific primer were design from the *AcVRN1* intron 1 sequence of one of onion cultivar sequence earlier.

A total of seven onion cultivars were amplified and sequenced using sanger sequencing. After eliminating the unreadable sequences, a fragment of 371 bp was obtained. Nucleotides alignment of intron 1 sequence of *AcVRN1* gene of all seven onion cultivars were done in Geneious software. Nucleotides alignment of *AcVRN1* intron 1 region did not show any polymorphism in all onion cultivars (Figure 5.6 – 1 to 180bp; Appendix 20 – 181 to 371bp).

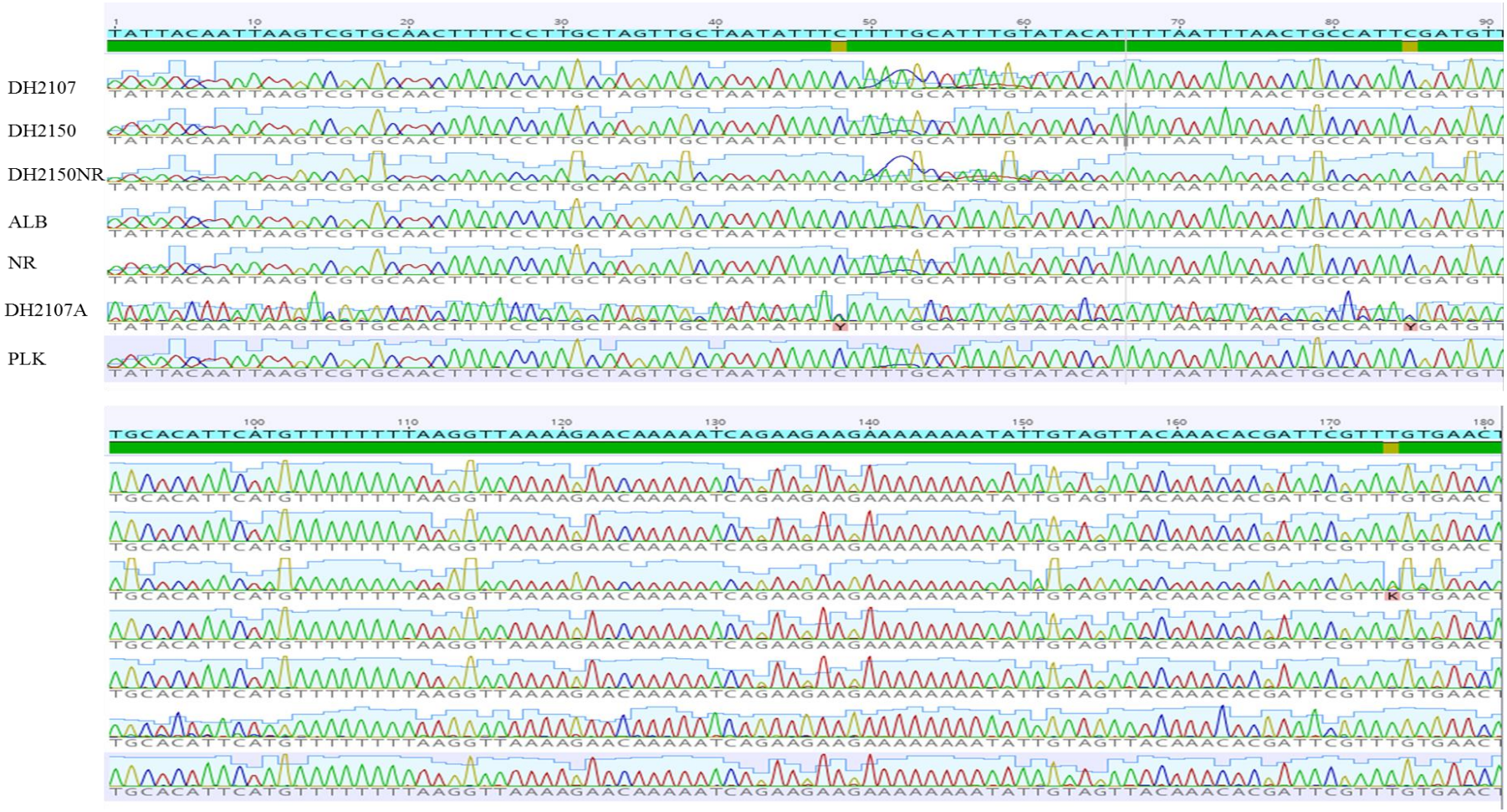

Figure 5.6 Nucleotides alignment of *AcVRN1* intron 1 region of different onion cultivar. DH2107A – cross between 'DH2107 x Albasile', DH2150NR – cross between 'DH2150 x Nasik Red', ALB – Albasile, NR – Nasik Red, and PLK – Pukekohe long keeper.

5.3 Discussion

Premature flowering (bolting) during bulb production is an undesirable trait and selected against by the breeder. In *Arabidopsis,* vernalization results in the repression of the floral repressor *FLC* and this allows the expression of *FT* gene, which triggers flowering (Song *et al.,* 2012). Lee *et al.,* (2013) discovered that *AcFT2* regulates flowering in onion. Baldwin *et al.,* (2014) mapped AcBlt1 locus on chromosome 1, which is responsible for premature bolting. Khosa (2018) in his study found that *AcVRN1* genes respond to cold and play an important role in vernalization. *AcVRN1* gene turns on by vernalization but optimum temperature for vernalization varies between LD and SD onions. Therefore, the objectives of this study were to identity SNPs and insertion-deletion (indels) in *AcVRN1* genes known to be involved in vernalization process and to perform association between *AcVRN1* gene and *AcBlt1* locus.

Genetic markers are important advancements in the field of plant breeding (Kebriyaee *et al.,* 2012). Despite of advances in next generation sequencing technologies, sequencing of onion remains a huge challenge because of its large genome size. Physiological basis of vernalization in onion has been studied extensively over the years, but not much importance has been given on the genetic and molecular basis (Brewster, 2008; Lee *et al.,* 2013; Baldwin *et al.,* 2014; Khosa *et al.,* 2016; Macknight *et al.,* 2016a). Single nucleotide polymorphisms are the most abundant and preferred genetic molecular markers, present in high number, evenly distributed and strong association to the traits of interest (Hayward *et al.,* 2012). However, it is difficult task to develop SNPs in onion due to large genome size and high levels of heterozygosity (King *et al.,* 1998). SNPs identification has been mainly focused on the transcriptome by sequencing doubled-haploid lines or highly inbred in order to avoid polymorphisms among paralogous sequences (McCallum *et al.,* 2008; Duangjit *et al.,* 2013; Kim *et al.,* 2014). A SNPs frequency of 1.7/Kb has been observed in onion and this SNPs have been validated across a wide range of germplasm (Duangjit *et al.,* 2013).

Promoter and intron region were selected because in previous study no polymorphisms were detected in coding region of *AcVRN1* gene in different LD and SD onion cultivars (Khosa, 2018). In this study also, no polymorphisms were detected in amplified sequence

of promoter (447 bp) and introl 1 (371 bp) region of *AcVRN1* gene. The results imply that the *AcVRN1* gene is highly conserved in onion and vernalization requirement are due to gene regulation, not to differences in the encoded promoter, intron, and exon sequence. The promoter and intron 1 regions of all seven onion cultivars studied – which represent LD and SD cultivars from the temperate and tropical groups are identical. One hypothesis would be that the *AcVRN1* gene is regulated by some another gene and this gene response differently in SD and LD onion. This gene in temperate onion turn on at low temperature, while in tropical turn on at high temperature. This study will help to understand the difference between early and late bolting onion cultivars prior to sowing using molecular markers.

