Environmental and genetic regulation of Juvenility in *Antirrhinum majus*

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In loving memory of my mother

Supawadee Amnuaykan

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DECLARATION

I would like to declare that the material presented in this thesis is my own work which has not been submitted for the award of any degree at any university. The material supported from the others is provided with acknowledgement.

SUMMARY

Whilst juvenile during vegetative growth, plants are incapable of initiating flowering when grown under floral inductive conditions. Understanding juvenility is important since it influences flowering time, which in turn impacts on scheduling of crop production. Daily light integral (DLI or LI) is the total photosynthetically active radiation received in one day and is calculated from light intensity and light period (Korczynski *et al*., 2002). It is one of the factors that could regulate juvenility.The project aims are to determine the effect of light integral on (i) phase development, (ii) photosynthetic assimilation and partitioning in relation to juvenility, (iii) the expression of key flowering time genes, including *FLOWERING LOCUS T* (*FT*) and genes involved in carbohydrate metabolism and (iv) the expression of microRNA156 in relation to juvenility. Transfer experiments have been carried out to generate material differing in juvenility length for molecular and biochemical analyses. These utilised antirrhinum plants grown under different light integrals and *Arabidopsis* mutants defective in specific carbohydrate related genes. cDNAs representing genes involved in starch synthesis (*PHOSPHOGLUCOMUTASE*(*PGM*), *PHOSPHOGLUCOISOMERASE*(*PGI*), *SUCROSE PHOSPHATE SYNTHASE*(*SPS*)) and degradation (*STARCH EXCESS1*(*SEX-1*), *BETA AMYLASE3*(*BAM-3*), *ALPHA AMYLASE*(*AMY*)) have been isolated from antirrhinum plants. Antirrhinum plants grown under reduced light integrals had extended juvenile and adult vegetative phases and reduced levels of *AmFT*, *AmPGM*, *AmPGI*, *AmSEX-1*, and *AmBAM-3* during juvenility. Juvenility was shown to be extended in mutants defective in both starch synthesis and starch degradation. This indicates that light integral influences the time plants need to accumulate enough level of carbohydrate to support phase change and levels of oligosaccharide, released during starch degradation, may influence the length of juvenility. Consistently, in both *Arabidopsis* and antirrhinum, the timing of *FT* induction was shown to correlate with the end of juvenility and interestingly, light integral has a greater effect on *AmFT* induction and phase transition than photoperiod. Moreover, the current study shows that LI also affects the length of JP when there are highly expression of miR156 which means that miR156 possibly acts downstream of LI in the regulation of flowering.

ABBREVIATIONS

%	per cent
<	less than
=	equals
>	greater than
°C	degrees Celsius
ABA	Abscisic acid
ADGase	ADP glucose pyrophosphorylase
adg-1	adp glucose pyrophosphorylase1 mutant
ADPGlc	ADPglucose
AGL	*AGAMOUS-LIKE*
AmFT	*Antirrhinum majus FT*
AmTEM	*Antirrhinum majus TEM*
AP1	*APETALA1*
AP2	*APETALA2*
AtAMY	*α-AMYLASE*
AtIDD8	*INDETERMINATE DOMAIN*
AVP	adult vegetative phase
bp	base pairs
BAM	*β-AMYLASE*
BLAST	Basic Local Alignment Search Tool
CO	Arabidopsis thaliana *CO*
et al	And others; to complete a list, especially of people, as authors of a published work.
bp	base pairs
cam1	*carbohydrate accumulation1* mutant
CCA1	*CIRCADIAN CLOCK ASSOCIATED 1*
CDF1	*CYCLING DOF FACTOR 1*
cDNA	complementary DNA
cm	centimetre
CO	*CONSTANS*
CO_2	Carbon dioxide

Col-0	Columbia
COP1	*CONSTITUTIVE PHOTOMORPHOGENIC 1*
CRY	cryptochrome
DLI	Daily light integral
DNA	deoxyribonucleic acid
EF1	α *ELONGATION FACTOR 1 ALPHA*
EMS	ethyl methane sulfonate
ESD1	*EARLY IN SHORT DAYS 1*
EST	Expressed sequence tag
EtBr	ethidium bromide
EtOH	ethanol
FD	*FLOWERING LOCUS D*
FKF1	*FLAVIN-BINDING, KELCH REPEATED*
FLC	*FLOWERING LOCUS*
FLD	*FLOWERING LOCUS D*
FLK	*FLOWERING LOCUS K*
FRI	*FRIGIDA*
FRK	fructokinase
Fru6P	Fructose 6-phosphate
FT	*FLOWERING LOCUS T*
FTIP1	*FT -INTERACTING PROTEIN 1*
FUL	*FRUITFUL*
g	grams
g	relative centrifuge force
GA	gibberellins
GAP	triose phosphate
GI	*GIGANTEA*
Glc1P	Glucose 1-phosphate
Glc6P	Glucose 6-phosphate
Glc6P	translocator2 glucose-6-phosphate/phosphate translocator 2
GWD	glucan water dikinase
H3K27	me3histone H3 modified by trimethylation on lysine 27
HL	high light integral

HOS1	*HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1*
h	hours
HXK	Hexokinase
IPTG	isopropyl/-D- thiogalactoside
kDa	kilodalton
LD	long days
LL	low light integral
LDHL	long day high light
LDLL	long day low light
LD	*LUMINIDEPENDENS*
LHP1	*LIKE HETEROCHROMATIN PROTEIN1*
LHY	*LATE ELONGATED HYPOCOTYL*
LI	daily light integral
JP	juvenile phase
Kb	kilobase
LFY	*LEAFY*
mA	miliampere
M	Molar
MEX	maltose transporter
MgSO4	magnesium sulphate
min	minutes
miR156	microRNA156
miR172	microRNA172
miRNAs	microRNA
mRNA	messenger RNA
mM	milimolar
NCBI	National Centre for Biotechnology Information
PAR	Photosynthetically active radiation
PCR	Polymerase chain reaction
PGI	phosphoglucoisomerase
PGM	Phosphoglucomutase
PHY	phytochrome
PIE1	*PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1*

PIFs	*PHYTOCHROMEINTERACTING FACTORS*
PP2C	Probable protein phosphatase 2C 33-like
PRR	*PSEUDORESPONSE REGULATOR*
PWD	phosphoglucan water dikinase
pri-miRNAs	primary transcripts microRNA
RACE	Rapid Amplification of cDNA Ends
RH	relative humidity
RNA	ribonucleic acid
RNASeq	Analysing and sequencing the entire RNAs
RP	reproductive phase
rpm	revolutions per minute
RuBisCO	Ribulose bisphosphate carboxylase
s	seconds
SAM	shoot apical meristem
SD	short days
SDHL	short day high light
SDLL	short day low light
SDW	Sterile Deionized Water
SEX-1	glucan water dikinase
SMZ	*SCHLAFMÜTZE*
SnRK1	Snf1-related kinase 1
SNZ	*SCHNARCHZAPFEN*
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CO1*
SPA1	*SUPPRESSOR OF PHYTOCHROME A*
SPL	*SQUAMOSA PROMOTER BINDING PROTEIN LIKE*
SUS4	sucrose synthesis enzyme
SVP	*SHORT VEGETATIVE PHASE*
T_0	50% of germination
T6P	trehalose-6-phosphase
TAE	buffer Tris-acetate-EDTA buffer
TFL1	*TERMINAL FLOWER1*
TOC1	*TIMING OF CAB EXPRESSION 1*
TOE	*TARGET OF EAT*

TOR	target of rapamycin
TPS1	Trehalose-6-phosphate synthase1
TSF	*TWIN SISTER OF FT*
UDPGlc	UDP Glucose
VIP	*VERNALIZATION INDEPENDENCE*
v/v	volume by volume
w/v	weight by volume
WT	wild-type
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
ZT	zeitgeber time
µg	micrograms
µl	microlitres
µM	micromolar
µmol	micromole

CHAPTER1. INTRODUCTION

1.1 Flowering in plants

In plants, the transition to flowering incorporates with the developmental phase change in their life cycle. The production of leaf from the apical meristem needs to alter to be the production of floral organ and floral meristem together with the change of growth pattern. This alteration is not an unplanned action, but happens in order to response to the inductive factors including environmental and internal stimuli which vary among different species.The environmental inductive condition cannot trigger flowering in plants immediately if the ingredient needed for floral initiation is not sufficient. There are several environmental factors which seemed to have an impact on flowering such as day length and temperature (Bäurle and Dean, 2006; Imaizumi, 2009).

The day length is the period of time when the light is available. Plants use day length to predict season so that they know their best time to flower. The optimal flowering time in plant is different among species. There are three types of day length response in plant, short-day plants (SDP), long-day plants (LDP), and day-neutral plants (DNP). SDPs flower when light period is shorter than a certain length within 24 h period. LDPs, in the other hand, are plants that need the light period to be longer than a certain length within 24h period. The certain lengths of light period vary between species. DNPs are plants that will flower at the same time regardless to the day length condition.SDPs and LDPs can be separated to be obligate (qualitative) or facultative (quantitative) type. Obligate plants need certain day length to flower which is not the case for facultative plants. The inductive day length only hastens flowering time in facultative plants and they can still flower under noninductive day length (Thomas and Vince-Prue, 1997).In fact, plants response to day length by measuring the length of dark not light period. Turning the light off didn't affect time to flower. In the opposite, interrupted dark period by light had an effect on flowering. SDPs had flowering time extended whereas LDPs had accelerated flowering (Bothwick *et al*., 1952).

1.2 Pathways control flowering time in plants

During early plant development, there is a change from an embryonic to postembryonic phase of growth followed by the transition from juvenile phase (JP) to adult phase (RP) within the vegetative phase (AVP) (Poethig, 2003) and subsequently the transition to reproductive growth (Bäurle and Dean, 2006). The transition to flowering in plants involves key developmental phase changes. Phase change in plants centres around changes that occur at the shoot apical meristem. The shoot apical meristem undergoes a transition to a floral meristem when plants reach a suitable developmental age.

There are several pathways that involve many genes that control the change of the shoot apical meristem from producing leaves to producing flowers (Bäurle and Dean, 2006) (figure 1.1). Once plants attain competence to flower, the genetic pathways that control flowering are complex. Regulation by environmental conditions involves the photoperiod and vernalization pathways (Araki, 2001; Srikanth and Schmid, 2011). The endogenous signals from the developmental stage involve the autonomous pathway. Another pathway that promotes flowering involves genes related to gibberellin (GA) biosynthesis and acts predominantly in short day (SD) conditions (Araki, 2001). CO_2 and carbohydrate metabolism also have an effect on flowering time (Springer *et al*., 2007). The response of plants to CO_2 concentration is species-specific. When CO_2 increases, the rate of photosynthesis is increased and followed by early flowering (Garbutt,1984; Garbutt,1990; Springer et al., 2007; Springer *et al*., 2008). CO_2 has an influence on plant growth and physiology. Interestingly, CO_2 concentration also affects carbon metabolism in the plant (McKee, 1994). Carbohydrates have an important role in flowering time control because of their signal molecule function (Springer *et al*., 2007).

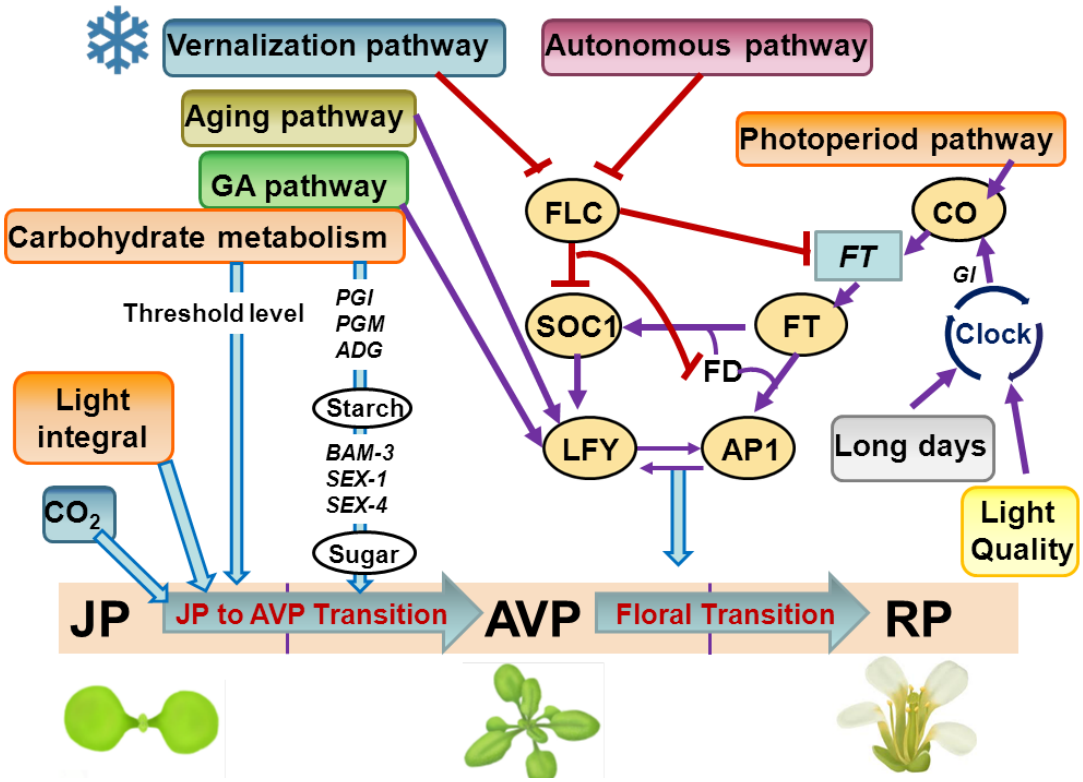


Figure1.1 Simple schematic representation of the signalling pathways controlling the floral transition in plants

1.2.1 *The photoperiodic pathway*

Photoperiodic flowering responses in plants involve the integration of light signals and the circadian system, which is linked to the expression of flowering genes like *CONSTANS* (*CO*) and *FT*.

CO is a nuclear protein with zinc fingers that is involved with floral induction (Robson *et al*., 2001). It is key for the day length discrimination mechanism in photoperiodic flowering. Expression of CO is circadian regulated. Under SD conditions, the peak of CO mRNA presents in the dark period and no peak of CO protein during the daytime. In contrast, under LD conditions, a high level of *CO* expression is present at the beginning and the end of the light period, which, as explained below, leads to stable CO protein during the day (Putterill *et al*., 2004).

After *CO* mRNA is transcribed, many regulatory components control the levels of CO protein depending on the light conditions (figure 1.2). Under LD

conditions, flowering time is controlled by how plants perceive and respond to the light. SUPPRESSOR OF PHYTOCHROME A (SPA1) and CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) together form a ubiquitin ligase complex that has the ability to degrade CO protein in the dark (Hoecker and Quail, 2001). In the early morning during the light period, phyB activates the degradation of CO protein via a COP1- independent parthway. HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1), a ubiquitin ligase, also degrades CO protein in the morning (Lazaro *et al*., 2012). At the end of the day, CRYPTOCHROME 2 (CRY2) interacts with the COP1-SPA1 complex and together with phyA results in the inhibition of COP-SPA1 complex. During the dark period, the COP1-SPA1 complex can freely trigger the degradation of CO (Liu *et al*., 2008). These controls from several regulatory factors lead to a low level of CO protein in the early morning followed by the peak level of the protein at the end of the light before the level is reduced again during the dark (Davis, 2002; Sawa *et al*., 2008). Also, *FKF1, GI*, and *CO* expression exhibit diurnal rhythms and when these rhythms coincide with the important environmental factor like light under LD, the transcribed proteins are able to activate *FLOWERING LOCUS T (FT)* and then help to induce flowering (Putterill *et al*., 2004; Valverde *et al*., 2004).

Under SD conditions, during the light period, the peak of GI and FKF1, which normally occur about ZT10, do not coincide with the light. Therefore GI and FKF1 cannot form a complex and repress the CDFs, the transcriptional suppressors of *CO*. Then the transcription of *CO* is repressed (Sawa *et al*., 2007). The expression of *CO* mRNA is hence low during the light and high only in the dark period. As a result, CO protein is not produced in the day time and is degraded by COP1-SPA1 complex during the dark period and the expression of *FT* is not activated (Suarez-Lopez *et al*., 2001; Imaizumi *et al*., 2003). However, GI alone can also trigger the photoperiodic control of flowering. It can act alone or cooperate with the function of *CO* and FT. Under SD conditions, the expression of *GI* in vascular bundles or mesophyll cells can activate *FT* expression without increasing the level of *CO* (Sawa and Kay, 2011).

There are two models, external and internal coincidence, to explain about photoperiodic responses in plants. In the external coincidence model, only one oscillation is generated from light signal. The duration of light is not necessary for

the photoperiodic response, but the presence of light at the correct time during 24 h is important for the photoperiod signals to be generated. In contrast, in the internal coincidence model, there are two different oscillations generated from light entrainment. One oscillation acts as a repressor to another (Kobayashi and Weigel, 2007). The regulation of *CO* described previously conforms to the external coincidence model. Only in LD photoperiod, high levels of *CO* expression coincide with light which leads to stabilization of CO protein to the activation of *FT*.

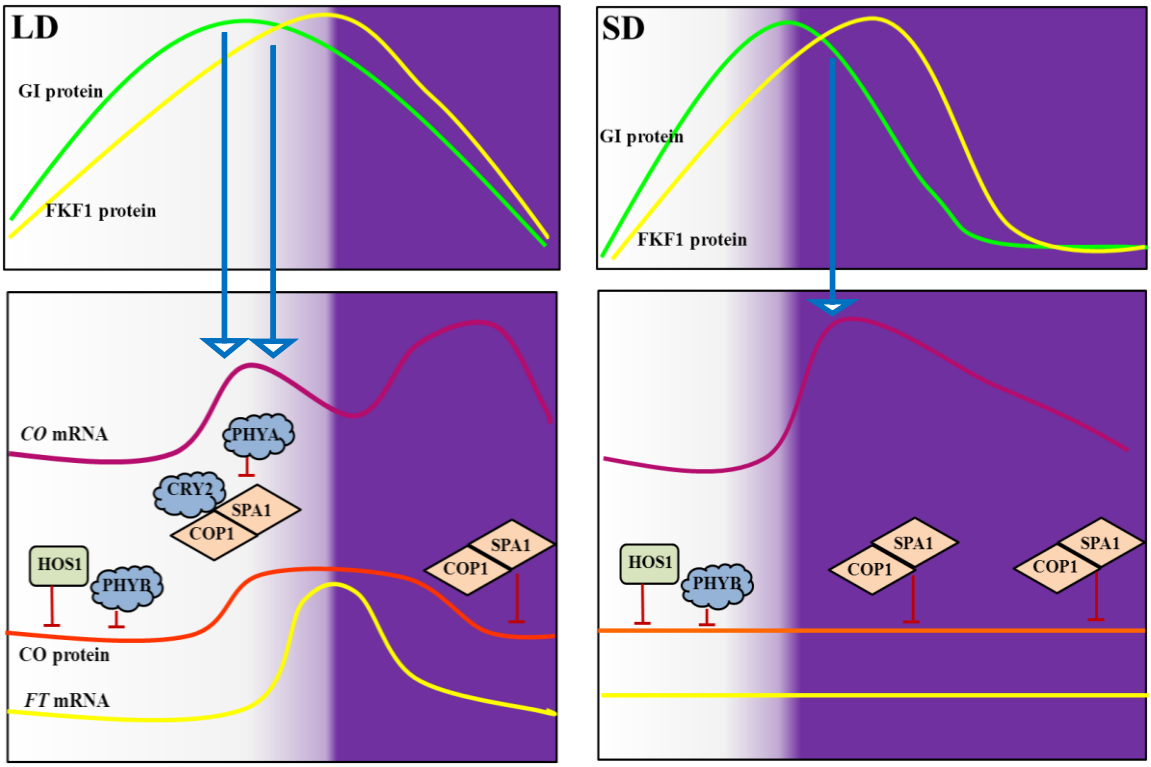


Figure 1.2 Schematic representation of the regulation of CO

The clock regulates the transcription of the regulatory genes CO, FKF1 and GI and provides the circadian rhythm of their expression. When the expression of CO coincides with light, the activation of FT and subsequently flowering occurs. Blue arrows indicate activation and red T-bars indicate inhibition. The complete nomenclature of the genes is described in section 1.2.2.

1.2.2 *The vernalization pathway*

The vernalization pathway is involved with the need for plants to be exposed to a period of cold to activate flowering (figure 1.3). FLOWERING LOCUS C (FLC) and SHORT VEGETATIVE PHASE (SVP) are MADS box proteins that act as repressors of *FT* transcription in the vernalization pathway (Helliwell *et al*., 2006; Lee *et al*., 2007). Both proteins bind *FT* at the CArG boxes which are located in its promoter (Helliwell *et al*., 2006). Since FLC and CO can bind to the same binding site of the *SOC1* promoter, lack of FLC protein allows CO to activate SOC1 by binding to its promoter (Hepworth *et al*., 2002). In the vernalization pathway FLC is positively regulated by several proteins such as FRIGIDA (FRI) (Michaels and Amasino, 1999; Michaels *et al*., 2004), EARLY IN SHORT DAYS 1 (ESD1) (Martin-Trillo *et al*., 2006), PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1 (PIE1) (Noh and Amasino, 2003), and VERNALIZATION INDEPENDENCE (VIP) (Zhang and van Nocker, 2002) FRIGIDA (FRI) activates FLC but, the activation can be stopped by the cold period (Massiah, 2007). When FLC is repressed, the floral transition is allowed.

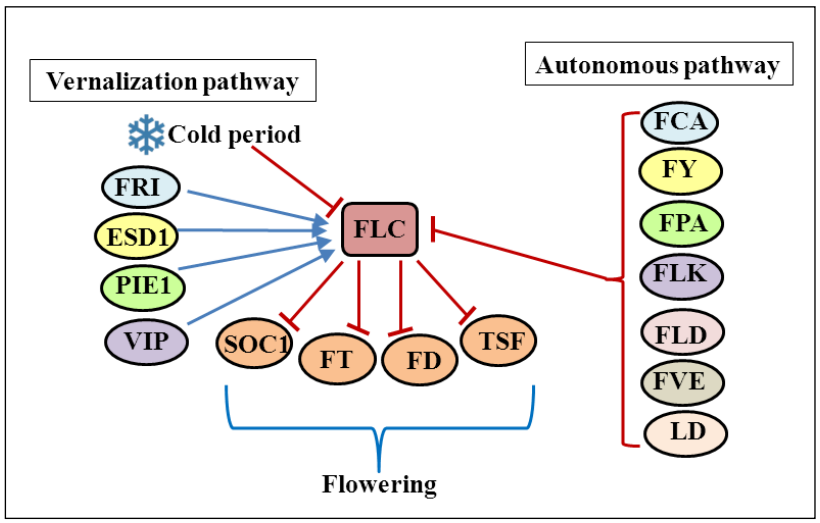


Figure 1.3 Simplified schematic representation of the vernalization and autonomous pathway
Blue arrows indicate activation and black T-bars indicate inhibition. The complete nomenclatures of the genes are described in section 1.3.1 and 1.3.2.

1.2.3 *The autonomous pathway*

The autonomous pathway is the endogenous regulator of flowering time in plants. This pathway functions independently of the photoperiod pathway. In the autonomous pathway, the repression of the floral genes above is needed for the meristem to remain vegetative when the plants are not ready to flower.

In the autonomous pathway, FLC is repressed by several proteins, employing different mechanism of action such as RNA binding FCA (Macknight *et al*., 1997), Flowering time control protein FY (Simpson *et al*., 2003; Razem *et al*., 2006), RNA binding FPA (Schomburg *et al*.,2001), FLOWERING LOCUS K (FLK) (Lim *et al*., 2004), FLOWERING LOCUS D (FLD) (He *et al*., 2003; Simpson, 2004), retinoblastoma-associated FVE (Ausín *et al*., 2004) and LUMINIDEPENDENS (LD) (Aukerman *et al*., 1999). Since *Arabidopsis* mutants of these genes are all late flowering due to the lack of FLC repression, the late flowering mutants can be rescued by cold exposure since it maintains repression of FLC.

1.2.4 *The gibberellin promotion pathway*

The gibberellin (GA) pathway acts predominantly in SD conditions (frigure1.4). Levels of the plant growth regulator (GA) in plants are increased when there is sufficient water, nutrient and light quality (Nakajima *et al*., 2006). It supports the floral transition in all rosette plants (Corbesier and Coupland, 2005). In its role in flowering time regulation it activates the expression of *LFY*, one of the flowering time integrators (Blázquez *et al*., 1998). The promotion of flowering by GA is not involved with FLC since it acts downstream of FLC (Sheldon *et al*., 2000). GA acts as the repressor of DELLA proteins (Cheng *et al*., 2004), which are inhibitors of flowering in plants (Dill *et al*., 2004). The DELLA proteins can repress PHYTOCHROMEINTERACTING FACTORS (PIFs), plant growth activators (Nozue *et al*., 2011), SPL, and miR172 (Galvão *et al*., 2012).

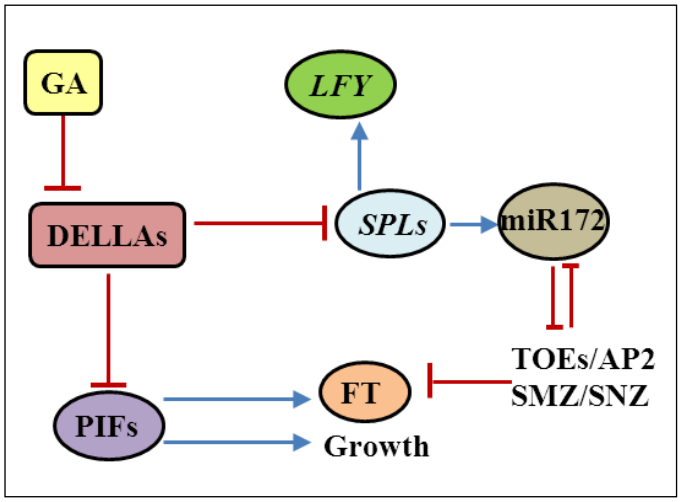


Figure1.4 Schematic representation of the gibberellin pathway

Blue arrows indicate activation and black T-bars indicate inhibition. The complete nomenclatures of the genes are described in section 1.3.3.

1.3 Carbohydrate metabolism and its involvement in flowering time of plants

1.3.1 *Carbohydrate metabolism*

Photosynthesis is an important activity for plants since they are autotrophic organisms that can synthesis nutrients from the environment to support themselves. Since the level of carbohydrates in the leaf are from the adjustment of the plant between photosynthesis and plant usage, the amount of carbohydrate accumulated in leaf is depending on the rate of photosynthesis (Atkin *et al*., 2000). The final products from photosynthesis are sugars, which integrate signals from metabolism inside and environmental factors outside. Sucrose is one of the important products from photosynthesis. This form of sugar is transported throughout the plant via the phloem and used for developmental process (Hitz and Giaquinta, 1987).

Plant growth is influenced by the light / dark regime (Graf *et al*., 2010). Growth is dependent on photosynthesis during the day and starch mobilization at night (Geigenberger, 2011). There are two main types of starch in the plant organs, reserve and transitory starch (Ritte *et al*., 2004). Reserve starch is stored in plant organs during a long period of time. During the daytime, the plant stores photosynthetic assimilates in the form of starch in the chloroplast. In the dark period, the transitory starch is mobilized by changing to soluble sugars, which are used for

metabolism by the plant (Yu *et al*., 2001; Yan *et al*., 2005).

In shown in figure1.5, the rate of starch breakdown at night is linear (Graf *et al*., 2010). In fact, starch is not completely degraded. There is a small amount of remaining starch by the end of the night (Graf *et al*., 2010; Stitt and Zeeman, 2012). When carbon supply to the plant is limited due to shorter photoperiods, lower light integral, or lower amount of CO_2, starch synthesis in the day time is increased followed by the reduction of starch degradation at night time. Therefore, in different diurnal regimes, there are different patterns of starch assimilation and degradation (figure 1.5). The starch synthesis rate is stimulated and starch breakdown rate is reduced under SD condition. In contrast, under LD condition, the starch synthesis rate is reduced and starch breakdown rate is stimulated. Therefore, In order to maintain carbon level at the end of the night to be similar to the plants in longer photoperiod, the activity of enzymes involved in carbohydrate metabolism in SD grown plants is reduced. Light and carbohydrate metabolism rates, including enzyme activities, affect developmental phase changes, carbohydrate metabolism could be key to the transition from JP to AVP.

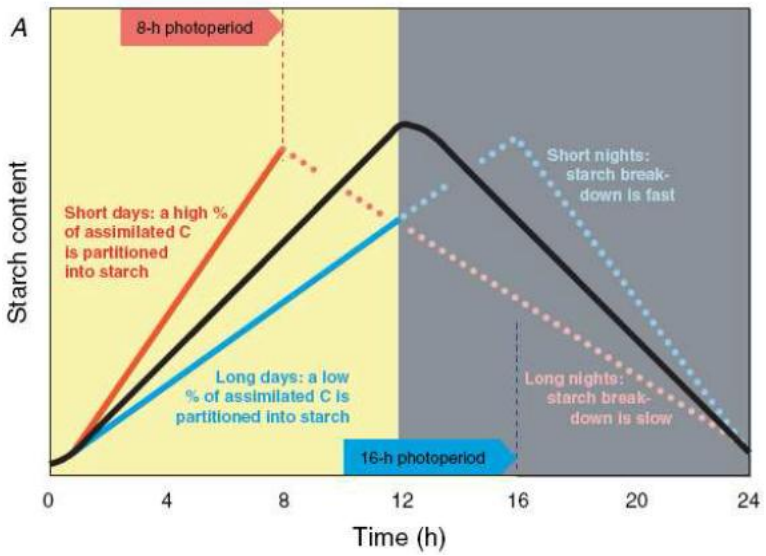


Figure1.5 A scheme illustrating the pattern of starch accumulation and utilization in Arabidopsis leaves during different diurnal regimes
The black line represents the near-symmetrical pattern observed under 12-h light/12 h dark conditions. The rates of starch synthesis and starch degradation respond in complementary ways to shortday (red) or long-day (blue) conditions, as indicated from Zeeman et al., 2007

The primary products of photosynthesis are starch and sucrose. During the day time, photophosphorylation occurs in the chloroplast thylakoid membrane in order to generate ATP (Geigenberger, 2011). The Calvin-Benson cycle provides

triose phosphate (GAP) as an intermediate for starch and sucrose synthesis (Ritte *et al*., 2000; Zeeman *et al*., 2007).

Starch is synthesised in chloroplasts using Fructose 6-phosphate (Fru6P) from The Calvin-Benson cycle (figure1.6) (Zeeman *et al*., 2007; Geigenberger, 2011). In figure1.6, there are two isozymes of phosphoglucoisomerase (PGI), plastid and cytosolic (Jones *et al*., 1986; Yu *et al*., 2000). Plastidal PGI is involved with starch synthesis whereas cytosolic PGI is associated with sucrose metabolism (Jones *et al*., 1986). Fru6P is converted to Glucose 6-phosphate (Glc6P) by PGI in the chloroplast (Yu *et al*., 2000; Geigenberger, 2011). Phosphoglucomutase (PGM) converts Glc6P to Glucose 1-phosphate (Glc1P) (Harrison *et al*., 1998; Manjunath *et al*., 1998; Periappuram *et al*., 2000). There are two isozymes of PGM present in two different locations, chloroplast and cytoplasm (Mühlbach and Schnarrenberger, 1978; Manjunath *et al*., 1998). Glc1P in chloroplasts is converted to ADPglucose (ADPGlc), which is the component of starch (Manjunath *et al*., 1998; Harrison *et al*., 1998). Glc1P in the cytosol is converted to UDP Glucose (UDPGlc), which is important for cellular component production, and in another direction it can be changed to Glc6P which is involved with glycolysis (Manjunath *et al*., 1998; Yu *et al*., 2000). Therefore Glc1P is the key to link the Calvin-Benson cycle to starch and sucrose metabolism (Manjunath *et al*., 1998). The different location of two PGM isozymes is important as one isoform in chloroplast is involved with starch synthesis and one in cytosol is associated with sucrose metabolism (Mühlbach and Schnarrenberger, 1978).

In photosynthetic tissue, GAP from Calvin cycle is the substrate for starch synthesis and degradation in the chloroplast in order to promote glucose to support plant metabolism or can be the substrate to produce sucrose and trehalose in the cytoplasm. Sucrose synthesis occurs in the cytoplasm using GAP obtained from the chloroplast (Zeeman *et al*., 2007).This process is driven by the sucrose phosphate synthase enzyme (SPS) (Geigenberger, 2011). Apart from sucrose, G6P can also produce trehalose-6-phosphate (T6P), when is subsequently converted to trehalose by another enzyme.

After being synthesised, sucrose is loaded into sink tissues (Zeeman *et al*., 2007). In sink tissues, sucrose is degraded either by invertase or sucrose synthase to become glucose and fructose (Sturm and Tang, 1999). Glucose and fructose are then

phosphorylated by Hexokinase (HXK) and fructokinase (FRK) respectively (Koch, 2004). Glc6P and Fru6P are then available to be used to support plant metabolism. In the dark period, the transitory starch in the chloroplast is converted to soluble carbohydrates to support metabolism (Ritte *et al*., 2000). Starch degradation is carried out by a network of reactions involving several enzymes. These include glucan water dikinase (SEX-1), phosphoglucan water dikinase (PWD), α-amylase (AtAMY), β-amylase (BAM) and debranching enzymes (Yan *et al*., 2005; Stitt and Zeeman, 2012). Maltose is the primary product from starch breakdown at night. It is synthesised in the chloroplast and exported to the cytoplasm by maltose transporter (MEX), which is located in the chloroplast envelope (Fulton *et al*., 2008). Phosphorylation is important for starch breakdown (Mikkelsen *et al*., 2004) since it is easier for the starch degradation enzymes to access into starch granules if the starch is phosphorylated (Ritte *et al*., 2004). R1 is an α -glucan water dikinase which is encoded by the *SEX1* gene (Ritte *et al*., 2002). This enzyme plays a role in amylopectin phosphorylation in order to destabilise granule formation and start the process of degradation (Ritte *et al*., 2000; Ritte *et al*., 2002). Amylose and amylopectin are two types of polyglucan included in the starch granules. In comparison with amylose, amylopectin is larger and contains more branches (Mikkelsen *et al*., 2004). Polyglucan size is important for starch phosphorylation. SEX-1 normally phosphorylates the long polyglucans and recognise the branch point in amylopectin, therefore phosphorylation occurrs in amylopectin rather than amylase (Mikkelsen *et al*., 2004). The starch degradation enzyme amylase is able to function easier when the substrate starch is phosphorylated. Therefore, lack of SEX-1 activity causes a reduction of starch degradation (Ritte *et al*., 2002; Ritte *et al*., 2004). Phosphorylation of transitory starch could occur during starch biosynthesis at the day time.

The rate of phosphorylation is much higher when starch is degraded at night (Ritte *et al*., 2004). AMY can hydrolyse glucans that packed in the starch chain to become maltose (Fulton *et al*., 2008; Stitt and Zeeman, 2012). However, there is also another pathway of minor starch breakdown processing by AMY3 and linear α-D1-4Glucan (LDA), which is the debranching enzyme. The products from this pathway usually accumulate in the chloroplast stroma and are thought to support chloroplast metabolism (Zeeman *et al*., 2007). In *Arabidopsis*, β-amylase, the chloroplast-

targeted isoform of BAM and debranching enzymes help to breakdown glucans which are packed at the starch granule surface (Zeeman *et al*., 2007; Stitt and Zeeman, 2012). In *Arabidopsis*, there are 9 genes in the *BAM* family, *BAM1* to *BAM9* (Fulton *et al.*, 2008). The *BAM3* gene encodes chloroplastid β-amylase, which is involved with starch degradation in photosynthetic tissues (Fulton *et al*., 2008; Francisco *et al*., 2010).

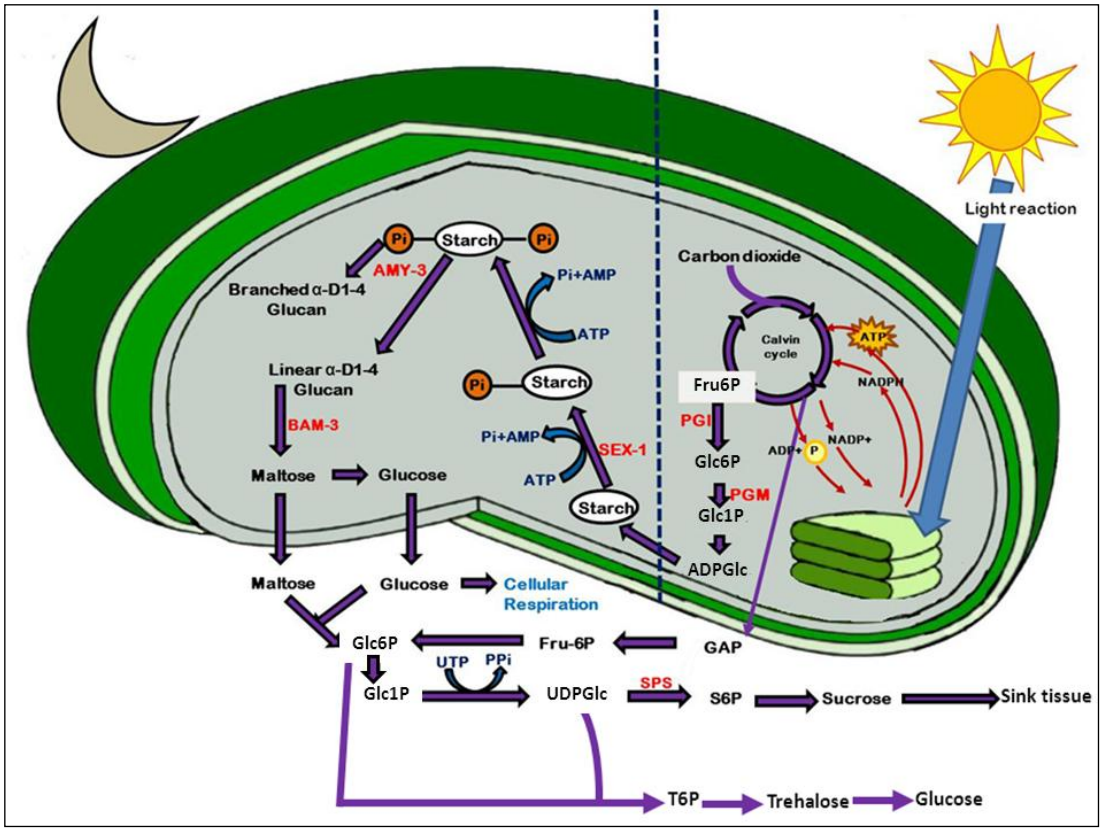


Figure1.6 Schematic representation of the carbohydrate metabolism pathway

The complete nomenclatures of the genes and carbohydrates are described in section 1.4.1.