Final Conclusions

Onion is grown throughout the world from tropical regions where the daylength is around 12 hours all year round and there is little seasonal differences in temperature, to temperate regions where there are large differences in seasonal daylength and temperatures. As onion uses seasonal changes in daylength and temperature to trigger bulbing and flowering, onions cultivars need to be breed to suit their local conditions. The poor adaptation of onion cultivars at different latitudes results in substantial losses due to reduced yield and precocious flowering.

The goal of this study was to begin to understand molecular and genetic difference between LD and SD onion cultivars, which is important for adapting new onion cultivars at different latitudes.

The daylength-dependent initiation of bulbing places a significant barrier for adapting onion cultivars at the different regions. In comparison to photoperiodic regulation of flowering in *Arabidopsis* and other plants, relatively little is known about genetic regulation of the bulbing process. Chapter 3 aimed to investigate genes responsible for the adaptation of onion under different daylength. The research hypothesis was that photoreceptor and circadian clock genes alter between LD and SD onion resulted in the adaptation to different latitudes. *AcFTs* regulate bulbing in both, LD and SD onions but respond differently under different daylength. The diurnal experiment of *AcFTs* showed that *AcFT1* and *AcFT4* involved in promoting and repressing bulbing respectively. Expression of the different photoreceptors, circadian clock, and output pathway genes was not altered under the SD condition suggesting they are not involved in the different bulbing behaviour of two LD and SD onion cultivars examined here. Future work should investigate the possibility that changes in the protein encoded by the photoperiodic pathway genes, rather than the expression of these genes might be responsible for the difference in bulbing between the LD and SD cultivars.

Genetic transformation is one of the most important method that are used for genetic improvement and for functional analyses of genes in plants. In Chapter 4, an efficient and reliable method was established to produce callus from two different onion genotypes. *A. tumefaciens* strain GV3101 and EHA105 were effective in induction of callus. Having a reliable way to transform onion callus cells will be important for future

work aimed at understanding how the different genes involved in bulbing function. For example, we are interested in if overexpression of the *AcAP1* gene is sufficient to activate *AcFT1*.

The final chapter focused on develop a molecular marker for the *AcVRN1* gene to identify bolting requirement of different onion cultivars. Promoter and intron 1 region did not show any polymorphism which suggest that the *AcVRN1* gene is highly conserved in different onion genotypes. Genetic analysis of onion cultivars by molecular markers will help in the understanding variation for bolting and vernalization requirement.

Overall, this study will help to understand genetic behind the adaptation of onion under different latitudes, which is important for breeding new onion cultivars in the future.

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Appendices

Appendix 1: List of primers used for RT-qPCR

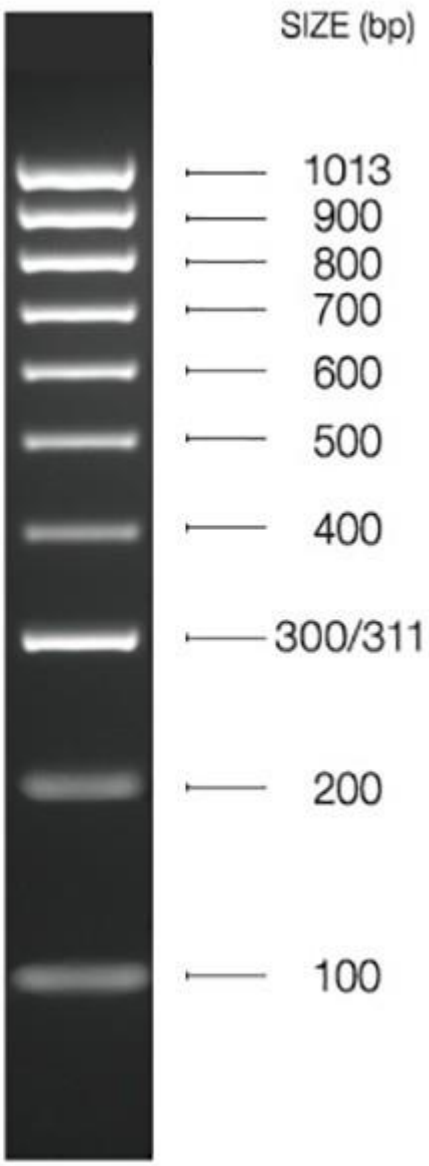
Primer Name	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
AcFT1	AAACCATCACAAATAACTCAGCA	GTTTCTCGCCCAAAGTTCG
AcFT4	TGAAATAGGAGGTGTACCAAGAAT	TTCCGAAACTACCATCCATATTTG
AcAP1	GGGAACAACAAGTCCAGCCA	GCAGCCCATCACTGACTCTT
AcCCA1	GGTTCAACGCGTTTACAAGA	GTACCTCCATCCATCCGTTT
AcPRR9	ATTCAACGCAACTTCTCTCT	CGGAATATGCAATGGGAGTA
AcPRR7	ATTTCCAAACAAACGGGTTC	TTGTCTTCGTGTGATGGAAT
AcFKF1	CCGGTGCAGTTGTTTATGTTG	TCCCACCCACCACACAGGTAC
AcGI	CACAGATGGATTGCTTGTTGA	ATTGGCTACGAGATGTGC
AcPHYA	CTTAAGGTCGCCCCATAGTT	CCCCCAAAGTCTTTTCCTCT
AcPHYB	GAGAGATAGGCGGTGATCTG	TCCTCCACTTCCAAAGCAAT
AcPHYC	TACACACGAATACAAGGCGA	TGACTCTAACTCCAGTCCCA
AcCO	GTATATCCACCATGGCCACA	TATGGGTACGGGTATAGGCA
Acß-tubulin	GTCTTCAGAGGCAAGATGAGCAC	TCAGTCCAGTAGGAGGAATGTCG
Actin	CTGGGATGACATGGAGAAGATT	GTTAAGTGGAGCCTCCGT