1.3.2 *Sucrose and flowering time*

Sucrose is one of the important signaling molecules derived from photosynthesis and transported from source tissues to sink tissues and can support flowering (Roldan *et al*., 1999). It regulates the phase transition in plants via

miR156. The level of sugar is very low during juvenility and this leads to high levels of miR156 (Huijser and Schmid, 2011; Yu *et al*., 2013). When the plants get older, there are enough products from photosynthesis to support developmental processes. Sucrose is transported to the sink tissues such as young leaves and the SAM (Roldan *et al*., 1999). It represses the expression of *MIR156A* and *MIR156C* primary transcripts. Sucrose is also converted to glucose in sink tissues, which degrades primary miR156 A and C and causes a reduction of miR156 level (Yu *et al*., 2013). Therefore sucrose has the ability to suppress miR156 at the transcription level whereas glucose acts as the miR156 repressor at the post transcription level.

INDETERMINATE DOMAIN (AtIDD8) is a transcription activator in *Arabidopsis* that is expressed in the leaf and stem. Its expression is present throughout the life cycle of the plant and is especially high during juvenility. Under LD, the *Arabidopsis idd8* mutant shows the late flowering phenotype and the overexpression of AtIDD8 mutant, 35S:IDD8, exhibits an early flowering. AtIDD8 is involved with flowering time control, which is related to the photoperiod pathway by direct activation of the sucrose synthesis enzyme (SUS4) (Seo *et al*., 2011). The level of *SUS4* expression is high in LD grown plants whereas the expression is low in SD grown plants. *SUS4* expression level is reduced in *phya* and *phyb* mutants. When the endogenous sugar level is low and not enough to promote the floral transition, AtIDD8 is repressed and the floral transition cannot proceed until the level of sugar is sufficient (Seo *et al*., 2011).

When both SD grown *Arabidopsis ft-1*mutants and the corresponding wild type are maintained under high light intensity, flowering times in both are accelerated. Hence, light intensity could play a role in photosynthetic control of flowering independently from *FT*. Since high light intensity increases sucrose content in the plant, sucrose could have a role in the promotion of flowering (King *et al*., 2008). Sucrose has the ability to activate *FT* expression but it does not act like florigen under LD condition where *FT* is the dominant florigen (Roldan *et al*., 1999; King, 2012). Therefore, plenty of sucrose is ineffective for flower activation if there is no *FT* expression in LD grown plants (Roldan *et al*., 1999).

1.3.3 *Trehalose-6-phosphate and flowering time*

Flowering in plants is regulated by the sugar content at the apex. Sucrose and T6P play key roles in regulation of flowering. The growth signaling molecule, T6P, is derived from the change of Glc6P and UDPGlc using Trehalose-6-phosphate synthase1 (TPS1) and is essential for the phase transition from JP to AVP and flowering in plants (Cabib and Leloir, 1958; Schluepmann *et al*., 2003). The *Arabidopsis tps1* mutant is defective in the flowering transition in that lack of TPS1 expression in *tps1* mutant causes the reduction of T6P and the delay of flowering time (van Dijken *et al*., 2004). TPS1/T6P or T6P pathway plays a role in regulating flowering in both leaf and shoot meristem (Wahl *et al*., 2013). The diurnal pattern of T6P level in *Arabidopsis*, which is similar to the pattern of sucrose, *CO*, *FT*, and *TSF* in leaf could support the role of T6P pathway in regulating floral transition together with the photoperiod pathway. In the shoot meristem, T6P facilitates the phase change in response to the aging pathway by repressing the expression of miR156 (Wahl *et al*., 2013).

Snf1-related kinase 1 (SnRK1) and target of rapamycin (TOR) kinase in plants are the regulators that connect the signal from carbohydrate and energy status to growth and development (Wullschleger *et al*., 2006; Polge and Thomas, 2007). At low sugar levels, SnRK1 plays a role in suppression of growth processes. At high sugar levels, T6P levels are also high and suppress SnRK1, the repressor of TOR (Zhang *et al*., 2009). TOR can then promote plant growth and development. To emphasize the importance of sugar signaling in phase transition in plants, the effect of SnRK1 on flowering time was studied in *Arabidopsis*. The result showed that the over expression of SnRK1 encoded gene (*AKIN10*) causes a late flowering phenotype (Baena-González *et al*., 2007).

1.3.4 *Hexokinase and flowering time*

HXK is a glucose phosphorylating enzyme in sink tissue. It acts as a glucose specific sensor to provide information on the availability of hexose which is very important for developmental processes in plants (Rolland and Sheen, 2005). The expression of *HXK1* is normally stimulated by glucose (Price *et al*., 2004). Lack of HXK1 expression in the *gin2-1* mutant exhibits the late flowering phenotype (Pourtau *et al*., 2006), which suggests that sugar signaling is essential for the flowering process. This emphasises the relationship between the sugar availability and flowering time in plants.

1.4 The regulation of FT and the induction of flowering

FT protein is an important component of florigen in the plant (Corbesier *et al*., 2007). The *FT* promoter consists of several important components that help to regulate *FT* expression regarding the photoperiodic response. In response to long days, CO activates the transcription of *FT* by binding to the promoter region near the site that the transcription starts (Tiwari *et al*., 2010). Moreover, FKF1, GI, and CDF1 are also able to bind to the *FT* locus around the promoter region (Sawa and Kay, 2011; Song *et al*., 2012). FKF1 together with GI allow the transcription of *FT* by eliminating CDF1protein from *FT* locus (Nelson *et al*., 2000; Imaizumi *et al*., 2003).

Apart from the transcriptional activators, the *FT* locus is also regulated by transcriptional repressors. LIKE HETEROCHROMATIN PROTEIN1 (LHP1) and histone H3 modified by trimethylation on lysine 27 (H3K27me3). LHP1 and H3K27me3 form Polycomb repressive complex together to repress the transcription of *FT* (Turck *et al*., 2007; Exner *et al*., 2009). *FT* locus without this repressive complex can provide the space on the chromatin for transcription factors to bind and function (Turck *et al*., 2007; He, 2012).

In addition to the proteins above, FT can also be controlled by other suppressors such as proteins in the MADS box family, AP2- like gene family. AP2-

like gene family is a small group of genes that plays a role in supressing *FT* at the transcription level (Aukerman and Sakai, 2003; Kim *et al*., 2006).This family consists of *SCHLAFMÜTZE (SMZ), TARGET OF EAT 1 (TOE1), TOE2, SCHNARCHZAPFEN (SNZ),* and *APETALA2 (AP2)* Kim *et al*., 2006) and *TEMPRANILLO (TEM1* and *TEM2). TEM1* is able to bind *FT* locus at the 5'untranslated region (Castillejo and Pelaz, 2008; Yant *et al*., 2009; Sgamma *et al*., 2014).

Apart from *FT*, there is *TWIN SISTER OF FT (TSF)* that has a similar function to *FT* (Yamaguchi *et al*., 2005). Since light is perceived in the leaf and flowers are formed at the shoot apical meristem (SAM), the signals from the leaf need to be transported to the SAM in order to promote flowering. FT protein is one of the important signals for flower induction. *FT* is expressed in the leaf phloem then FT protein moves to the shoot meristem through the sieve tube of the phloem (Huang *et al*., 2005; Corbesier *et al*., 2007). FT‑INTERACTING PROTEIN 1 (FTIP1) is required for the transportation of FT to the SAM (Liu *et al*., 2012).

At the SAM, MADs box transcription factors are essential for controlling flowering (figure 1.7). FT protein is required to interact with FLOWERING LOCUS D (FD) to stimulate the expression of flowering genes (Abe, *et al*., 2005) such as *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Borner *et al*., 2000), *FRUITFULL* (*FUL* or *AGL8* (Teper-Bamnolker and Samach, 2005), *SQUAMOSA BINDING PROTEIN LIKE* (*SPL*), and *APETALA1* (*AP1*) (Cardon *et al*., 1997). *SOC1* and *SPLs* are the targets of FD and FT in shoot meristem. The FT-FD complex and *SPLs* stimulate *FUL* which activate SOC1 (Jung *et al*., 2012). SOC1 acts with another MADs box protein, AGAMOUSLIKE 24 (AGL24), in order to activate *LEAFY* (*LFY*), the meristem identity gene that regulates the development of the flower (Lee and Lee, 2010). Another meristem identity gene, *AP1*, which is required for normal flower development is another target of the FT-FD complex and *SPLs* (Pose *et al*., 2012). *AP1* and *LFY* can activate each other indirectly (Wagner *et al*., 1999; Hempel *et al*., 2000). In addition to its interaction with FT, FD can also combine with TERMINAL FLOWER1 (TFL1) to repress the expression of *AP1* and *LFY* (Abe, *et al*., 2005). AP1 and LFY in turn repress the transcription of TFL1 when the young floral primordia are formed (Liljegren *et al*., 1999).

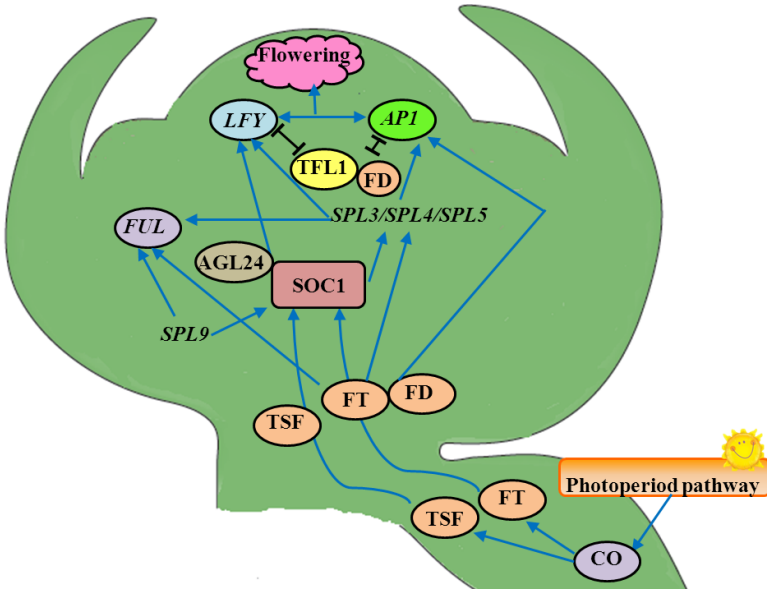


Figure 1.7 Schematic representation of the regulation of FLOWERING LOCUS T (FT) and the induction of flowering in the shoot apex meristem

FT protein is transported from the leaf to shoot apex in order to promote the apical flowering pathway. Blue arrows indicate activation and black T-bars indicate inhibition. The complete nomenclature of the genes is described in section 1.2.3.

1.5 Juvenility

1.5.1 *Definition of Juvenility*

The juvenile phase (JP) is the early stage where plants are not competent to flower. The plants are not able to be induced to flower by environmental signals and other factors that promote flowering in adult plants (Thomas and Vince-Prue, 1997; McDaniel *et al*., 1992; Amasino, 1996). The competence to flower in plants occurs when the shoot meristem can be induced by the inductive signals, which is when plants are in the adult vegetative phase (AVP). In the reproductive phase (RP), the meristem is changed from producing leaves to flowers, which is the floral evocation. Floral evocation can occur in juvenile plants which are not induced to flower by grafting an induced leaf on to it (McDaniel, *et al*., 1992). Therefore, floral induction and floral evocation are separate processes. Floral induction occurs in leaf whereas

floral evocation occurs in shoot meristem with the support of signals from induced leaves. The factors that have an influence on JP in plants could be both environmental and developmental (Koornneef *et al*., 1998). The factors could estimate the time of the year that plant can produce flower, which helps the plant to survive and have reproductive success.

The length of JP varies among species. The plants that have longer life cycles tend to have longer JP length. For example, in beech, oak, orchid, and rose, the JP lengths are around 30-40 years (Rezq Basheer, 2007), 25 -30 years (McGowran and Parkinson, 1998), 4-7 years (Hew and Young, 1997), and 20-30 days (Hackett, 1985) respectively.

1.5.2 *MicroRNAs control JP*

MiRNAs are short, non-coding RNAs, of approximately 20 bp. They are generated from the cleavage of hairpin structure primary miRNA (Jones-Rhoades et al., 2006). They regulate the expression of genes in plants (Bartel, 2009). Studies of the genetic regulation of transitions in *Arabidopsis* and maize reveal that SPL transcription factors are the target of microRNA 156 (miR156) (Wu and Poethig, 2006) and that activate genes that regulate flowering such as *FRUITFUL* (*FUL*), *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), *LEAFY* (*LFY*), *AGAMOUS-LIKE* (*AGL*), and *APETALA1* (*AP1*). The level of miR156 is changed in each developmental phase and regulates the level of SPL. There are eight gene members of the miR156 family located in the *Arabidopsis* genome, MIR156A to MIR156H (Schwab *et al*., 2005; Wu and Poethig, 2006).

During the JP, there are high levels of miR156 whereas levels decrease in the adult phase (Proveniers, 2013). Reduction in the levels of miR156 in the AVP raises the level of SPL3 (Wu and Poethig, 2006; Wang *et al*., 2009). Therefore, the expression of miR156 and repression of SPL may be the key to maintaining the juvenile phase. MicroRNA 172 (miR172) is also involved in developmental transitions in plants. Expression of miR172 is repressed by miR156 is activated by SPL (Meyers *et al*., 2008). MiR172 acts in the adult phase. It suppresses the AP2-like family of transcription factors, which are repressors of FT. Therefore, miR172

promotes the transition from juvenile to adult vegetative phase (Fornara and Coupland, 2009). However the simple model based on miRNAs does not explain the change in juvenility due to environmental signals.

1.5.3 *The characteristics of JP*

Apart from reproductive competence, changes in plant morphology have been used to distinguish the JP from other phases. Plants that show obvious differences in characteristics between JP and AVP are heteroblastic plants whereas plants that present only small distinguishable characteristics are termed homoblastic plants (Goebel, 1900; Zotz *et al*., 2011). For example, in *Arabidopsis*, leaf morphology is one of the marks for the phase transition from JP to AVP. When plants are in the JP, their leaves are round, not serrated, and no trichomes are produced on the leaf abaxial sides whereas in the AVP, their leaves are elongated, serrated and contain abaxial trichomes (Araki, 2001; Wu *et al*., 2009). In antirrhinum, the length, width, and area of leaves are increased when plants age (Costa *et al*., 2012). *Hippuris vulgaris* is an aquatic plant, where leaf morphology changes dramatically from shoots to aerial leaves when the phase transition occurs (Goliber and Feldman, 1989). In the shrub plants *Melicope simplex*, the juvenile leaf shape is trifoliate whereas the adult leaf shape is simple (Gamage and Jesson, 2007). Not all the plants display changes in their morphology when they enter the AVP. Therefore attainment of reproductive competence is the most reliable measure of the transition (Adams *et al*., 2003).

1.5.4 *Measuring JP*

In photoperiodic plants, the JP and RP are not sensitive to photoperiod, whereas the AVP is sensitive to photoperiod (Thomas and Vince-Prue, 1997). Since plants are not always sensitive to photoperiod throughout their developmental phases, the length of the photoperiod sensitive phases can be measured using reciprocal transfer experiments by transferring of plants from SD to LD conditions

and vice versa at regular intervals after germination (Wang *et al*., 1997; Adam *et al*., 2001; Adam *et al*., 2003). In this study, plants were transferred from SD to LD and the numbers of leaves at flowering are recorded in order to generate the modelling graph of the different growth phases (figure 1.8). Number of leaves at flowering in each plant is not affected by photoperiod when plants are in the photoperiod-insensitive growth phases. The number of leaves at flowering is stable when plants are in JP and RP. This indicates that photoperiod does not have an effect on flowering time during these growth phases.

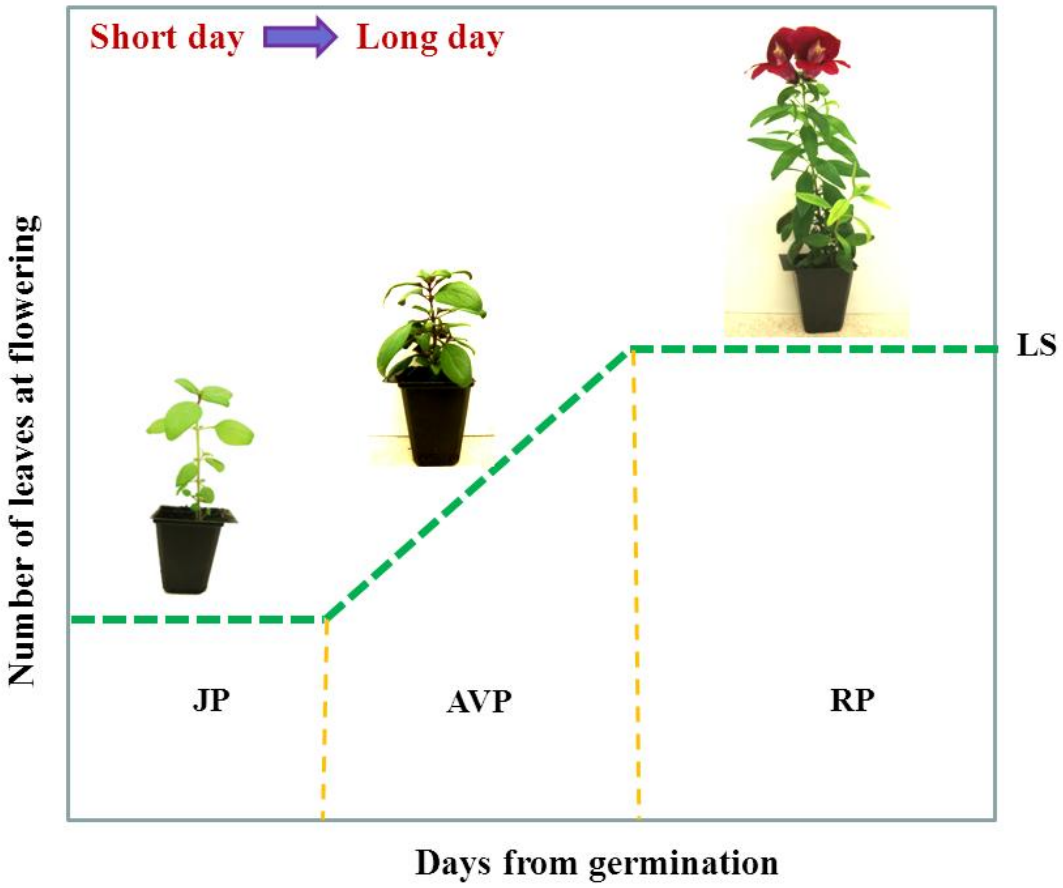


Figure 1.8 Effect of photoperiod on the phases of photoperiod sensitivity in antirrhinum
Flowering time of plants generated from number of leaves below the inflorescence data.Green line represents the plants transferred from SD to LD conditions at regular intervals from germination. Abbreviations: JP, a photoperiod-insensitive juvenile phase; AVP, photoperiod-sensitive adult vegetative phase; RP, photoperiod-insensitive phases; LS, the number of leaves produced under continuous SD. Figure adapted from Adams et al. (2003).