Appendix 2: List of primers used for TAIL-PCR and transformation

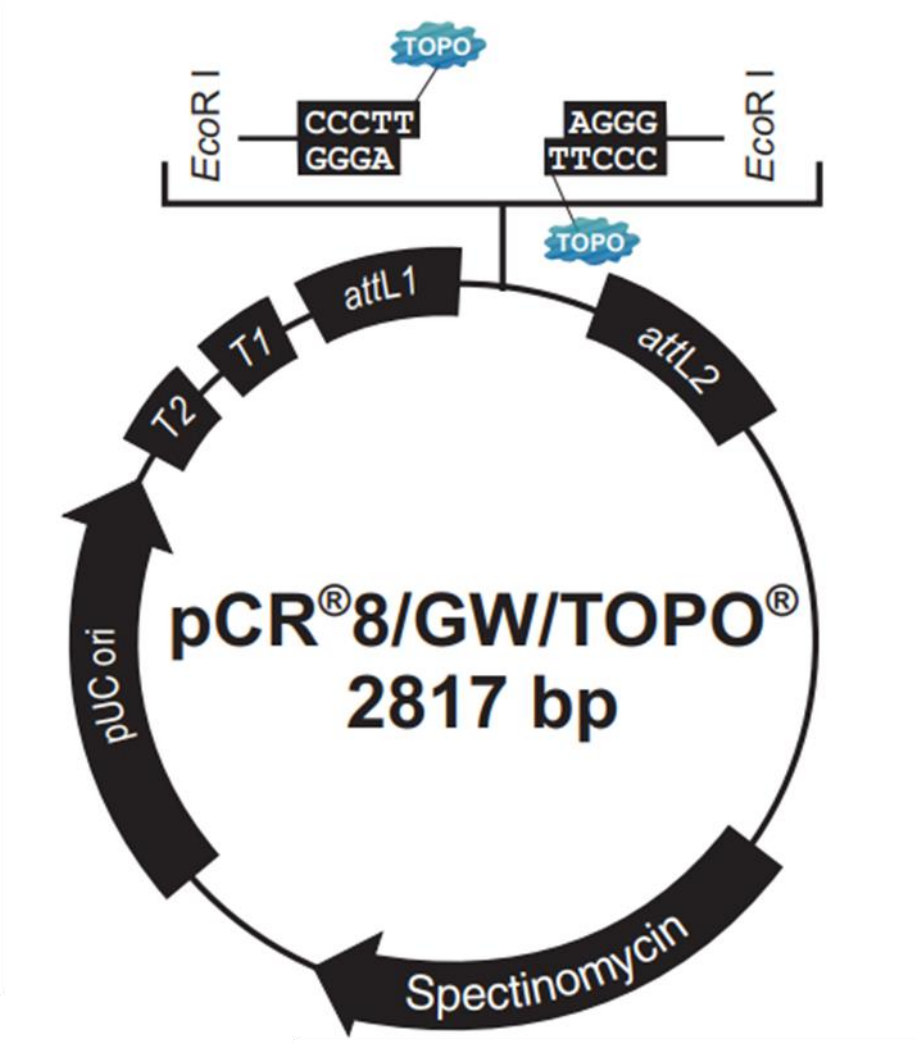
Primer Name	Primer Sequence (5' to 3')
AD1 (Forward Primer)	NGT CGA SWG ANA WGA A
AD5 (Forward Primer)	NTC GAS TWT SGW GTT
AD6 (Forward Primer)	WGT GNA GWA NCA NAG A
AcVRN1TAIL2 (Reverse Primer)	AAG CAA AGT GGT TGC CTT TGA
AcVRN1TAIL3 (Reverse Primer)	TGG AAA TTA TGG GAA ACA GAC A
AcVRN1TAIL9 (Reverse Primer)	TGT CAG TCG ATG TGA ATG ATT
AcVRN1TAIL10 (Reverse Primer)	CAT AAA ATC CAT CTC AGT GCG A
*35S promoter (Forward primer)	CACTGACGTAAGGGATGACG
*AcAP1 (Reverse primer)	GCAGCCCATCACTGACTCTT

Footnotes: * used for onion transformation

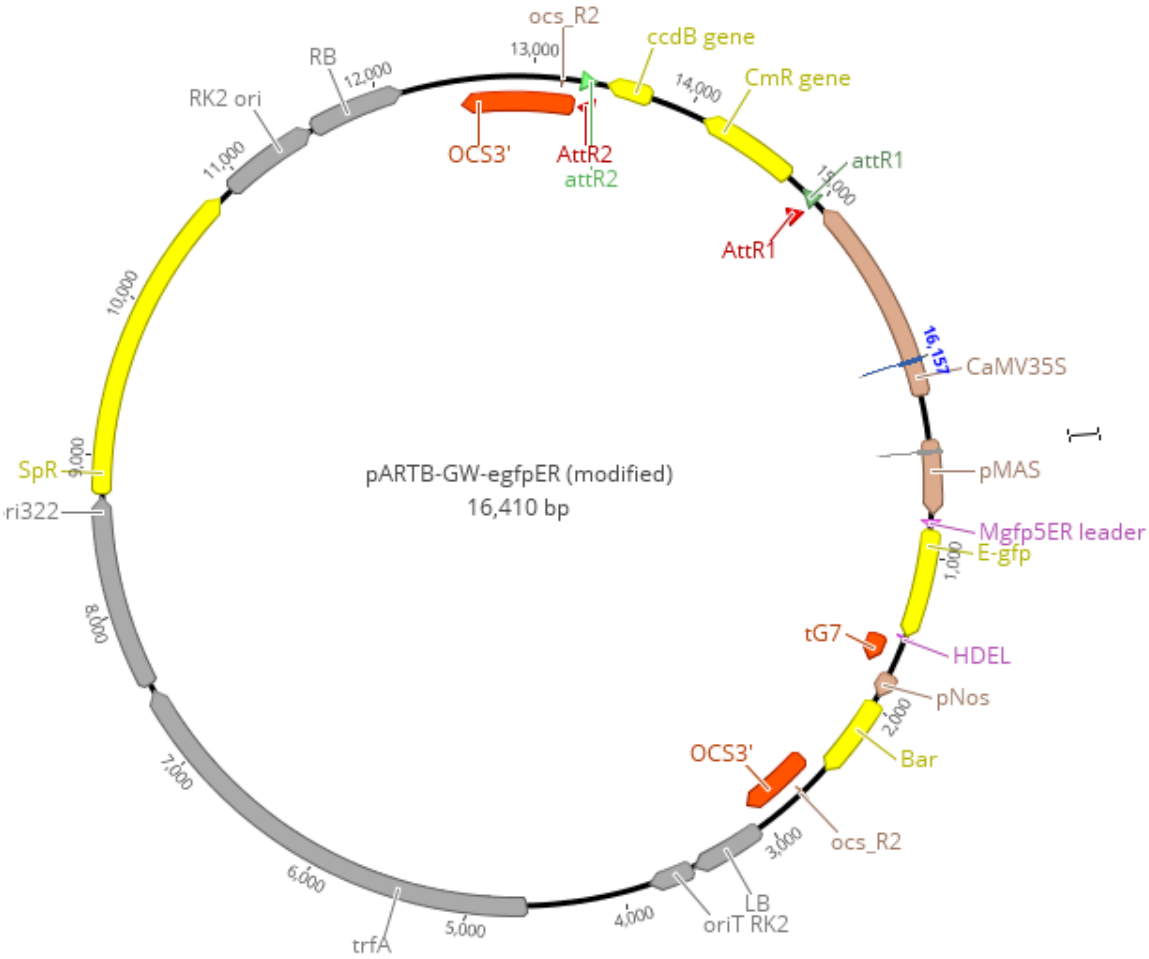
Appendix 3: DNA Ladder (HyperLadder™, Bioline)



Appendix 4: Plasmid of pCR™8/GW/TOPO. The entry vector used for gateway cloning

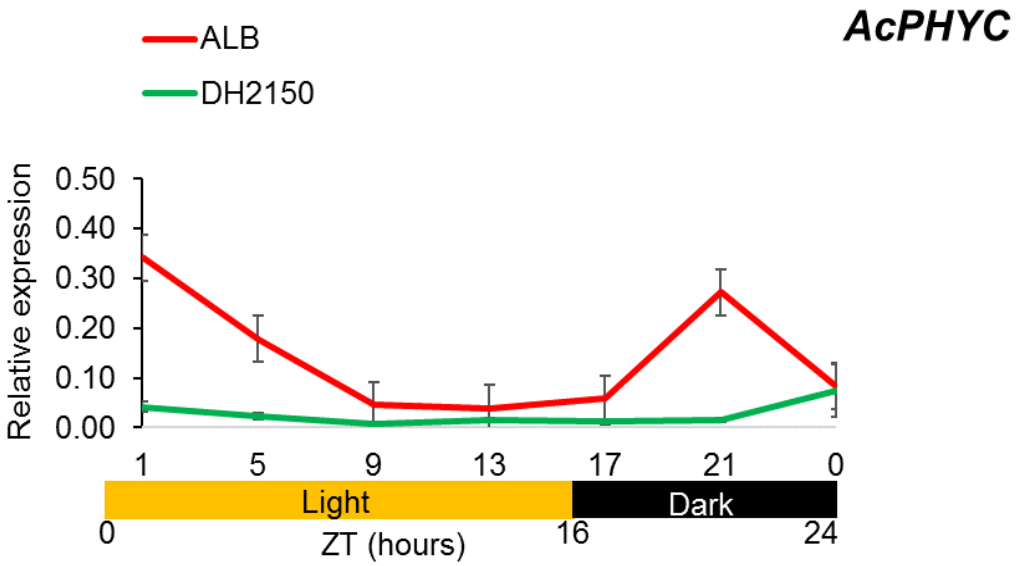


Appendix 5: Plasmid of pART-GW-efgpER. Destination vector used for gateway cloning