1.6 Model plants for the study of flowering

1.6.1 *Arabidopsis thaliana*

Arabidopsis is a native plant from Africa and Eurasia (O'Kane and Al-Shehbaz, 1997). It is a member of the Brassicaceae family (Koch *et al*., 2001). It is a facultative photoperiodic plant, which flowers quicker in long photoperiod conditions compared to short photoperiod (Park *et al*., 1999). In this study, *Arabidopsis* is used to study the effect of carbohydrate related genes on the JP because its life cycle is very short with an average of 6-8 weeks (Somerville and Koornneef, 2002). The biggest advantage of *Arabidopsis* for this study is that there is a wide range of mutants available to support this study. These includes mutants produced using ethyl methane sulfonate (EMS) to promote single base changes in DNA, X-rays to induce DNA damage (Koornneeff *et al*., 1982), or using transformation technology, T-DNA mutagenesis (Feldmann, 1991; Koncz *et al*., 1992).

1.6.2 *Antirrhinum majus*

Antirrhinum is originally from western Mediterranean and has long been used to study plant molecular biology. The flowering genes isolated from antirrhinum are quite similar to those of *Arabidopsis* including *DEFICIENS*, *GLOBOSA*, *FLORICAULA*, *SQUAMOSA*, *FIMBRIATA*, and *CENTRORADIALIS* which are orthologues of the *Arabidopsis* gene *APETALA3*, *PISTILLATA*, *LEAFY*, *APETALA1*, *UNUSUAL FLORAL ORGANS*, and *TERMINAL FLOWER* respectively (Schwarz-Sommer *et al*., 2003; Davies *et al*., 2006). Moreover, many MADS-box proteins, the important protein that involve with plant developmental including flowering, were found in antirrhinum (Davies *et al*., 1996 Egea-Cortines *et al*., 1999). Similar to *Arabidopsis*, antirrhinum is a facultative LDP. It has been used to

study the environmental effects on flowering time (Cremer *et al*., 1998; Khan *et al*., 2009). In antirrhinum, photoperiod and light integral (LI) affect flowering time and the number of leaves at flowering. Shorter photoperiod and lower LI resulted in an extension of flowering time and increased leaf number at flowering (Adams *et al*., 2003; Munir *et al*., 2004).

In this study, antirrhinum was used to study the effect of LI on JP because the JP is longer than in *Arabidopsis*, which is long enough to see the effect of LI on the plant and make it a suitable model for crop species.

1.7 Project aims

The overall mechanism of how carbohydrate metabolism affects the length of the JP is still unclear. The relationship between the juvenile to adult phase change and carbohydrate metabolism could be the key to the mechanism of the juvenility. Hence, this study of the expression of the genes that control carbohydrate metabolism in relation to light and juvenility was conducted.

The length of the JP is important since it impacts on flowering time. Flowering time is important since it is associated with seed yield and several crop production systems in the market. Understanding and manipulating the JP is a key objective in developing strategies for managing flower production to match market requirements. The physiology and molecular mechanisms underlying JP in annual species have been studied by Professor Brian Thomas's group, the University of Warwick. They found that plants are not able to synthesis FT during the JP (Sgamma *et al*., 2014). The PhD study of Matsoukas (2010) revealed a better understanding of factors associated with the JP to AVP transition. He showed that the length of the JP is influenced by light level and CO2 concentration. In addition, a correlation between the developmental transitions and photosynthetic assimilate levels was revealed. In order to advance from juvenile to adult vegetative growth, it appears that a certain level of carbohydrate is required. The intention of my work was to build on the observations of Matsoukas to gain a better understanding of how the environment, specifically LI, influences JP length and its effect on phase transition in antirrhinum.

Therefore the overall objective for this study is to investigate the mechanism by which environmental factors regulate juvenility in the model plant, *Arabidopsis thaliana*, and the crop plant, *Antirrhinum majus.*

1.7.1 *In Antirrhinum majus*

- To establish a relevant experimental system to investigate the effect of light integral on phase development
- To determine the effect of light integral on photosynthetic assimilation in relation to juvenility
- To determine the effect of light integral on the expression of key genes in relation to juvenility

1.7.2 *In Arabidopsis thaliana*

- To determine the influence of specific carbohydrate related genes on JP
- To determine the effect of miR156 on flowering time
- To determine the effect of LI on the expression of miR156 in relation to JP

CHAPTER2. GENERAL MATERIALS AND METHODS

The descriptions in this chapter are for the common materials and methods that appear in more than one result chapter. The more specific materials and methods will be explained later in their related chapters.

2.1 Plant materials

The *Antirrhinum majus* seeds (Bells Red F1) were obtained from Colgrave Seeds, UK (Goldsmith Seeds, Inc.).

2.2 Plant growth conditions

Antirrhinum seeds were sown with two seeds per cell into Plantpak P40 trays (HSP, Essex, UK) containing seed and modular compost (Levington F2 +Sand). After sowing, the seeds were watered heavily and the trays covered with a 634x413 mm clear autoclave bag (CPS Flexible Ltd.) to maintain the humidity. The trays were put into the appropriate growth cabinet for each experiment. After 50% of germination (T_0), autoclave bags were removed from the trays and seedlings thinned to one per cell and the remaining seedlings were used for specific experiments.

All growth cabinets used in this study were set with day/night temperatures of 22°C with no humidity setting. Transfer experiments and experiments performed to generate leaf material for RNASeq were conducted in four MLR-351 Versatile plant growth (SANYO Electric Co., Ltd.) cabinets. For other experiments (section 3.2.1, 3.2.3, and 5.2.4), three MLR-352 Versatile plant growth (Panasonic Co., Ltd.) cabinets were used.

For the Sanyo cabinets, the short day high light (SDHL) and short day low light (SDLL) cabinets with 8 h photoperiod were set to provide light integral by fluorescent bulbs (General Electric 60W, HU). The long day high light (LDHL) and

long day low light (LDLL) cabinets with 16 h photoperiod were set to provide 8 h high light integral by fluorescent bulbs and 8 h light extension by tungsten bulbs (Philips 32W, NL). Light levels and light integrals were provided in table 2.1.

For the Panasonic cabinets, SDHL and SDLL cabinets with 8 h photoperiod were set to provide light integral by fluorescent bulbs. LDHL and LDLL with 16 h photoperiod was set to provide light integral by fluorescent bulbs. Light levels and light integrals were provided in table 2.2.

Light condition	**Light level**		**Light integral**
	8h photoperiod by fluorescent bulbs ($\mu mol\ s^{-2}\ m^{-1}$)	**8h photoperiod extension by tungsten bulbs ($\mu mol\ s^{-2}\ m^{-1}$)**	**Total light integral per day ($mol\ s^{-2}\ d^{-1}$)**
SDHL	95	N/A	2.7
SDLL	31.5	N/A	0.9
LDHL	104	16.7	4.1
LDLL	32.8	17.3	1.4

Table 2.1 The light conditions set up in Sanyo cabinets

Light condition	Light level by fluorescent bulbs (8h photoperiod for SD condition and 16h photoperiod condition) ($\mu mol\ s^{-2}\ m^{-1}$)	Total light integral per day ($mol\ s^{-2}\ d^{-1}$)
SDHL	124	3.6
LDHL	35.6	1
LDHL	126	7.3
LDLL	36.2	2

Table 2.2 The light conditions set up in Panasonic cabinets

2.3 Diurnal time course experiments

Plants were grown under three growth conditions, LDHL, LDLL and SDHL in growth cabinets as described in section 2.2. Plants were grown under each light regime until they reached the late adult vegetative phase. Then first expanded leaves were harvested at 2 h intervals for two consecutive days. Seven plants were harvested for each sample.

2.4 Antirrhinum and *Arabidopsis* transfer experiments

2.4.1 *Transfer experiments to generate plant materials for molecular and biochemical analyses*

After sowing, antirrhinum plants were initially grown under SDHL conditions at T0. Then the plants were moved to two different SD cabinets, SDHL and SDLL. Starting one week after germination, transfer from each SD cabinet to LDHL was carried out weekly with seven plants per transfer at ZT7 (Zeitgeber time). At the end of the first LD experienced, leaves were harvested from the transferred plants with the replication size of seven (figure 2.1).

In transfer experiments using *Arabidopsis* carbohydrate gene mutants, seven plants were transferred for each mutant from the SDHL to LDHL cabinet daily. The next day after germination, transfers took place at ZT7 and the leaves were harvested at ZT14 on the day of transfer (figure 2.2).

Leaf material was harvested into aluminium foil packages (Terinex Limited., UK) and broken up into smaller pieces under liquid nitrogen and pooled. These were separated into two batches, one for molecular and another for biochemical analysis and stored at -80°C.

2.4.2 *Transfer experiments for determination of growth phases*

For both antirrhinum and *Arabidopsis*, the transfer experiments were carried out at the same time and following the same procedure as described in section 2.4.1. For the control plants in each experiment, seven plants were grown in each cabinet used without transfer. Growth rates and flowering times were recorded for both transferred and control plants. In antirrhinum, the number of true leaves below the flower on the main stem was counted weekly to assess growth rate and the flowering time in days from germination was observed. Flowering time was assessed by number of leaves at flowering since no more leaves are produced on the main stem once flowers are initiated. In *Arabidopsis*, the number of rosette leaves was counted weekly to assess growth rate and flowering time recorded by number of rosette leaves at 1 cm bolt length.

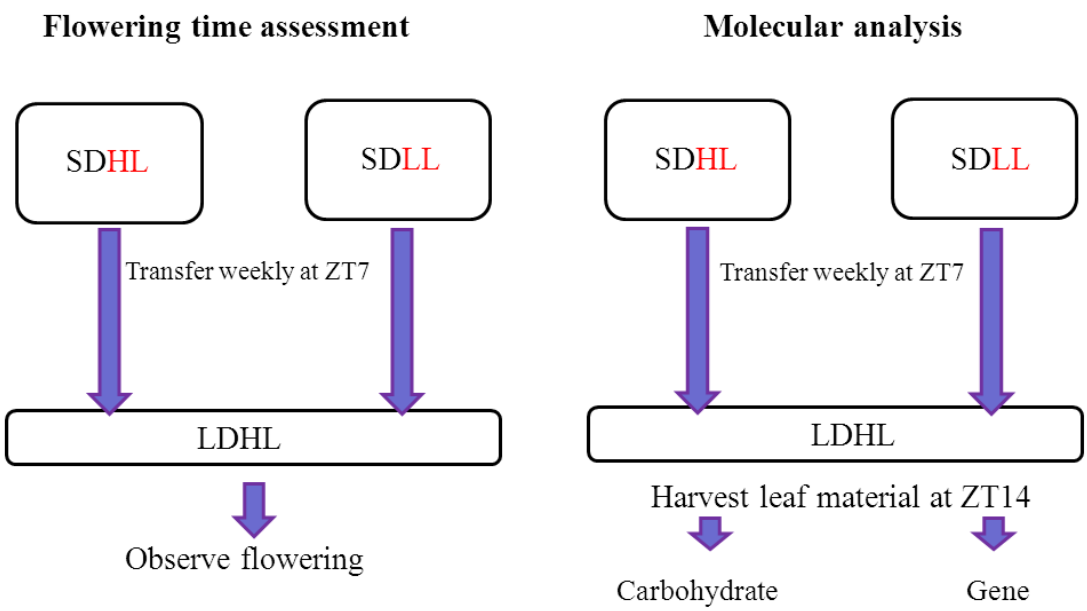


Figure2.1 Schematic representation of the transfer experiment with antirrhinum under different light integrals*.To determine the effect of different LI and expression of carbohydrate and flowering genes on JP, the plants under SDLL and SDHL were transfer to LDHL weekly to assess flowering time and generate plant materials. Abbreviations: SDHL, short day high light; SDLL, short day low light; LDHL, long day high light*

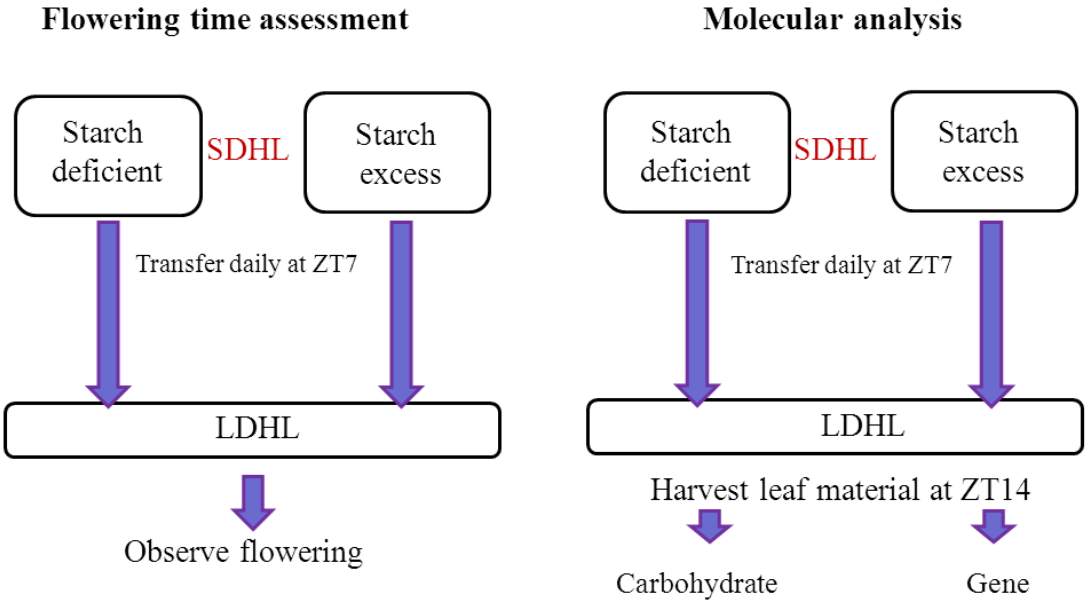


Figure2.2 Schematic representation of the transfer experiment with Arabidopdsis carbohydrate gene mutants*.To determine the effect of specific carbohydrate genes and expression flowering genes on JP, the starch deficient and starch excess mutants under SDHL were transfer to LDHL daily to assess flowering time and generate plant materials. Abbreviations: SDHL, short day high light; LDHL, long day high light*

For analysis, curves were generated using Microsoft® Excel® (Microsoft Corporation, 2007) to plot average number of leaves at flowering versus ages from germination. To determine the different growth phases, GenStat 13th Edition software package (Payne *et al.*, 2009) was used. The length of each developmental phase was calculated from the number of leaves on the main stem at flowering that fitted in the logistic curve (black line) and the maximum slope (green line) was created followed by the lag time lines (light blue line) and the stationary phases (dark blue line) fitting which were obtained from the lower and upper logistic curve asymptote respectively. The purple dotted lines distinguish between each developmental phase (figure 2.3).

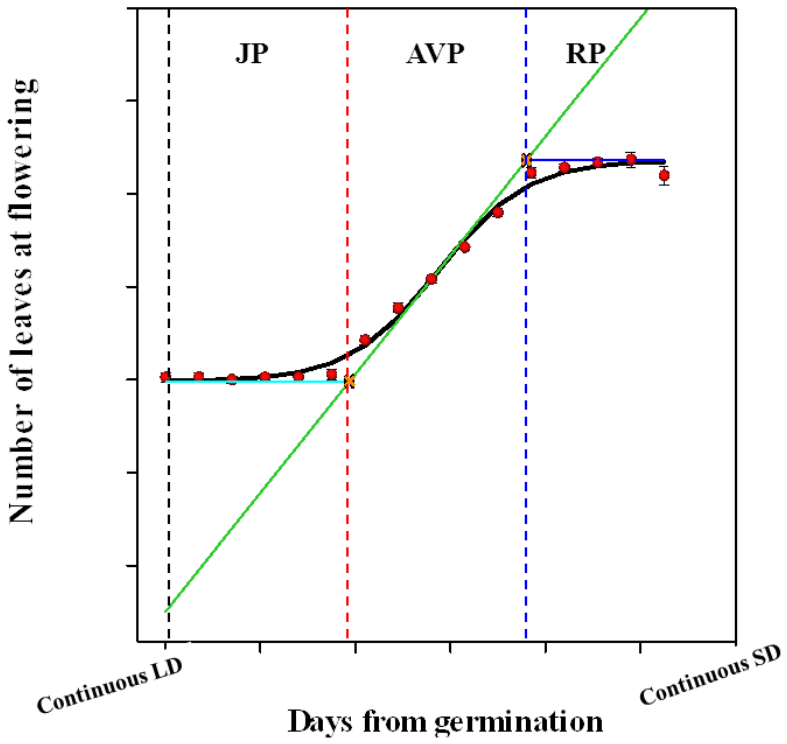


Figure 2.3 Determination of developmental growth phases of the plant using the GenStat 13th Edition software package

The number of leaves at flowering in plants transferred from SD to LD conditions at the regular interval from germination. JP: juvenile phase, AVP: adult vegetative phase, RP: reproductive phase, SD: short day condition, LD: long day condition, black curve line represents logistic curve, green line represents maximum slope, light blue line represents lag time, dark blue line represents stationary phase, red dotted line represents the limit of JP, blue dotted line represents the limit of AVP, vertical and horizontal error bars represent the standard error of the mean of the leaf number at flowering and the phase length respectively.

2.5 RNA Extraction and cDNA synthesis

Total RNA of antirrhinum and *Arabidopsis* materials was isolated using TRIZOL reagent (Invitrogen, USA), following the manufacturer's guidelines. After isolation, in order to measure the quality and quantity of total RNA, the Thermo Scientific NanoDrop^{TM} 1000 Spectrophotometer (NanoDrop Technologies, Inc., USA) was used and an aliquat of total RNA was run on a1% (w/v) agarose gel to check the quality (section 2.9). Total RNA was treated with TURBO™ DNase from the TURBO DNA-free treatment kit (Ambion, USA), following the manufacturer's guidelines, in order to eliminate contaminant genomic DNA. To ensure all gDNA was removed, RNA was used as a template in a PCR (section 2.7) using housekeeping gene primers (appendix, table A.2).