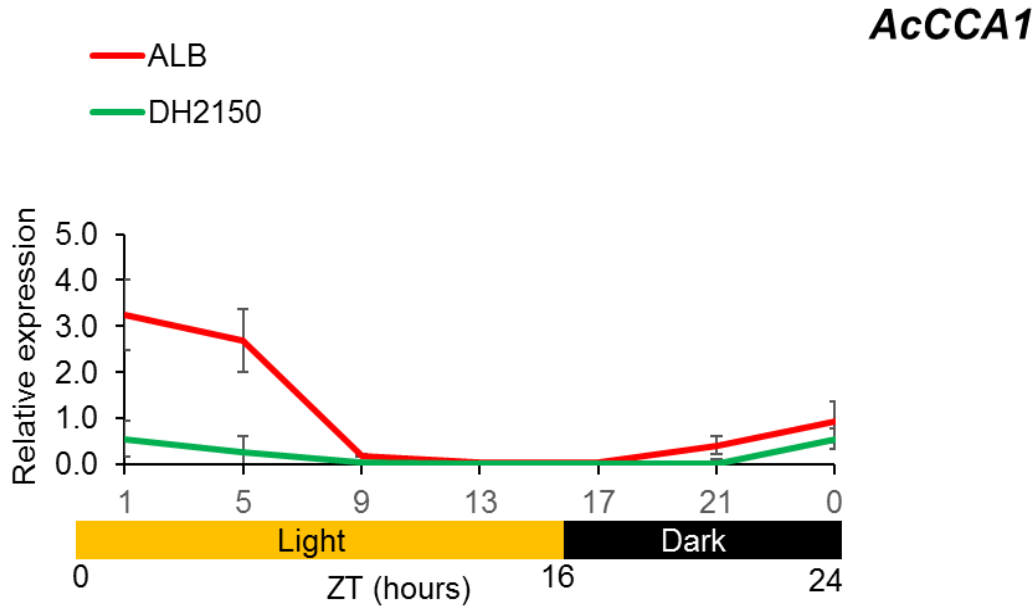


Appendix 6: Primer efficiency of all the primers used in RT-qPCR

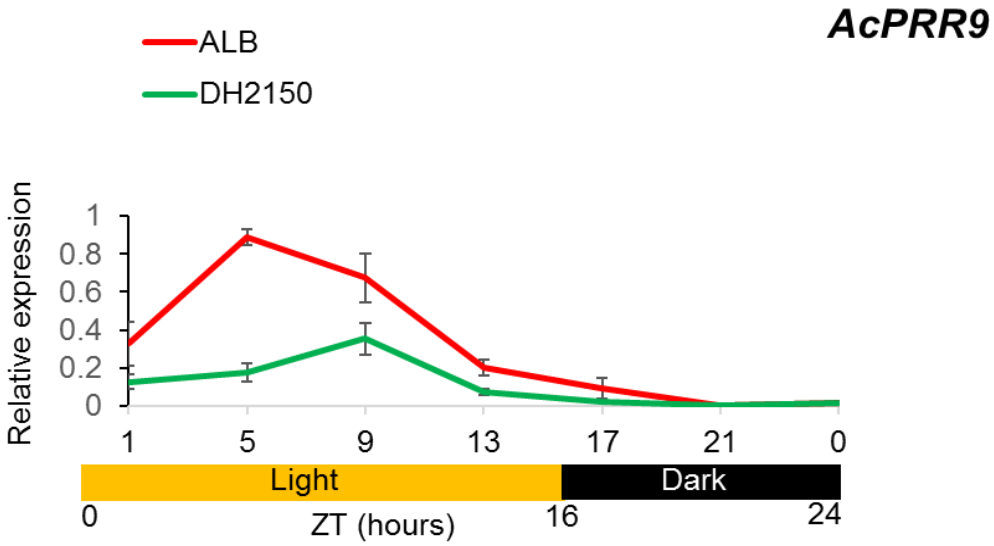
Primer Name	Slope	R Squared value	Efficiency (%)
AcFT1	-3.2393	0.9875	103.57
AcFT4	-3.4220	0.9948	95.99
AcAP1	-3.1300	0.9891	108.68
AcPHYA	-3.4075	0.9969	96.56
AcPHYB	-3.1124	0.9916	109.14
AcPHYC	-3.1247	0.9575	108.95
AcCCA1	-3.1580	0.9983	107.33
AcPRR7	-3.1568	0.9932	107.15
AcPRR9	-3.3807	0.9990	97.61
AcGI	-3.2170	0.9941	104.57
AcFKF1	-3.1090	0.9914	109.72
AcCO	-3.2076	0.9864	104.08
ß-tubulin	-3.1153	0.9994	109.41



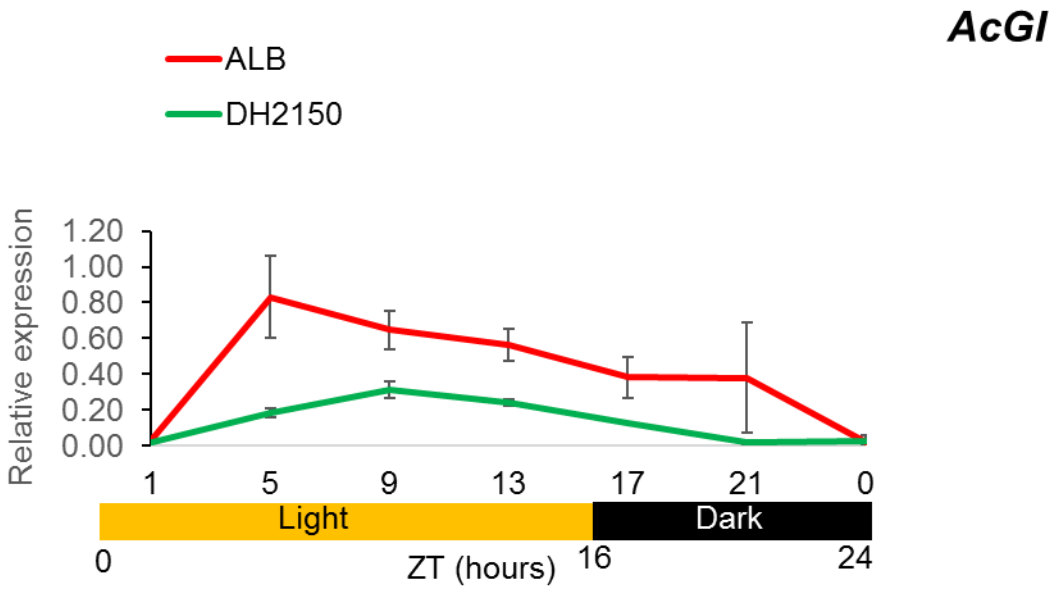
Appendix 7: Diurnal expression of *AcPHYC* in SD and LD onions under LD condition. *AcPHYC* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring the plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.



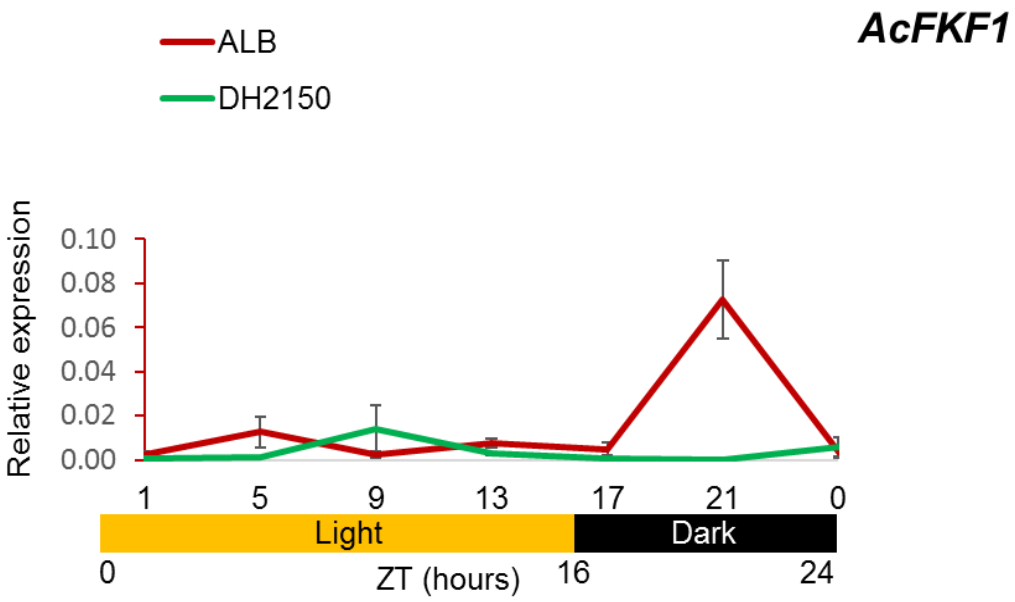
Appendix 8: Diurnal expression of *AcCCA1* in SD and LD onions under LD condition. *AcCCA1* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring the plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.



Appendix 9: Diurnal expression of *AcPRR9* in SD and LD onions under LD condition. *AcPRR9* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring the plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

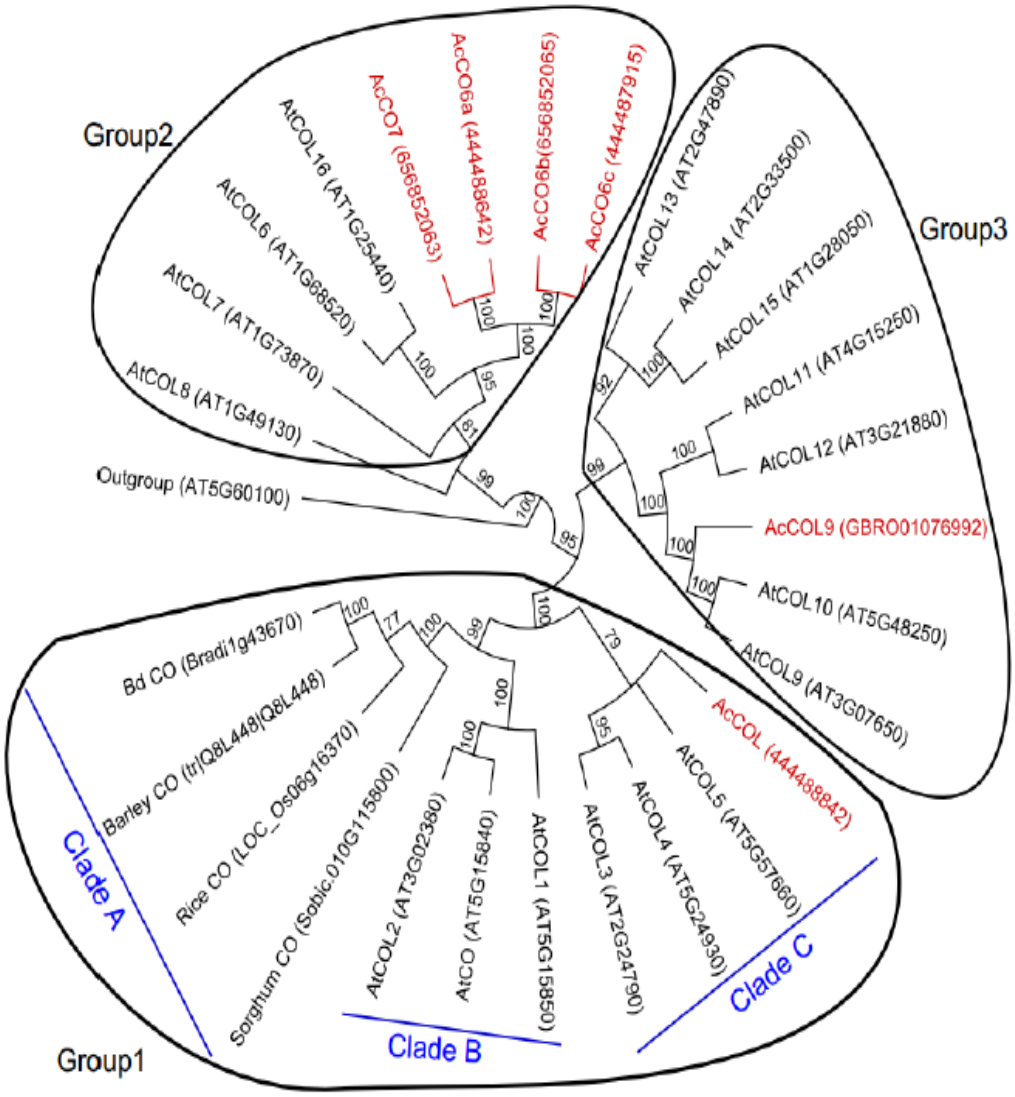


Appendix 10: Diurnal expression of *AcGI* in SD and LD onions under LD condition. *AcGI* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring the plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