Two microgram of total RNA was used for cDNA synthesis. ThermoScript™ Reverse Transcriptase (RT) from ThermoScript™ RT-PCR Systems kit (Invitrogen Ltd., USA) was used to synthesis cDNA for carbohydrate gene isolation and iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Ltd., UK) was used to produce cDNA for real time PCR quantification. The manufacturer's instructions were followed for both kits.

2.6 Primer design

Primers for PCR and Real-time PCR qualification were designed using Primer3Plus (http://www.bioinformatics.nl/cgi bin/primer3plus/primer3plus.cgi). For Real-time PCR, the primers were checked for binding in the amplicon in a region free of secondary structure using the mfold (http://mfold.rna.albany.edu/?q=mfold). Primers for Real-time PCR with carbohydrate genes in antirrhinum were designed using newly identified sequence for *AmPGM* and *AmPGI* and partial EST sequence for the other genes (section 4.3.1).

2.7 Polymerase Chain Reaction (PCR)

The KOD Hot Start DNA Polymerase kit (Merck Chemicals, Germany) was used for general PCR reaction, following the manufacturer's guidelines. A standard 10 µl reaction consisted of 1 µl of 10X KOD Hot Start DNA Polymerase reaction buffer, 0.6 µl of 2 mM MgSO4, 1 µl of 0.2 mM dNTPs, 0.5 µM of each forward and reverse primer, 0.4 µl of template DNA from cDNA synthesis reaction (section 2.5), 0.2 µl of KOD Hot Start DNA Polymerase (1U/ µl), and made up volume to 10 µl using Sterile Deionized Water (SDW). There were four steps of amplification. The first step was Polymerase activation at 95°C for 2 minutes, later was denaturation at 95°C for 20 seconds followed by annealing at the specific primer temperature for 10 seconds, and the last step was extension at 70°C for 10 seconds. Steps 2 to 4 were repeated for between 20 to 40 cycles. All primer details and expected PCR product sizes are shown in the appendix, table A.1 and A.2.

2.8 Real-time PCR quantification

Total RNA was isolated from plant materials and cDNA synthesized from each RNA sample (section 2.5). Real-time PCR quantification was performed using the ICycler® and CFX384 TouchTM Real-time PCR machines from BioRad (Bio-Rad Laboratories Ltd., UK).When the ICycler® was used, each PCR reaction consisted of 7.5 µl of SYBR green (Eurogentec Ltd., UK), 0.5µl of cDNA, between 0.1 to 0.4 µM of specific forward and reverse primers and the volume made to 10 µl by SDW. When the and CFX384 Touch™ was used, each PCR reaction contained 5 µl of SsoAdvanced™ SYBR® Green Supermix (Bio-Rad Laboratories Ltd., UK), 0.4µl of cDNA template, between 0.1 to 0.4 µM of specific forward and reverse primers and the volume made to 10 µl by SDW. The protocol is provided in table 2.3 and all primer details are presented in the appendix, table A.5.

The Real-time data obtained were analysed using qbase plus software (Biogazelle, Belgium). In antirrhinum, expression of target genes was normalized

against *ACTIN* (HQ853640) and *ELONGATION FACTOR 1 α* (AJ805055) expression levels. In *Arabidopsis*, expression of each target gene was normalized against the expression of *ACTIN2* (BE038458) and *β-TUBULIN* (AY040074). After PCR amplification, all products were sequenced (section 2.11) in order to confirm the product.

Step	Temperature (°C)	Time (minute)
1	95.0	3:00
2	95.0	0:10
3	Dependent on the gene of interest	0:30
	Plate Read	
4	GOTO Step 2, 39 more times	
5	95.0	0:10
6	Melt Curve 65.0°C to 95.0°C: Increment 0.5°C 0:05	
	Plate Read	

Table 2.3 The thermal cycling conditions for Real-time PCR quantification

2.9 Agarose gel electrophoresis of RNA and DNA

Agarose gels were composed of 1-2% (w/v) ultra-pure agarose (Invitrogen, USA) with 1x Tris-acetate-EDTA buffer (TAE buffer) (VWR Interbational, UK) and either 0. 2 μg/ml of ethidium bromide EtBr (VWR International, UK) or 1 μl/ml of GelRed™ Nucleic Acid Gel Stain 10,000x in water (VWR International, UK). One volume of Orange G loading buffer (Sigma-Aldrich, UK) was added to five volumes of either RNA or DNA prior to loading each on a gel. 1Kb plus DNA ladder (Invitrogen, USA) was also loaded each gel for product size estimation. The electrophoresis was carried out at 100 mA in tanks with 1x TAE running buffer for the electrode distance at room temperature (25 °C) for 40 to 80 minutes. After

electrophoresis, the samples on the gel were visualised and recorded by a G: BOX gel documentation system (Syngene, UK).

2.10 DNA extraction and purification from gel

After gel electrophoresis, DNA products were isolated and purified from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN), following the manufacturer's guidelines.

2.11 DNA Sequencing

The ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, UK) was used for the PCR reactions. For each 10 µl reaction, 4 µl of Terminator Ready Reaction mix was added together with 10 ng of PCR product, 3.2 pmol of primers and SDW to to added to a volume of 10 µl. PCR reactions were carried out with the first step of 96°C for 10 seconds, second step of 50°C for 5 seconds and last step of 60°C for 4 minutes repeating for 25 cycles. The PCR products were sent to Genomic Resource Centre, School of Life Sciences, University of Warwick for sequencing.

To sequence the products from Real-time PCR quantification, all products from Real-time PCR reaction were purified using the QIAquick PCR Purification Kit (QIAGEN) following the manufacturer's guidelines and sent for sequencing using the LIGHTrun sequencing service (GATC Biotech AG, Germany) following the service quidelines.

Sequencing data were interpreted using Chromas and analysed using LASERGENE® SeqMan™ (LASERGENE 9).

2.12 Examination of carbohydrate levels at each developmental phase

2.12.1 *Sample preparation*

Frozen plant material for biochemical analysis was homogenised using a homogeniser (Dremel Workstation, Dremel Europe) for 2-3 min followed by adding 1 ml of 80% ($^{v}/_{v}$) EtOH (ethanol) per 100 mg of plant tissue and mixed before incubating at 75°C for 5 min. After that, samples were centrifuged (Biofuge, Heraeus instruments) at 3,000 x g for 10 min. Supernatants were transferred to new 1.5 ml eppendorf tubes. Added 1 ml of 80% (v/v) ethanol to each pellet and mixed before incubating at 75°C for 5 min. Pellets were centrifuged at 3,000 x g for 10 min and supernatants were removed and transferred to1.5 ml tubes that contained supernatants from the previous spin. Supernatants containing soluble sugars were analysed as described in section 2.12.2. Pellets were kept in 0.5 ml 40% (v/v) EtOH at 2°C for starch analysis (2.12.3).

2.12.2 *Soluble sugar determination by enzymatic assay*

Glucose, fructose, and sucrose concentrations were determined using the Glucose (HK) Assay Kit (Sigma-Aldrich Co. LLC., UK), Fructose Assay Kit (Sigma-Aldrich Co. LLC., UK) and Sucrose Assay kit (Sigma-Aldrich Co. LLC., UK) respectively, following the manufacturer's guidelines but reducing each reaction volume. The assay volumes for glucose, fructose, and sucrose were 220 µl, 222 µl, and 220 µl respectively. The enzymatic assays were carried out in 96 microplates (Microtest^{TM} 96, Becton Dickinson Labware Europe) using the extracted supernatants (section 2.12.1).The concentration of soluble sugars were determined by measurement of the absorbance at 340 nm using Varioskan Flash Multimode Reader (Thermo Fisher Scientific Inc., USA). The absorbance data and standard curve were interpreted using Skanlt software (Thermo Fisher Scientific Inc., USA).

2.12.3 *Enzymatic assay for starch analysis*

Pellets obtained from section 2.12.1 were added to 1 ml of Sterile Deionized Water (SDW) and centrifuged at 3,000 x g for 5 min. Supernatants were removed and this pellet washing step repeated twice. Pellets were air-dried for 5 min before dissolving in SDW. SDW was added to pellets in the proportion of 2.5 ml per 100 mg of original frozen plant tissue and mixed. Dissolved pellets were transferred to falcon tubes (Aktiengesell schaft & Co.) and boiled for 3 min. Samples were autoclaved (Classic 2100 Standard, Prestige Medical UK) at 135°C for 1 hr followed by addition of 2.5 ml of SDW to each sample tube.

Starch concentration was determined using the Starch (HK) Assay Kit (Sigma-Aldrich Co. LLC., UK), following the manufacturer's guidelines but reducing each reaction volume from 1.2 ml to 200 µl. The assays were carried out in 96 microplates (Microtest^{TM} 96, Becton Dickinson Labware Europe) using 100 µl of dissolved pellet of the EtOH extract in each assay before measuring the concentration of starch at 340 nm using Varioskan Flash Multimode. The absorbance data and standard curve were interpreted using Skanlt software.

CHAPTER3. INFLUENCE OF LIGHT INTEGRAL ON DEVELOPMENTAL EXPRESSION OF FLOWERING TIME GENES

3.1 Introduction

The ability to detect environmental signals is important for plants in order to estimate the best timing for important developmental process such as flowering. Plants need to estimate the right time to ensure they can produce offspring and survive in specific environmental conditions. Daylength is one of the factors that integrates flowering time to seasonal changes. Since changes in daylength throughout the year are predictable, plants can adjust themselves to the change by linking daylength information to their developmental processes.

Light absorption is important for plants since it occurs with photosynthesis, which is essential for metabolism, growth and developmental processes including flowering. Phase transition in several plants is affected by photoperiod (Amasino, 1996; Chuck and Hake, 2005; Bäurle and Dean, 2006; Imaizumi, 2010). In *Arabidopsis*, light signals perceived by plant have an important role in plant circadian clock regulation (Imaizumi and Kay, 2006). The clock drives rhythmic expression of key genes which is necessary for protein production to peak at the right time. Therefore the clock helps to measure the suitable environment for plant to develop their growth. Plant biological processes are not controlled by the outside environmental factors directly but need the circadian clock to mediate the system. Since environmental conditions like dark and light period can be predicted, preparation of the plant for the expected environment is essential and can provide an immediate response from the plant rather than waiting until the plant is exposed to some environmental condition and then responding.

An important aspect of the photoperiodic response in plant is the diurnal rhythm of specific genes such as *FKF1*, *GI*, and *CO*. In *CO*, when these rhythms coincide with light under LD, the translated proteins are able to activate *FT* which leads to the induction of flowering (Putterill *et al*., 2004; Valverde *et al*., 2004; Mizoguchi *et al*., 2005; Imaizumi and Kay, 2006).

The importance of light on plant developmental processes has been studied for many years. There are several studies on the relationship between photoperiod and plant development but not many focused on the effect of light integral on plant development. Light integral has been shown to have a bigger effect on the rate of carbohydrate production in cucumber leaf compared to that of photoperiod (Obbins and Pharr, 1987) and the carbohydrate content in plants was increased when plants were moved from LL to HL growth condition (Schmitz *et al*., 2014). Studies in antirrhinum have shown that growth of plants in either shorter photoperiods or at lower light integrals increase the length of time taken to flower and the number of leaves present on the plant at flowering time (Cremer *et al*., 1998; Schwarz-Sommer *et al*., 2003). Therefore, studying the effect of differences in light integral has on the expression of flowering time genes could clarify how light integral plays a role in juvenility and flowering time. The objective for this chapter was to establish a relevant experimental system to investigate the effect of light integral on phase development, the length of juvenility, and the expression of flowering time genes in relation to juvenility in *Antirrhinum majus*.

3.2 Materials and Methods

The descriptions in this chapter are for materials and methods specific for this results chapter. For the common materials and methods, the descriptions are in chapter 2. For the transfer experiments, plants were grown under the same environmental conditions at the same time to generate material for flowering time assessment and molecular and biochemical analysis.

3.2.1 *Transfer experiment with antirrhinum grown under different light integrals*

To generate antirrhinum materials that differed in juvenile phase length, antirrhinum seeds (section 2.1) were sown with two seeds per cell and thinned out to be one seedling per cell after T_0 following the procedure in section 2.2. The seedlings were divided equally between SDHL and SDLL cabinets. The light condition and cabinet setting are described in section 2.2. Leaf materials were harvested as stated in section 2.4.1.This material was used for developmental expression analysis of flowering time genes (section 3.2.4) and carbohydrate genes (section 4.3.2).

To assess the flowering time of each group of plants, SDHL and SDLL, antirrhinum seeds (section 2.1) were sown with two seeds per cell following the procedure in section 2.2. After plants reached T_0, seedlings were put in SDHL, SDLL, and LDHL cabinets as controls. The other seedlings were divided equally to put in SDHL and SDLL cabinets and flowering time assessment was carried out as described in section 2.4.2.

3.2.2 *Transfer experiment with antirrhinum to determine the effect of the number of long days (LD) received after transfer of plants from SDLL on the expression of AmFT*

Antirrhinum seeds (section 2.1) were sown with two seeds per cell following the procedure in section 2.2. After plants reached T_0, the excess seedings in each cell were removed.To make six transfer groups, SDLL to LDHL, SDLL to LDLL, SDLL to SDHL, and SDHL to LDHL, SDHL to LDLL and continuous SDHL as controls. The seedlings were divided first into two equal groups to put in SDHL and SDLL cabinets (figure 3.1). The plants were grown until 1 week after juvenility ended (about five and six weeks after germination for SDHL and SDLL grown plants respectively). Later, plants in each condition were divided into three groups. For SDHL conditions which were the control for this experiment, plants in group one and two were transferred to and kept under LDHL and LDLL conditions respectively. Plants in group three remained under SDHL. For SDLL condition, plants in group one, two, and three were transferred to and kept under LDHL, LDLL, and SDHL conditions respectively. Light conditions and cabinet settings are described in section 2.2. For plants grown under SDHL and SDLL transferred to the LDHL condition, at the end of each consecutive LD experienced for a total of seven days, the first expanded leaves were harvested every day at the end of each LD (ZT14). For the other four transfer groups, SDLL to LDLL, SDLL to SDHL, SDHL to LDLL, and continuous SDHL, plants were transferred to LDHL condition at the end of each consecutive LD experienced for 24h before harvesting. The harvested leaf materials were processed followed section 2.4.1. These materials were used for *AmFT* expression analysis in section 3.2.4.

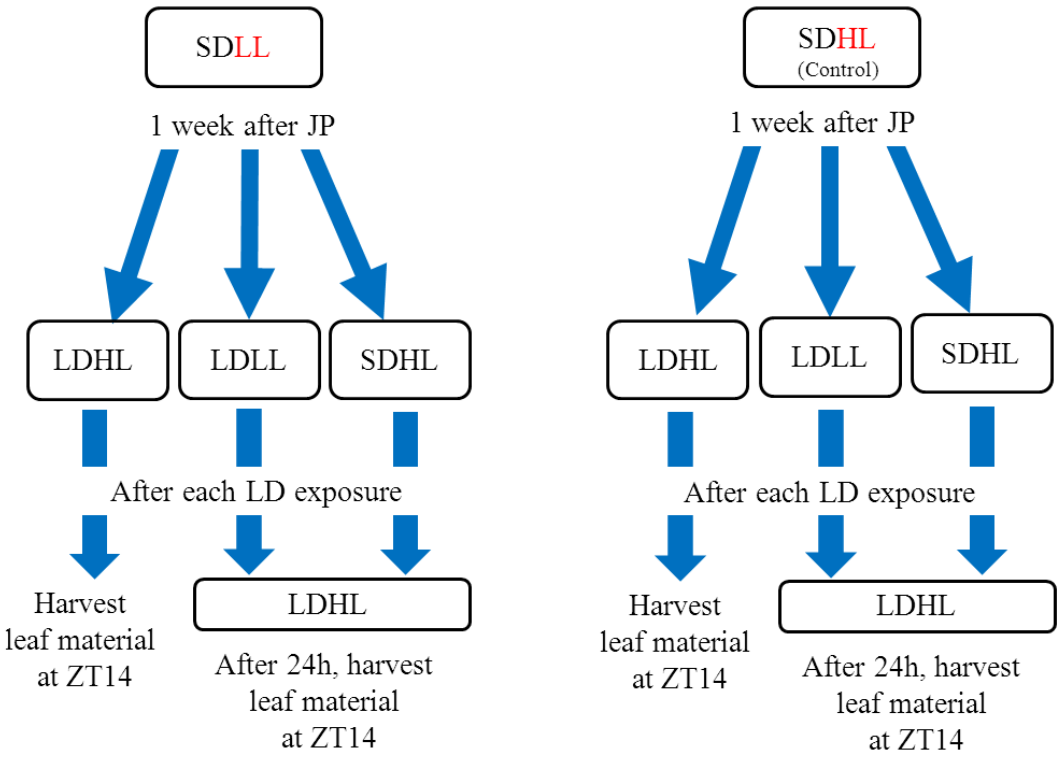


Figure3.1Schematic representation of the transfer experiment with antirrhinum under different light integrals*.To determine the effect of number of LD receive after transfer of plants from SDLL on the expression of AmFT.There were six groups of transfer, SDLL to LDHL, SDLL to LDLL, SDLL to SDHL, SDHL to LDHL, SDHL to LDLL, amd continuous SDHL . After each LD plants obtained, first expanded leaves were harvested at ZT14 for seven days. Abbreviations: SDHL, short day high light; SDLL, short day low light; LDLL, long day low light; LDHL, long day high light*

3.2.3 *Transfer experiment to assess flowering time of each transfer group of plants, SDLL to LDLL, SDHL to LDLL, and SDLL to SDHL*

Antirrhinum seeds (section 2.1) were sown with two seeds per cell following the procedure in section 2.2. After plants reached T_0, seedlings were put in SDLL, SDHL, and LDLL as controls. The other seedlings were divided into three transfer groups, SDLL to LDLL, SDHL to LDLL, and SDLL to SDHL (figure 3.2).Transfers were carried out weekly at ZT7. At the end of the first LD experienced, the first expanded leaves were harvested at ZT7 following with the harvesting procedure described in section 2.4.1. Flowering time assessment was carried out as described in section 2.4.2.

To determine the leaf production rate, the number of leaves > 1.5 millimetres length, not including cotyledons on the main stem were counted weekly for all plants.