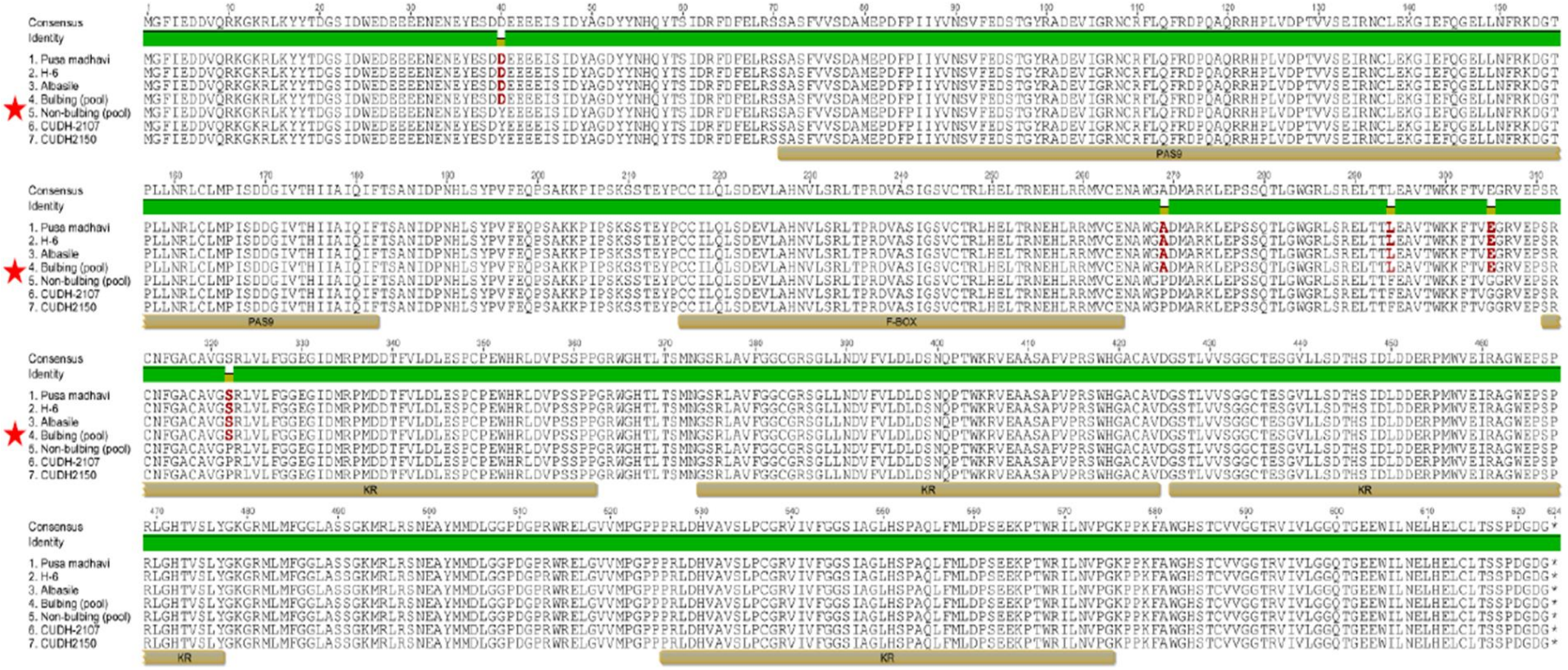


Appendix 11: Diurnal expression of *AcFKF1* in SD and LD onions under LD condition. *AcFKF1* expression was measured using RT-qPCR in SD (Albasile) and LD onions (CUDH2150), 5 days after transferring the plant to white light supplemented with far-red light. Error bars represent the ±SEM of 3 replicates, with transcripts normalized to *ß-tubulin*. Leaf samples were taken over 24 hours period at the 4 hours intervals.

Appendix 12: Phylogenetic relationship of bulb onion CO family with other plants

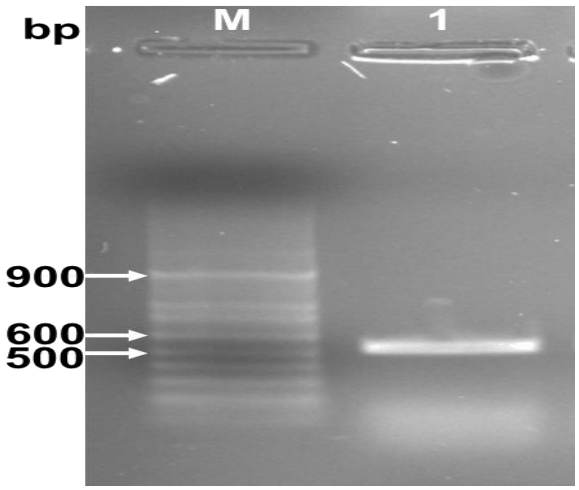


Taken from Khosa, 2018



Appendix 13: *AcFKF1* amino acid alignment of SD and LD onion. In SD onions (Pusa Madhavi, H-6, and Albasile) *AcFKF1* have missense mutations (shown by red star) but not in LD onions (CUDH-2150 and CUDH-2107). Further, bulbing bulks derived from the F2 population (Albasile x CUDH 2107) show SD response and show the same mutation occurring in SD onions, whereas in non-bulbing bulk *AcFKF1* has been similar to LD onions. (Taken from Khosa, 2018)

Appendix 14: Gel electrophoresis of the secondary TAIL-PCR product of promoter region of *AcVRN1* used for extraction and purification of DNA fragment.



Lane M – Ladder, Lane 1 – products of the secondary TAIL-PCR using AD1 and AcVRN1TAIL3 primers

Appendix 15: Nucleotide sequence of the promoter region of the *AcVRN1* gene

GCATACTGATGCTTCGAAGGTTGGTATTGGAGGAGTTCTGAGTCAGAATGG
GAAGCCAGTTGCTTATTTYAGYGAAAARTTGTCAGGGGCAAAGATGAGATA
CAGTACATATGACGTTGAATTTTATGCTGTTGTWCARGCTGTTAAGCATTG
GAGACATTAYYTATTTCAGAAGGAATTTGTTCTKTACACTGATCAYGAAGC
WTTGAARCATTTACAGGGGCAGGATAAAATTTCAGCRAGACATGCATCATG
GATTGCWTATTTACAGCAGTTTACTTTTGTCGTTAAGCACAAATCAGGGGT
GACAAATCGRGTAGCTGATGCTTTGAGTAGGAGGAGTCATTTAYTAACGAC
AATGAAGGTTGAAGTGACGGGGTTTGAAATGTTGCCAGAGATGATTGAAG
AAGATCCTTACTTTGTGAAATATGTTGAGAAAGTGAAGGA

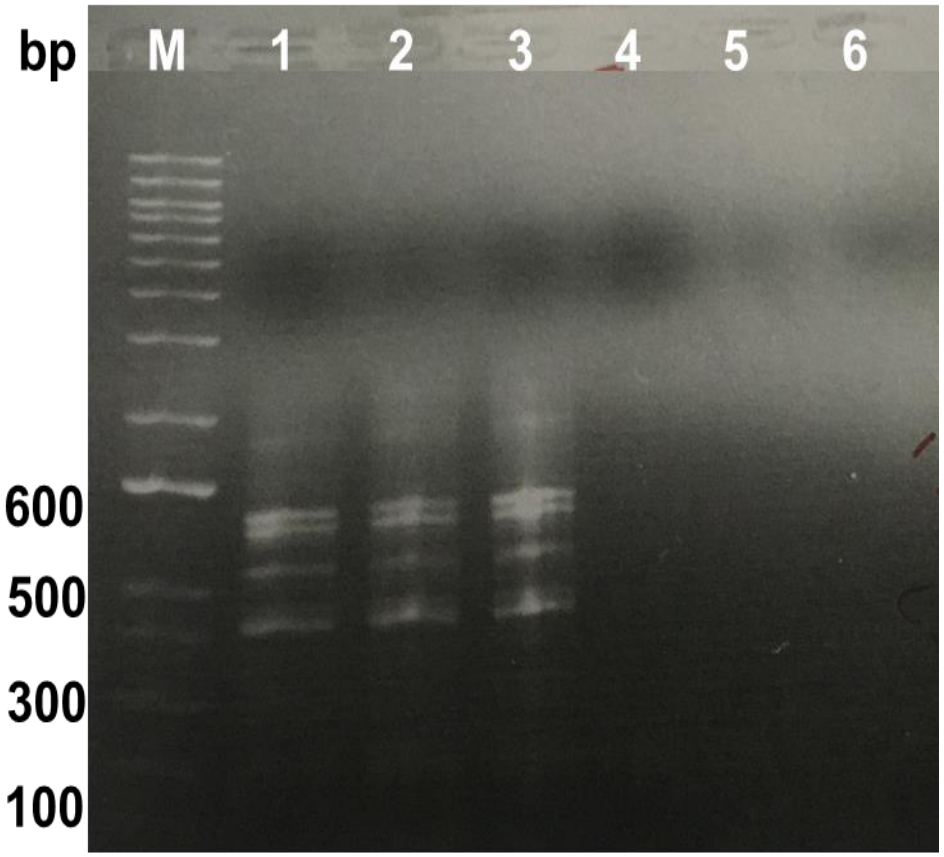
* total 447 bp

Appendix 16: Nucleotide sequence of the intron 1 region of the *AcVRN1* gene

TATTACAATTAAGTCGTGCAACTTTTCCTTGCTAGTTGCTAATATTTCTTTTG
CATTTGTATACATTTTAATTTAACTGCCATTCGATGTTGCACATTCATGTTTT
TTTTAAGGTTAAAAGAACAAAAATCAGAAGAAGAAAAAAAAATATTGTAGT
TACAAACACGATTCGTTTGTGAACTTACACTGTTAATGGTAGAAACATGAA
ATGGTTTTCTTTATGATGTTCATCAAAGTTAAATAAATACATGGACCTTACT
TTGTTATATCATATAGGATACAAACTATACATATAAATGGCTTGTATGACAC
ACACACACGCTGACTGTTAATGAATTATGTATTGTTTGTGTTATGTAGTATG
GAAAAAAT

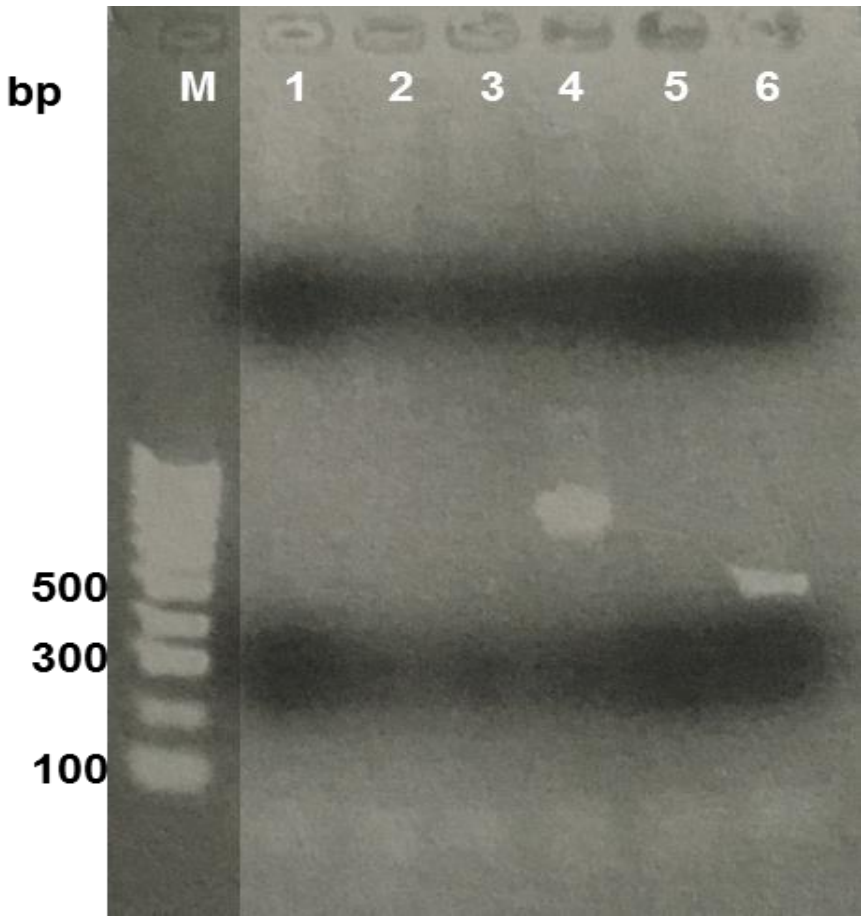
* total 371 bp

Appendix 17: Gel electrophoresis of the primary TAIL-PCR products of intron 1 region of *AcVRN1*

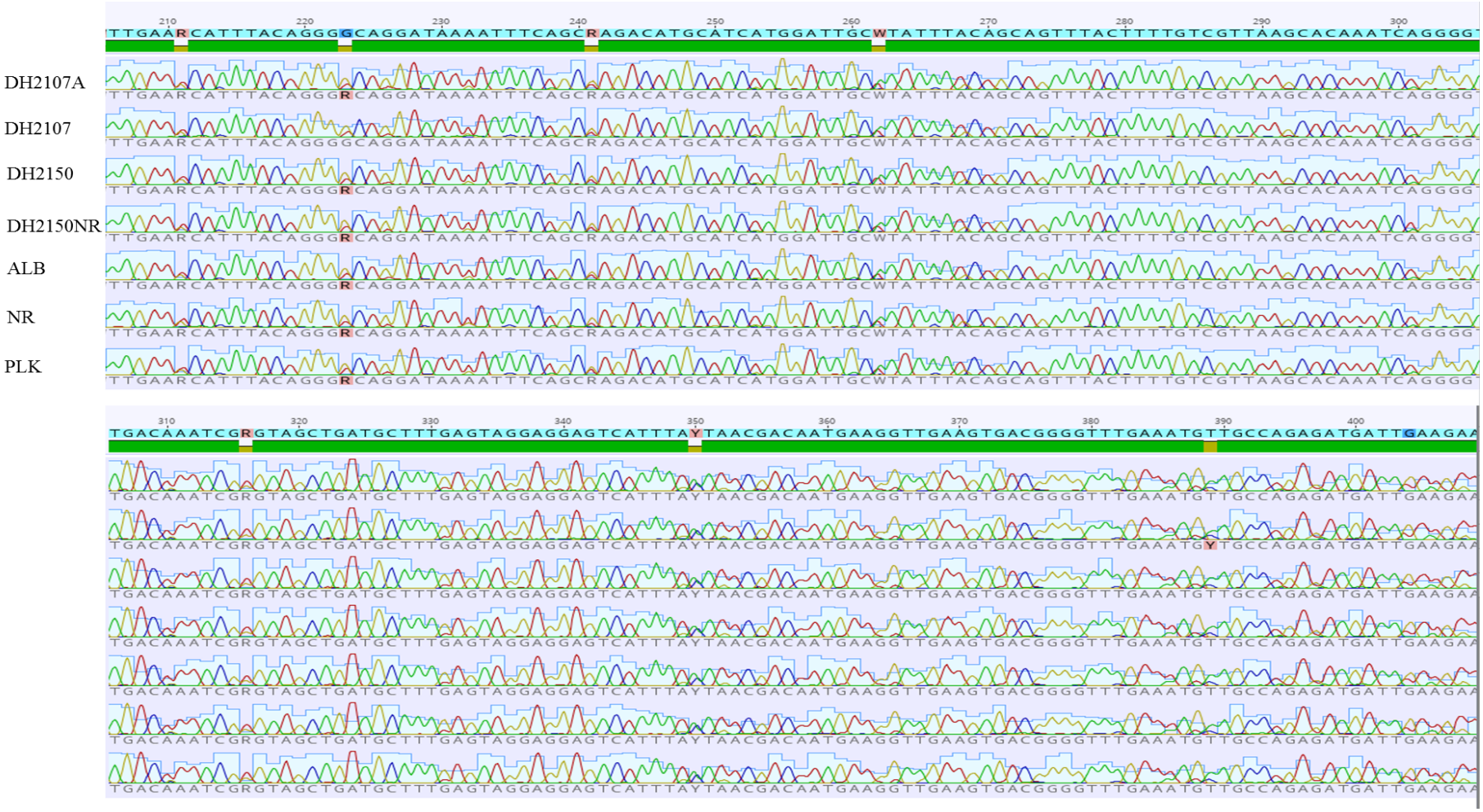


Lane M – ladder, Lane 1,2,3 – products of the TAIL-PCR using AD1, AD5, and AD6 primers respectively, with AcVRN1TAIL10 as a specific primer, Lane 4,5,6 – negative control (water).