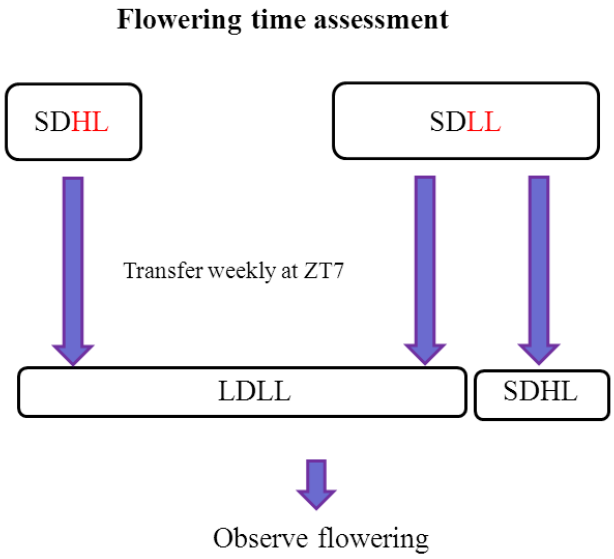


Figure3.2 Schematic representation of the transfer experiment with antirrhinum under different light integrals*.To determine the effect of different LI on JP, the plants were transferred from SDHL to LDLL, SDLL to LDLL, and SDLL to SDHLweekly to assess flowering time.Abbreviations: SDHL, short day high light; SDLL, short day low light; LDLL, long day low light*

3.2.4 *Generating antirrhinum materials for diurnal analysis of AmFT expression in plants grown under different light conditions*

Plants were grown under three growth conditions, LDHL, LDLL and, SDHL (section 2.2) until they reached the late adult vegetative phase close to flower initiation before harvesting. LDHL, LDLL, and SDHL grown plants were harvested at 45, 70, and 45 days from germination respectively. The plants were harvested at two hour intervals to complete a 24 h diurnal time course for the LDHL treatment and for two consecutive days for LDLL and SDHL. All plants materials were stored at -80 °C until required for analysis.

3.3 Results

3.3.1 *Impact of light integral on Juvenile phase length and flowering time gene expression in antirrhinum*

3.3.1.1 Effect of light integral on juvenile phase length

Photoperiod transfer experiments were performed using two different light integrals, short day high light (SDHL) and short day low light (SDLL), to determine the impact of light integral on juvenility. Plants were grown at either PAR 2.7 $s^{-2}d^{-1}$ (SDHL) or PAR 0.9 $s^{-2}d^{-1}$(SDLL) and were transferred to the same long day high light condition (LDHL) PAR 2.9 $s^{-2}d^{-1}$ in first eight hours and PAR 0.4 $s^{-2}d^{-1}$ in the second eight hours (section 3.2.1). Lower LI and shorter photoperiod cause the delay in flowering (appendix, figure A.1). The graphs show the effect of light integral on the different phases of growth (figure 3.3). The JP and AVP length of SDHL and SDLL grown plants were presented in table 3.1, indicating that light integral has an affect on juvenility. Juvenility was extended when plants were grown under low light condition. Notably, the length of the AVP phase was also extended and plants grown under SDLL condition flowered later relative to those grown under SDHL.

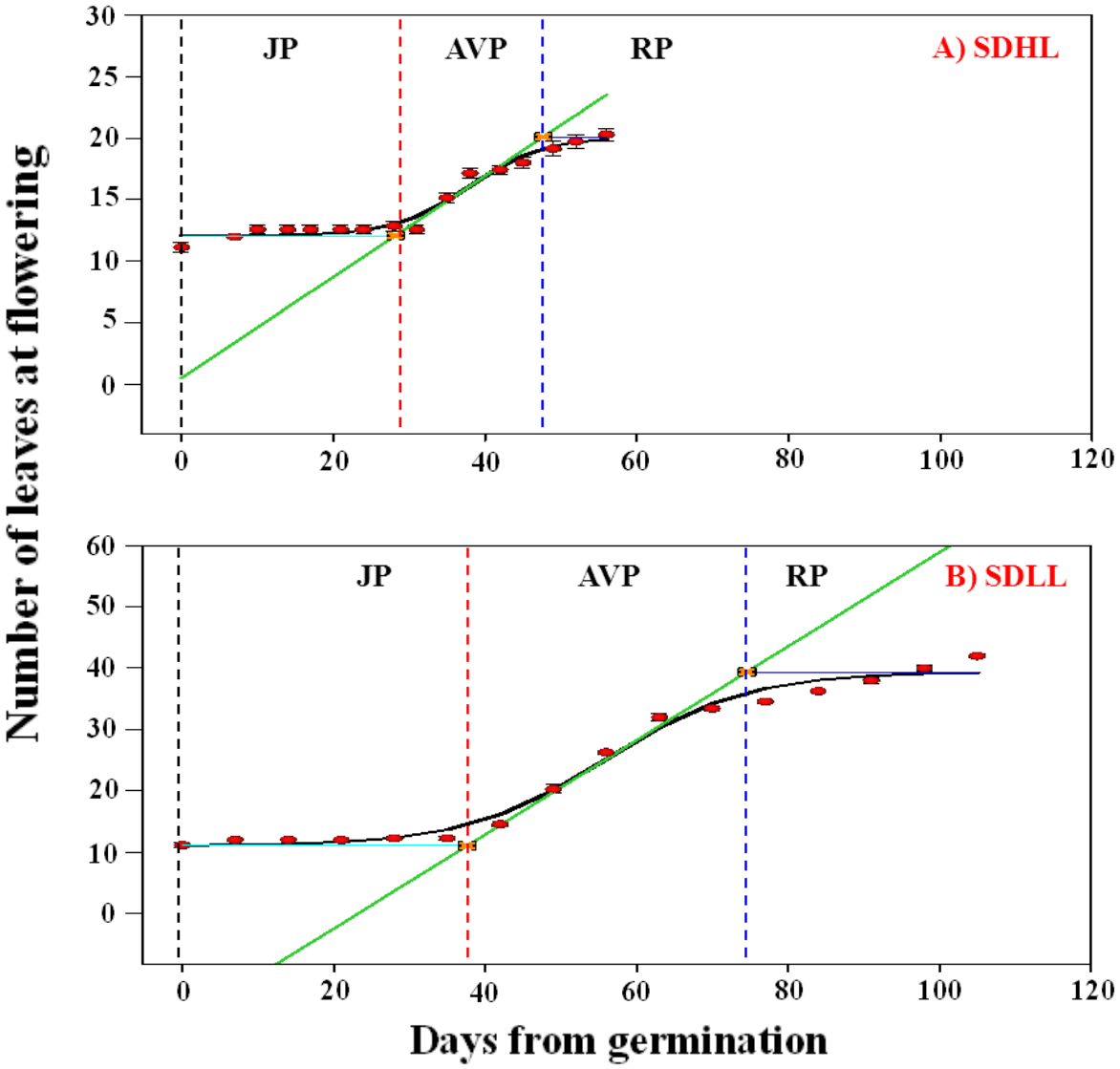


Figure 3.3 Effect of light integral on the phases of photoperiod sensitivity in antirrhinum

Flowering time of plants transferred from SDHL to LDHL (A) and SDLL to LDHL (B) at regular intervals from germination. Fourteen transfers were carried out twice a week for SDHL treated plants and weekly for SDLL treated plants with 7 plants per transfer.Vertical error bars indicate the standard error of the mean of the leaf number. Horizontal error bars show the standard error of the mean of the phase length. The black curve, green line, light blue line, dark blue line, and purple dotted line represent the logistic curve, maximum slope, lag time, stationary phase, and boundaries of the different phases respectively. Abbreviations: JP, Juvenile Phase; AVP, Adult Vegetative Phase; RP, Reproductive Phase.

Light integral	Total flowering time (day)	JP length (day)	AVP length (day)	Significantly different compared between LL and HL	
				JP	AVP
HL	47.7+/- 0.8	28.16 +/- 0.8	19.54+/- 0.8	P>0.05	P>0.05
LL	74.55+/- 0.88	37.62+/- 0.88	36.93+/- 0.88		

Table 3.1 JP and AVP lengths in antirrhinum grown under different light integral

Flowering time of plants transferred from SDHL and SDLL to LDHL at regular intervals from germination. Abbreviations: JP, Juvenile Phase; AVP, Adult Vegetative Phase; +/-, Standard error of the mean of the phase length

3.3.1.2 Impact of light integral on leaf production rate during juvenility

To ensure that observed differences in juvenile phase length are not due to differences in growth rate, leaf production rates of continuous SDLL and SDHL grown plants were compared. Although plants grown under different light integrals presented differences in juvenile phase length, adult vegetative phase length, and flowering time, leaf production rates during juvenility of both plants grown under the different light integrals were comparable for the first 28 days (figure 3.4). It is likely that the late flowering that occurred in plants grown under SDLL was not because of a reduced rate of plant development. Plants grown under SDHL flowered early with leaf numbers less than 20. For those grown under SDLL, leaf production continued for much longer with leaves totalling up to 35.

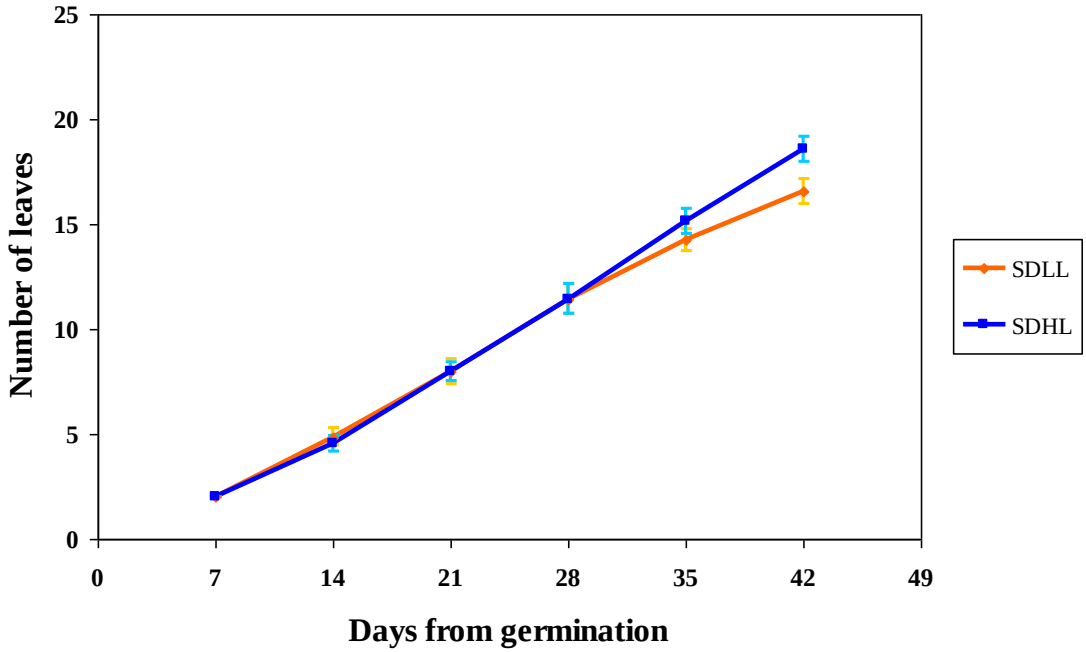


Figure 3.4 Comparison of leaf production rate during juvenility of antirrhinum plants grown under different light integrals

Leaf production rates are displayed as the number of leaves greater than 1.5 mm produced at weekly intervals from germination. Plants were either kept continuously under SDLL (orange line) or SDHL (blue line). Abbreviations: SDHL, short day high light; SDLL, short day low light

3.3.1.3 Effects of light integral on the expression of flowering genes

In order to determine the effect of light integral on the expression of flowering time genes, the developmental expression of *AmFT* and *AmTEM* were analysed using the antirrhinum leaf materials obtained from the experiment described in section 3.2.1. Leaf materials were harvested at ZT14. Developmental expression of *AmFT* in plants grown under high and low light integral is presented in figure 3.5A and B. Similar patterns were observed in that the levels of expression increased around the end of the juvenile phase and peaked in the AVP. During juvenility irrespective of its length, *AmFT* expression levels were low. Although the patterns of *AmFT* expression were similar, expression levels were very different in low and high light grown plants. In LL grown plants, the *AmFT* expression level took a longer time to increase compared with that in HL grown plants. Moreover, the

level of *AmFT* expression was significantly lower in plants grown under LL compared to HL.

The patterns of expression of *AmTEM* in plants grown under HL and LL conditions are presented in figure 3.5 C and D. In HL grown plants, *AmTEM* levels dropped from early in development with lowest levels preceding the end of juvenility. In plant grown under LL, there were maximal levels around the end of juvenility until the beginning of the AVP. The reduction of *AmTEM* expression level in HL grown plant occurred earlier compare to that in LL grown plants. Throughout the developmental phases from JP to RP, the level of *AmTEM* expression in LL was higher relative to that in plants grown under HL.

The patterns of expression of miR156 in plants grown under SDHL and SDLL condition are presented in figure 3.6A and B (Alison Jackson, School of Life Sciences, University of Warwick). The experiment was conducted in the same way with specific transfers and harvesting regime of the procedure in section 3.2.1. Since this experiment was carried out in the Panasonic cabinets that has different light integral compared to Sanyo cabinets, the length of growth phases in this experiment cannot be shown and compared with the results described in section 3.3.1.1. In both condition, the levels of miR156 expression were high in young plants and they declined as the plants aged. The reduction of miR156 in HL grown plants was earlier compared to that in LL grown plants.

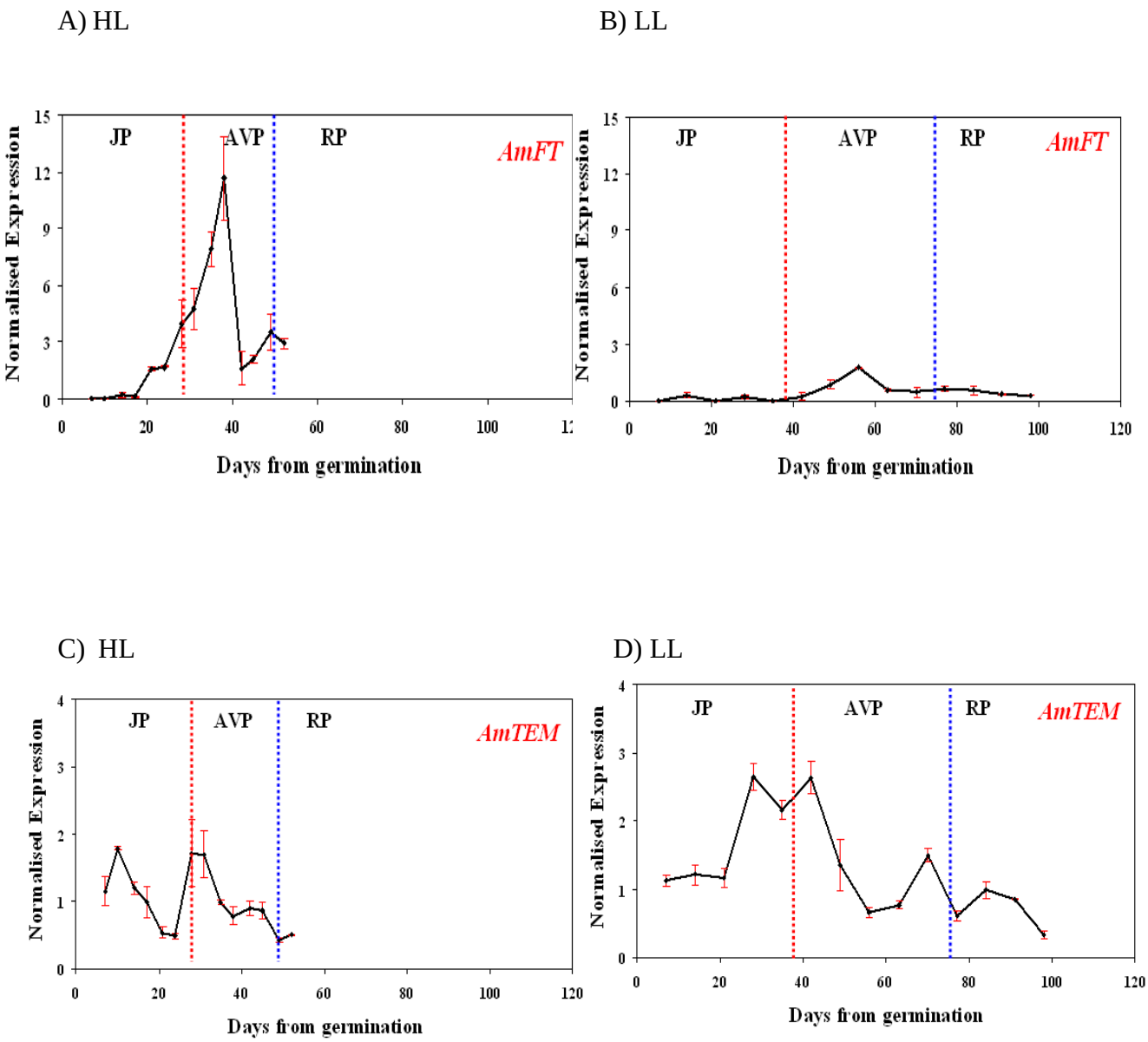
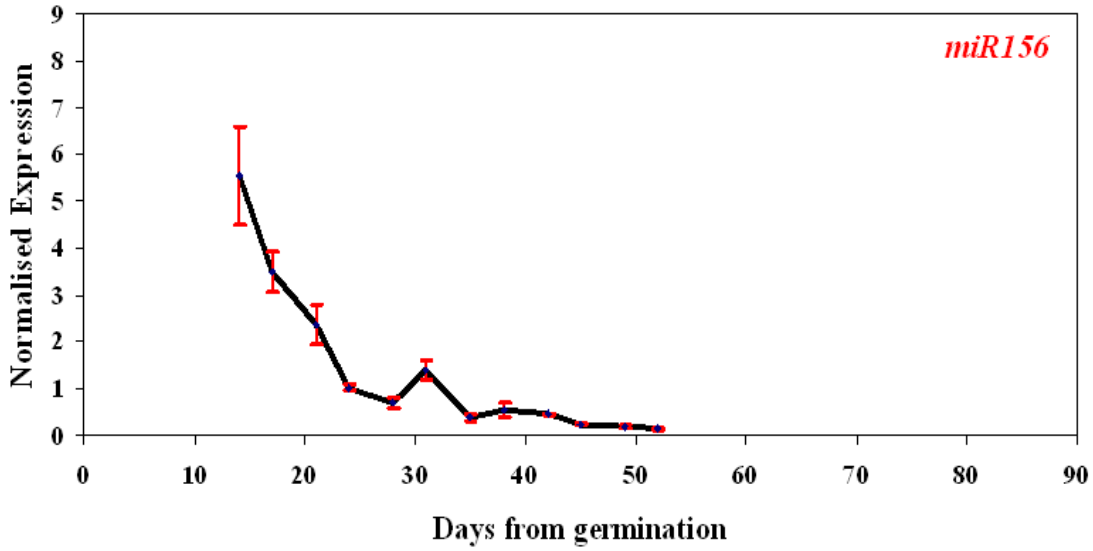


Figure 3.5 Developmental expression of AmFT and AmTEM in antirrhinum plants grown under different light integrals

Plants were grown under SD at HL (A and C) or LL (B and D) and transferred to LDHL conditions. Fourteen transfers were carried out twice a week for SDHL treated plants and weekly for SDLL treated plants with 7 plants per transfer. Expression of each gene in leaves harvested at ZT14 in the first LD experienced after transfer was normalised against the geometric mean of Actin and Elongation factor 1 α expression levels .Vertical error bars denote the propagated standard error of the mean of the gene expression obtained using three technical replicates of each gene at each data point. Red and blue dotted lines indicate the end of juvenility and adult vegetative phase respectively. Abbreviations: HL, high light integral; LL, low light integral; JP, Juvenile Phase; AVP, Adult Vegetative Phase; RP, Reproductive Phase

A) HL



B) LL

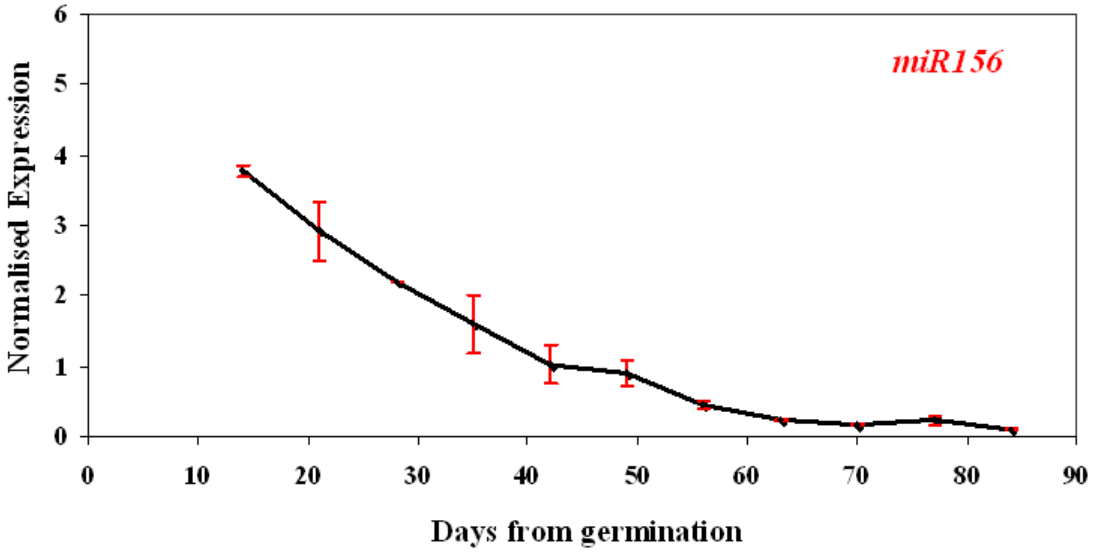


Figure 3.6 Developmental expression of miR156 in antirrhinum plants grown under different light integrals

Plants were grown under SD at HL (A) or LL (B) and transferred to LDHL conditions. Expression of miR156 in leaves harvested at ZT14 in the first LD experienced after transfer was normalised against the geometric mean of small nuclear RNA U6 expression level following the method in Plant Methods (Varkonyi-Gasic et al., 2007). Vertical error bars denote the propagated standard error of the mean of the gene expression obtained using three technical replicates of each gene at each data point.

3.3.2 *The effect of the number of LDs plants receive after transfer from SDLL or SDHL to LDHL or LDLL conditions on the expression of AmFT*

In the study in section 3.3.1.3, plants grown under SDHL or SDLL were exposed to only one LD before harvesting materials for analysis which might be not enough to trigger *AmFT*, especially when plants were grown under low light integral. Therefore, further experiments were carried out to determine whether longer exposure of SDLL grown plants to LDs would result in higher levels of *AmFT* expression. This would provide better understanding of how light integral affects *AmFT* expression and flowering time in relation to photoperiod.

AmFT expression varied depending on the starting and ending light conditions before harvesting (figure 3.7). In SDLL to LDHL condition (A), the expression was very low for the first four LDs and then increased but not too high level. In SDHL to LDHL condition (B), the level of expression throughout was higher than that in SDLL to LDHL condition (A) and it took only three LDs before the level of *AmFT* increased. In SDLL to LDLL condition (C), the level of *AmFT* expression remained low throughout seven LDs, with LDLL not being sufficient to induce *AmFT*. Transfer of plants from SDHL to LDLL (D) resulted in a different pattern of expression. The starting level of expression was roughly the same as the starting level of *AmFT* in SDHL to LDHL condition (B) and remained the same until four LDs were obtained and then the level dropped to quite low. Interestingly, the pattern of expression in SDHL to LDLL (D) differed from that in continuous SDHL condition (F). In SDHL to LDLL condition (D), the expression level declined whereas in continuous SDHL (F) condition the expression level increased after obtained four LDs. In SDLL to SDHL condition (E), the starting level of expression was low and then increased after four LDs. The pattern of this expression was quite similar to that in SDLL to LDHL condition (A) but it took more LD to reach the expression level compared to that in SDLL to LDHL (A). In continuous SDHL condition (F), the starting expression level was similar to those in SDHL to LDHL and LDLL condition (B and D). It increased after plant received three LDs and then fluctuated between four and six LDs. These data show that although both

photoperiod and light integral seem to be important to induce *AmFT*, light integral has a greater effect on *AmFT* expression.

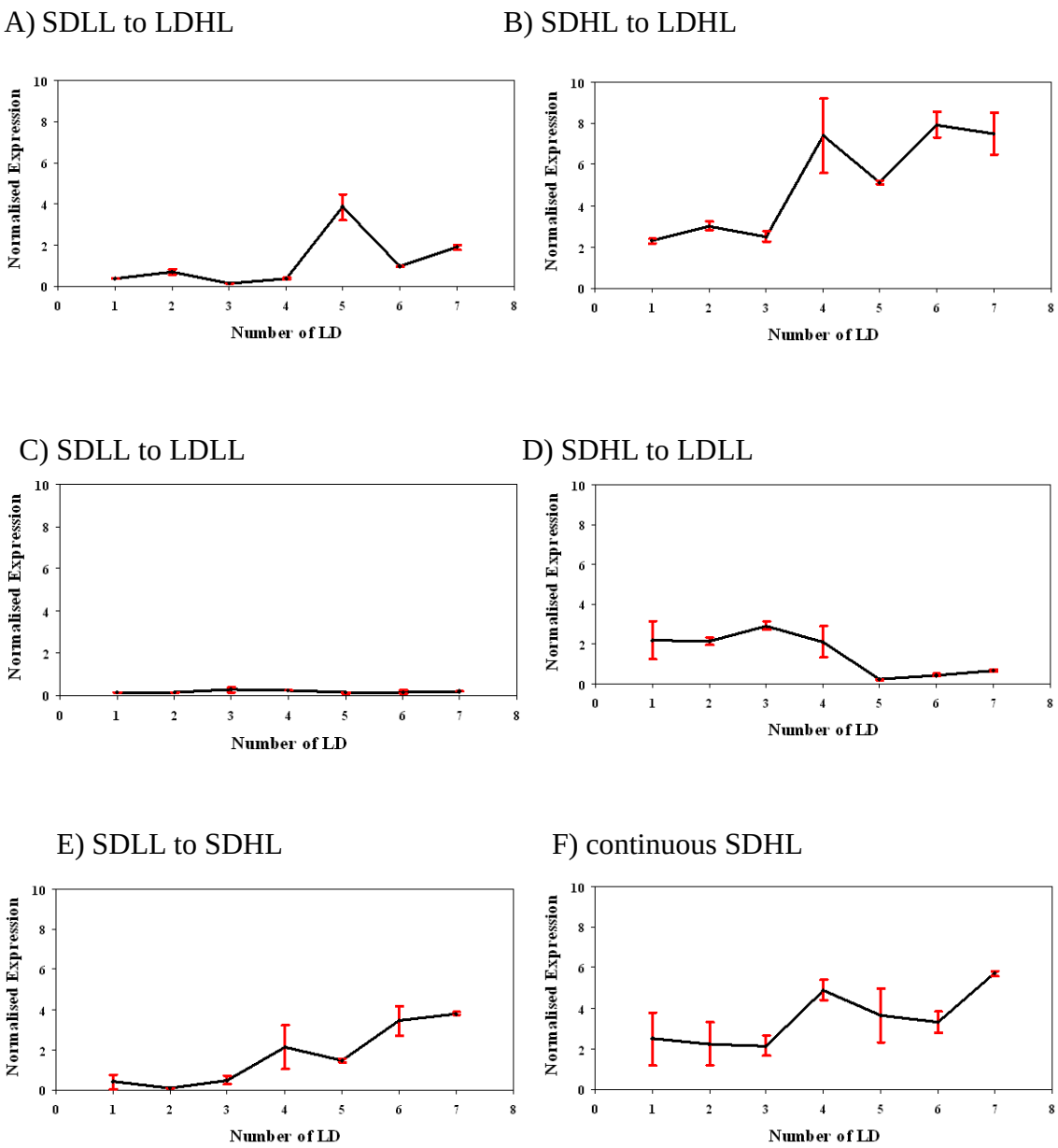


Figure 3.7 Effect of number of LDs received after transfer from SDs on AmFT expression in antirrhinum plants

Adult plants were transferred from SD conditions to either LDHL or LDLL. After each consecutive LD, first expanded leaves were harvested at ZT14 for seven days. FT expression at each data point was normalised against the geometric mean of Actin and Elongation factor 1 α.Vertical error bars denote the propagated standard error of the mean of the gene expression obtained using three technical replicates of each gene at each data point. Abbreviations: LD, longday; SDLL, shortday with low light integral; SDHL, shortday with high light integral; LDLL, longday with low light integral; LDHL, longday with high light integral

3.3.3 *Photoperiod transfer experiment to measure JP in different light condition*

As data in figures 3.7 indicated that the effect of LI on *AmFT* expression was more significant than the photoperiod, further experiments were carried out under different conditions of photoperiod and LI (section 3.2.1). Figure 3.8 shows the effect of photoperiod and light integral on the length of JP. The JP and AVP lengths of plants transferred from SDLL to LDLL, SDHL to LDLL, and SDLL to SDHL are presented in table 3.2. Interestingly, the logistic curve for plants transferred from SDHL to LDLL (figure 3.8B) was reversed from those of SDLL to LDLL and SDLL to SDHL (figure 3.8A and C). This indicated that the longer the plant was in LDLL compared to SDHL, the later the flowering time. In the other words, SDHL grown plant was more responsive to the change in LD received or photoperiod compared to LDLL grown plants. When compared to the flowering time data shown in figure 3.1, these results show the most effective condition to induce flowering was LDHL, SDHL, LDLL, and SDLL respectively. The *AmFT* expression results (figure 3.7) and the flowering time results (figure 3.8) show that light integral has a greater effect on *AmFT* expression and flowering time than photoperiod in antirrhinum.

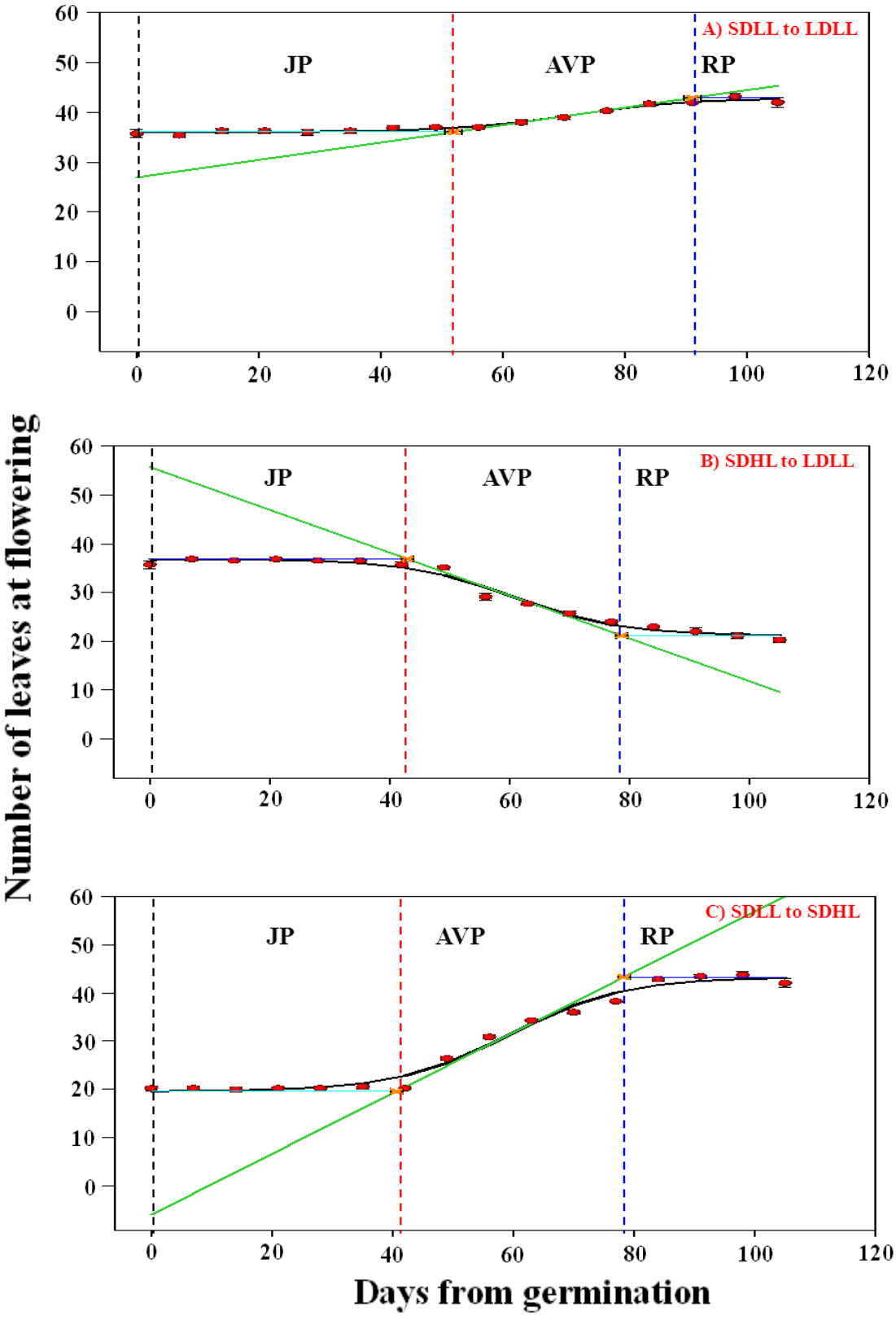


Figure 3.8 Effect of light integral and photoperiod on the phases of photoperiod sensitivity in antirrhinum. *Flowering time of plants transferred from SDLL to LDLL (A), SDHL to LDLL (B), and SDLL to SDHL(C) at regular interval from germination. Fourteen transfers were carried out weekly with 7 plants per transferFigure legend as stated in figure 3.1.*

Transfer experiment	Total flowering time (day)	JP length (day)	AVP length (day)
SDLL to LDLL	88.79 +/- 1.38	51.96 +/- 1.38	36.83 +/- 1.38
SDHL to LDLL	60.45 +/- 1.03	42.93 +/- 1.03	17.52 +/- 1.03
SDLL to SDHL	77.83 +/- 0.98	40.62 +/- 0.98	37.21 +/- 0.98

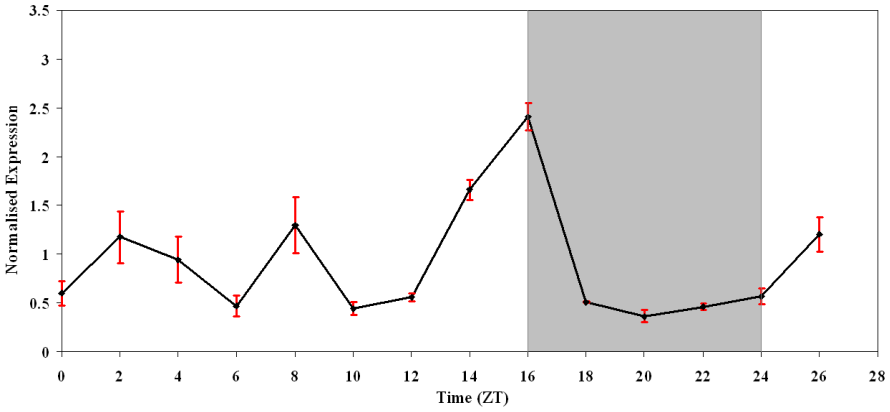
Table 3.2 JP and AVP lengths in antirrhinum grown under different light integral

Flowering time of plants transferred from SDLL to LDLL, SDHL to LDLL, and SDLL to SDHL at regular intervals from germination. Abbreviations: JP, Juvenile Phase; AVP, Adult Vegetative Phase; +/-, Standard error of the mean of the phase length

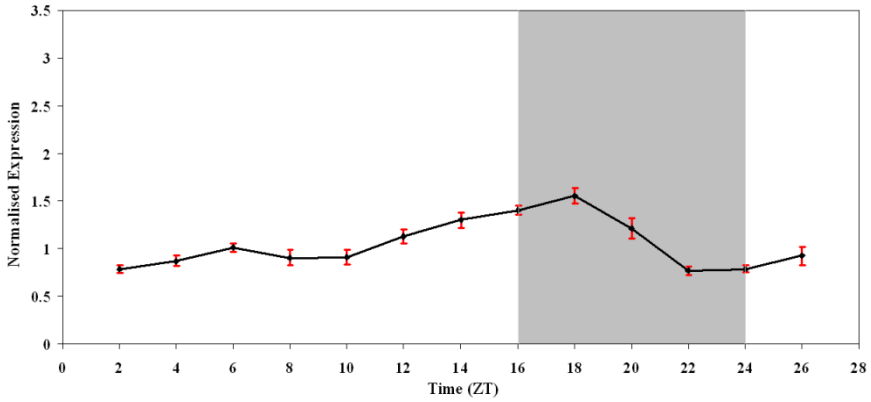
3.3.4 *Diurnal AmFT expression pattern under different light conditions*

In the previous experiment, it was found that HL promoted flowering in SD. We therefore determined the diurnal patterns of *AmFT* expression to test whether *AmFT* was expressed in SD. Plants were grown under continuous LDHL, LDLL, and SDHL to determine the diurnal pattern of *AmFT* expression. *AmFT* exhibited diurnal expression patterns in plants grown under each of the three light conditions (figure 3.9). In plants grown under both HL and LL long-day conditions, the level of expression started to increase toward the end of the light period with levels peaking at a lower level and later in plants grown under LDLL compared to LDHL (figure 3.9A and B).Whereas in SDHL grown plants, the level of expression remained low during the light period and peaked at high levels in the middle of the dark period. In plants grown under LDLL, the main peak of expression occurred during the dark period at ZT18 whilst in LDHL grown plant it was at the end of the light at ZT16 (figure 3.9B). Although there was a small peak of *AmFT* expression at ZT2 in SDHL grown plants, the main peak of expression was at ZT16 in the dark period. It appeared that the light integral has a greater affect on *AmFT* expression levels than photoperiod since LDHL and SDHL grown plants had higher expression peaks than that in LDLL and interestingly the peak of expression was at 16 to 18 hours for all conditions.