Appendix 18: Gel electrophoresis of the secondary TAIL-PCR products of intron 1 region of *AcVRN1*

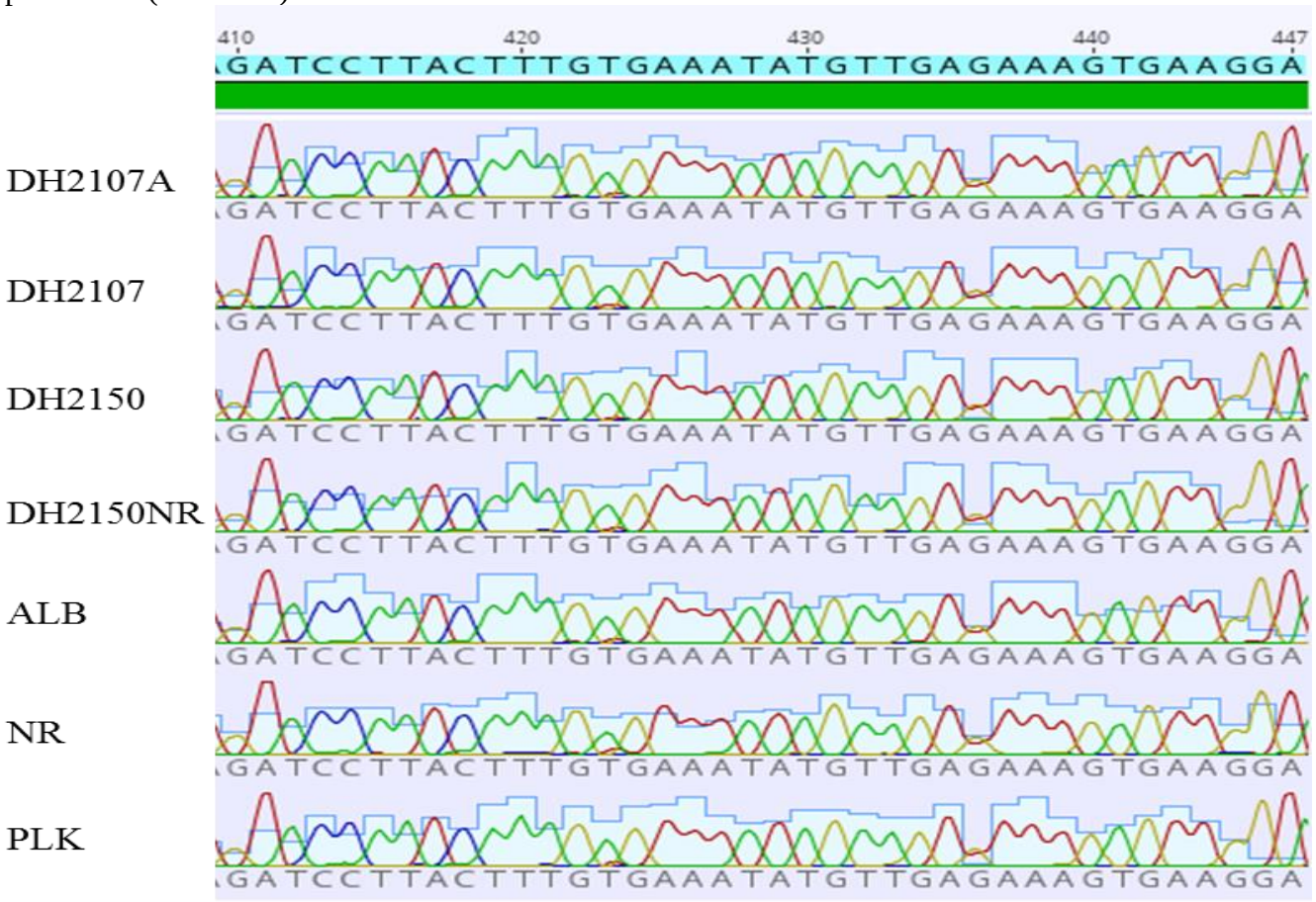


Lane M – ladder, Lane 4,5,6 – products of the TAIL-PCR using AD6, AD5, and AD1 primers respectively, with AcVRN1TAIL9 as a specific primer, Lane 1,2,3 – negative control (water).

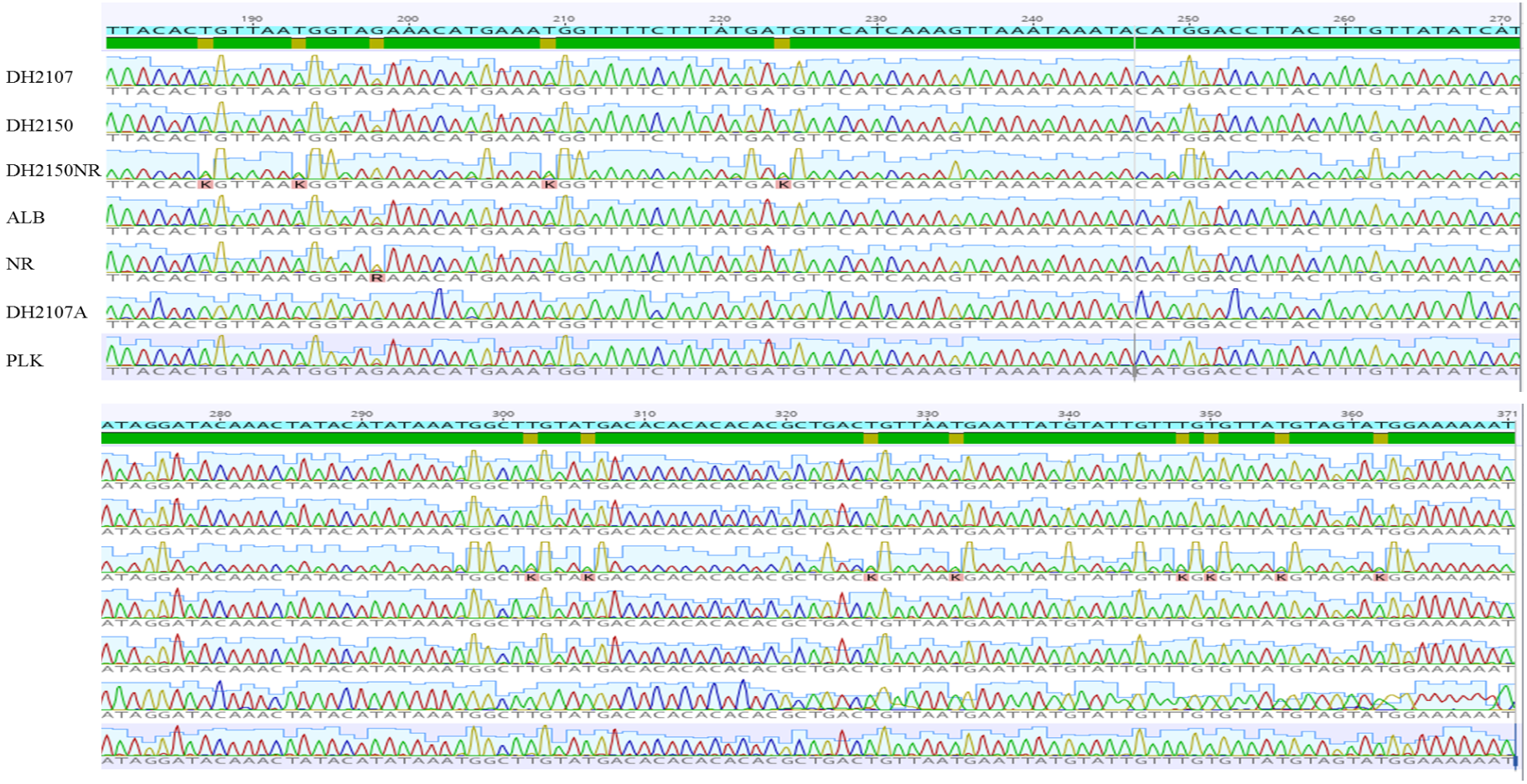


Appendix 19 continues

Appendix 19: (continues)



Appendix 19: Nucleotides alignment of AcVRN1 promoter region of different onion cultivar. DH2107A – cross between 'DH2107 x Albasile', DH2150NR – cross between 'DH2150 x Nasik Red', ALB – Albasile, NR – Nasik Red, and PLK – Pukekohe long keeper.



Appendix 20: Nucleotides alignment of AcVRN1 intron 1 region of different onion cultivar. DH2107A – cross between 'DH2107 x Albasile', DH2150NR – cross between 'DH2150 x Nasik Red', ALB – Albasile, NR – Nasik Red, and PLK – Pukekohe long keeper.

Appendix 21: Types of callus on selection media