A) LDHL



B) LDLL



C) SDHL

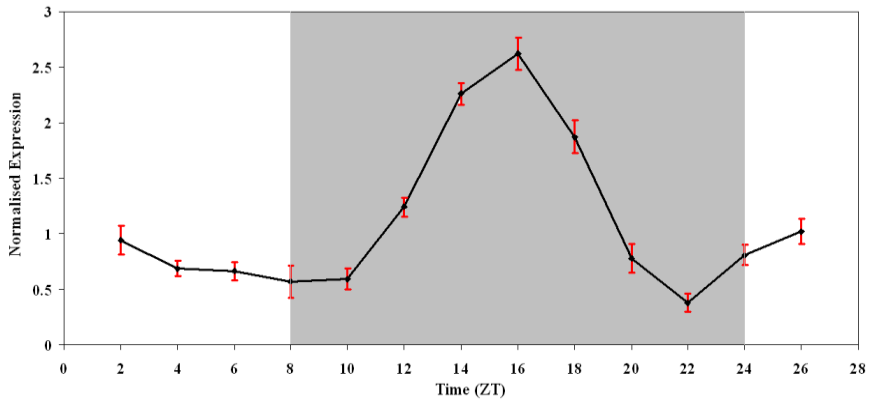


Figure 3.9 The pattern of diurnal expression of AmFT in antirrhinum plants grown under different light conditions

AmFT expression under LDHL (A), LDLL (B), and SDHL (C) is normalised against the geometric mean of Actin and Elongation factor 1 α expression levels. Vertical error bars denote the propagated standard error of the mean of the gene expression obtained using three technical replicates of each gene at each data point. The grey and white area represent the dark and light period respectively.

3.4 Discussion

The antirrhinum transfer experiments from short day high and low light to long day high light conditions were carried out in order to assess the impact of LI on the length of the JP and to determine the effect on the expression of flowering time genes. The JP was extended when plants were grown under low light condition. Notably, the length of the AVP phase, when plants are capable of responding to the inductive environment, was also extended. Keeping plants in low light seems to alter this response and extended the AVP by extending the length of the JP and delays the onset of the RP. The result supports the idea that the transition from the JP to AVP in plants is affected by environmental signals (Amasino, 1996; Chuck and Hake, 2005; Bäurle and Dean, 2006; Imaizumi, 2010). Since The AVP is the phase that plants establish their flowering competence (Poethig, 2003),this illustrates that plants grown under LL take longer time to develop floral competence compared to the plants grown under HL which is why they flower later and longer to commit flowering under SD.

Although plants grown under different LIs differed in juvenile phase length and flowering time, the rate of leaf production of both plant groups were comparable (section 3.3.1.2) indicating that the difference in the JP length and flowering in plants grown under the different conditions was not due to the development or plant growth rate.

The patterns of *AmFT* expression were similar in SDHL compare with SDLL grown plants. During juvenility, expression was low and increased during the AVP with a decline in the reproductive phase. The reason why plants become competent to flower during the AVP is because the *FT* gene that encodes the flowering signal is expressed (Srikanth and Schmid, 2011). Thus the AVP is the phase that plants has competence to respond to environmental factors that trigger flowering (Poethig, 2003). Growing plants under LL compared to HL extended both the JP and AVP and, although the general patterns of expression were similar, *AmFT* induction was delayed and the levels of *AmFT* expression were reduced. These data indicate that the LI affects *AmFT* induction and expression level. This causes plants grown under LL conditions to flower later relative to the plants grown under HL conditions. The correlation between *AmFT* expression, the length of JP, and time to flower supports

the importance of *FT* in the regulation of flowering time.

TEM plays a role in suppressing *FT* at the transcription level (Castillejo and Pelaz, 2008) and *AmTEM* has a role in determination of JP (Sgamma *et al*., 2014). Interestingly, a reciprocal relationship was observed between levels of *AmTEM* and *AmFT* expression irrespective of the light integral that plants were grown under.Therefore LL causes a delay in the decline of *AmTEM* expression, which results in repression of *AmFT* for longer and hence flowering is delayed in LL.

miR156 is involved with phase change in plants. The reduction of miR156 expression causes the transition from the juvenile to the adult phase of growth (Schwab *et al*., 2005). When antirrhinum plants were young, the level of miR156 was high and gradually reduced when the plants got older in order to allow the floral inductive factors to stimulate flowering. miR156 is accumulated mostly during juvenility and this leads to the inhibition of the transcription factor, *SQUAMOSA* promoter binding protein-like3 (SPL3) (Cardon *et al*., 1997; Rhoades *et al*., 2002). Interestingly the reduction of miR156 level was delayed in SDLL grown plants compared to SDHL. Therefore the late flowering in SDLL grown plants shows the involvement between flowering time, light integral and miR156. This could be connected to carbohydrate metabolism since miR156 is regulated by sugar (Proveniers, 2013). This observation led to investigation of the effect of miR156 knockout in *Arabidopsis* on JP length (section 5.3.3, chapter5).

The light integral that plants were exposed to prior and after transfer from SD to LD was shown to influence *AmFT* expression patterns. For SDLL and SDHL grown plants that were transferred to LDLL, the induction of *AmFT* was delayed and the level of expression was lower than those transferred to LDHL. For SDLL and SDHL grown plants that were transferred to LDLL, there was no *AmFT* induction in SDLL grown plants and the *AmFT* expression level was reduced in SDHL grown plants. According to the results, the LDLL condition is inefficient for *AmFT* induction.

In order to see how this correlated with how the plants developed, transfer experiments between specific photoperiods and light integrals were carried out to determine the different phases of growth in section 3.3.3. Juvenile and adult vegetative phase lengths of plants transferred from SDLL to SDHL were shorter than those of plants transferred from SDHL to LDLL and SDLL to LDLL respectively.

The results show that the higher light integral had a stronger effect on *AmFT* expression and flowering time acceleration compared to the longer photoperiod, even though antirrhinum is a facultative longday plant. This strongly supports the idea that higher LI triggers the expression of *AmFT*.

The diurnal pattern of *AmFT* under LDHL conditions was similar to that in *Arabidopsis* which is also a facultative long-day plant. The diurnal expression of *FT* in *Arabidopsis* Columbia wild type under LD condition is low throughout the light period before peaking at the end of the light period (Imaizumi *et al*., 2003; Galvao *et al*., 2012). In *Arabidopsis*, the pattern of diurnal *FT* expression under LD depends on the expression of *CO* (Ma, 1998; Imaizumi and Kay, 2006; Sawa *et al*., 2008). When plants get the light signals, GI and FKF1, the component of plant circadian clock, is activated after blue light is perceived (Putterill *et al*., 2004; Imaizumi and Kay, 2006; Sawa *et al*., 2008; Mcwatters *et al*., 2011).These components induce the degradation of CDFs, the repressors of *CO* and leads to the high expression of *CO* mRNA at the end of LD and continue in the dark. When high level of *CO* expression coincides with light under LD, CO protein is stabilised by CRY1 and 2, and PHYA and is able to activate *FT* which induce flowering. Therefore a peak in *FT* expression at the end of the light period coincides with high level of stable CO protein at the end of the light period. Although the patterns of diurnal *AmFT* expressions in all conditions are similar and the peak of expressions are presented at the same time of the day (from ZT14 to ZT16), the level of expression throughout the day are different. The level of *AmFT* expression was higher in LDHL and SDHL grown plants compared to LDLL. The diurnal results are correlated with the results in section 3.3.2 which shows that *AmFT* expression in SDHL grown plants is higher than that in LDLL grown plants and SDHL is more effective for plants to induce flowering compared to LDLL.

The diurnal *AmFT* expression pattern in antirrhinum plants grown under SDHL was different from that observed in *Arabidopsis*. In *Arabidopsis*, *FT* remains low in SD grown plants throughout the light dark cycle (Imaizumi *et al*., 2003). This is because in *Arabidopsis*, CO protein is degraded in the dark by SPA1 and COP1 protein complex and thus *FT* cannot be induced in the dark (De Montaigu *et al*., 2010). Plants are able to distinguish the difference between LD and SD photoperiods by the circadian clock and plant photoreceptors (Marcelo and Steve, 2003; Imaizumi and Kay, 2006). The reason why *Arabidopsis* and antirrhinum can still flower under

SD photoperiod although flowering is delayed is that the photoperiod-responsive flowering could be regulated by several mechanisms that could be independent from *CO* expression. Under SD condition, the expression of *GI* in *Arabidopsis* vascular bundle or mesophyll could activate *FT* expression without increasing the level of *CO* (Sawa and Kay, 2011). Apart from photoperiod pathway, *Arabidopsis* contains autonomous parthway that induce flowering irrespective of environmental changes (Michaels and Amasino, 1999; Simpson, 2004), vernalization parthway that induces *FT* (Amasino, 2005), and giberellin synthesis partway that also help to promote flowering (Wilson *et al*., 1992; Blázquez *et al*., 1998; Srikanth and Schmid, 2011). From the diurnal *AmFT* expression results, light integral affects the level of diurnal *AmFT* expression whereas the photoperiod affects neither the *AmFT* peak induction nor the level of expression. Therefore LI influences flowering time since it affects the daily expression of *FT*. From diurnal data, it could be confirmed that to investigate *AmFT* expression throughout development, plants could be harvested between ZT14 to ZT 16 when the expression levels were high which is had done in this study.

CHAPTER4. INFLUENCE OF LIGHT INTEGRAL ON DEVELOPMENTAL EXPRESSION OF CARBOHYDRATE GENES AND CARBOHYDRATE LEVEL IN ANTIRRHINUM

4.1 Introduction

The role of carbohydrates on flowering has been studied for several years in many plant species. An early study in citrus showed that girdling activates flowering due to an accumulation of carbohydrates in the girdled branch (Goldschmidt *et al*., 1985). Later a study in avocado stated that higher starch accumulation within the tree provides greater flower and fruit production (Scholefield *et al*., 1985). A study in Red Clover also showed that the carbohydrate status in leaves and apices is important for flowering (Jones, 1990). In *Sinapis alba L*, soluble sugar levels increased during the transition from the vegetative to the reproductive phase, so sugar content could play a role in cellular activities to induce flowering, together with other factors that help to promote flowering at the apex (Bodson, 1997). Starch accumulation plays an important role in floral induction in *Arabidopsis* since all the starchless mutants, *pgi1*, *pgm1*, and *adg1*, exhibit late flowering when grown under short day photoperiods (Yu *et al*., 2000). In the flowering plant chrysanthemum, the level of sucrose is higher in florally induced compared to non-induced vegetative shoot tips (Lee *et al*., 2004). Also in the long-juvenility plant Oncidium orchid, carbohydrates, annan starch and pectin accumulated in young pseudobulbs prepared for floral initiation (Wang *et al*., 2008). Moreover, there is a correlation between early flowering, leaf temporary starch content and plant growth in *Arabidopsis Landsberg erecta* × *Kondara* recombinant plants (El-Lithy *et al*., 2010).These studies provide a strong correlation between soluble sugars from carbohydrate metabolism and floral initiation suggesting that the plants need to accumulate sufficient levels of carbohydrate in order to support the flowering process.

In addition to supporting energy to the plant, sugar can also act as a signalling molecule to measure if the plant contains suitable amounts of carbohydrate or metabolites needed to proceed though their development (Koch, 2004; Gibson, 2005; Rolland *et al*., 2006; Wind *et al*., 2010). There is a complex

association between flowering time control, carbohydrate metabolism, carbohydrate content, plant metabolite, signaling molecule and plant hormones. Sucrose is one of the important signaling molecules from photosynthesis which can support flowering (Roldan *et al*., 1999). High levels of miR156 prevent the juvenile to adult phase in plants (Wu *et al*., 2009; Wang *et al*., 2011; Yang *et al*., 2011). Since sucrose represses miR156 expression (Proveniers, 2013; Yang *et al.,* 2013; Yu *et al*., 2013). This points to a role of sugars in regulating juvenility.

Apart from sucrose which is the major product from photosynthesis, hexose products like glucose and fructose and their metabolites can also play a role in signalling for growth and development. HXK, the glucose phosphorylating enzyme in sink tissue, is also the glucose specific sensor that can act as the signalling molecule to provide information if there is enough hexose for plant metabolism which is very important for developmental processes in plants (Rolland and Sheen, 2005; Rolland *et al*., 2006). T6P promotes flowering by combining the signals from carbohydrate metabolism, photoperiod and the aging pathway (Wahl *et al*., 2013). The T6P pathway can regulate the level of *FT* expression in the photoperiod pathway and the level of miR156 in the aging pathway (Lastdrager *et al*., 2014).

The hormone auxin is involved in regulation of flowering in plants (Fisher and Loomis, 1954; Salisbury, 1955; Thingnaes *et al*., 2003). Sucrose activates the level of auxin and also stimulates the transportation of auxin in the plant (Aloni, 1980; Cheng *et al*., 1992; Woodward and Bartel, 2005).Gibberellins (GAs) are the plant hormones that has effects on many developmental processes of the plant including flower induction (Nanda *et al*., 1969; Porri *et al*., 1012; Goldberg-Moeller *et al*., 2013). GAs, sucrose, and FT have been confirmed as components of florigen that control flowering. They might work together in complex mechanisms integrating environmental factors (King, 2012).

During the day, plants store the photosynthetic assimilates in the form of starch in the chloroplast. In the dark period, the transitory starch is mobilized and converted to soluble sugars, which are used for plant metabolism (Yu *et al*., 2001; Yan *et al*., 2005; Zeeman *et al*., 2007).

Light and carbohydrate metabolism rates including enzyme activities affect growth in *Arabidopsis* (Gibon *et al*., 2009). As the growth of the plant is related to the phase transitions, clarification of the relationship between juvenile to adult phase

change and carbohydrate metabolism could provide a link to the mechanism of the juvenility. In chapter 3, it was shown that reduced light integral delayed the juvenile phase and also impacted on the pattern of flowering time gene expression. To gain a better understanding of the relationship between juvenility and carbohydrate metabolism, a study of the expression of the genes that control carbohydrate metabolism in relation to light and carbohydrate levels throughout developmental phases in antirrhinum were conducted and described in this chapter.

There were three aims for the studies described in this chapter: (i) to identify and isolate genes involved in starch and carbohydrate synthesis (*PGM*, *PGI*, and *SPS*) and starch breakdown (*SEX-1*, *AMY*, and *BAM-3*), (ii) to determine the effect of light integral on the expression of genes involved in carbohydrate metabolism in relation to juvenility and (iii) to determine the effect of light integral on photosynthetic assimilation and partitioning in relation to juvenility.

4.2 Materials and Methods

The descriptions in this chapter are materials and methods specific for this results chapter. For the common materials and methods in more than one result chapter, the descriptions are in chapter 2. The experiments discussed in this chapter are the same as described in chapter 3 for plants treated with different LIs and the antirrhinum samples used for genetic and biochemical analysis were the same batch as those used in chapter 3.

4.2.1 *Antirrhinum leaf materials*

Two sets of *Antirrhinum majus* (Bells F1 Red) leaf material were used in the gene identification and isolation study. The first set was obtained from Dr Tiziana Sgamma (School of Life Sciences, University of Warwick). There were 4 types of material that were pooled: (i) cotyledons harvested from 3-week old plants grown under LD conditions, (ii) the second fully expanded leaves harvested from plants that were transferred from SD to LD conditions at week seven from germination, (iii) the seventh fully expanded leaves harvested from 7-week old plants grown under LD conditions, and (iv) cotyledons harvested from 5-week old plants grown under LD conditions. All leaf material was frozen in liquid nitrogen and stored at -80 °C before use. These materials were used for the isolation of starch synthesis genes. The second set of leaf material was the first fully expanded leaves from 3-week old plants grown under LD conditions, the leaves were harvested during the dark period (ZT 19) and were used for the isolation of starch break down genes.

For the analysis of developmental carbohydrate gene expression and carbohydrate levels, the plant material was taken from the same batch of materials generated for flowering gene expression analysis, which is described in section 3.2.1. Total RNA extraction and cDNA synthesis were followed as described in section 2.5

4.2.2 *Gene Identification and isolation*

4.2.2.1 Carbohydrate gene identification

To identify carbohydrate genes in antirrhinum, the *Arabidopsis* genes were blasted against the antirrhinum database (http://antirrhinum.net/) to identify sequences with high sequence homologies.

4.2.2.2 Carbohydrate gene isolation

A partial sequence of each antirrhinum carbohydrate gene was isolated by PCR of cDNA which was synthesised from mRNA extracted from leaf materials described in section 4.2.1 using primers generated from EST sequences (appendix, figure A.2) in section 4.2.1.1. The primers of all six carbohydrate genes were designed as described in section 2.6.The primers detailed are stated in the appendix, table A.1. PCR amplifications were carried out with 10 µl reaction as described in section 2.7. The annealing temperatures used and product sizes generated using each primer pair are listed in the appendix, table A.1. PCR products were visualized on agarose gels (section 2.9) and extracted from gel (section 2.10) before sequencing (section 2.11) to confirm the product identity. After that, the EST sequences were used for primer design for real time PCR quantification.

4.2.2.3 Generating full-length cDNA for carbohydrate genes

Rapid Amplification of cDNA Ends (RACE)-PCR was carried out using the GeneRacer^{TM} RACE Ready cDNA Kit (Invitrogen Ltd., UK), following the manufacturer's guidelines.

The gene specific and nested primers for generating 5′ and 3′ fragments of each cDNA were obtained from the EST sequences (section 4.2.2.1) and following RACE primer design description in GeneRacer™ RACE Ready cDNA Kit manufacturer's guidelines for primer characteristics. The details of each primer are shown in the appendix, table A.3.

To amplify the 5′-end cDNA, mRNA was prepared from total RNA of the first set of antirrhinum materials (section 4.2.1) and reverse transcribed using GeneRacer™ 5′1 Primer (homologous to the GeneRacer™ RNA Oligo) and 5′ gene specific primer (GSP) following by PCR using 5′nested primer of each gene.

The same mRNA template for 5'- end amplification was used to obtain the 3'-end cDNA by reverse transcription using GeneRacer™ 3′1 Primer (homologous to the GeneRacer™ Oligo dT Primer) and 3′ gene specific primer (GSP) following by PCR using 3′nested primer of each gene.

Products of the expected size were visualized on agarose gels (section2.9), extracted and purified from gel (section2.10). The full-length cDNAs obtained were used for primer design for real time PCR quantification.

4.2.2.4 Cloning the partial cDNAs obtained from gene isolation

In order to obtain sequence information for the partial length cDNAs, the PCR products representing the full cDNA of each gene (section 4.2.2.3) was ligated into the pGEM-T Easy vector (Promega, Australia), following the manufacturer's guidelines. Transformation into *E. coli* was carried out by transferring 20 µl of electrocompetent EC100 cells (Cambio Ltd., UK) into the microcentrifuge tube containing 2µl of the ligation reaction. Then, 22 µl of the mix was transferred into electroporation cuvettes (Geneflow Ltd., UK) and electroporated using the Bio-Rad Micropulser following the manufacturer's instructions. After electroporation, 950 µl of SOC medium was added immediately before the mix was transferred to a new microcentrifuge tube. Products were shaken at 150 rpm for 1 h at 37°C before pouring on to the surface of solid LB (Luria-Bertani) growth media (VWR International, UK) containing ampicillin (100 µg/ml), IPTG (isopropyl/-D-

thiogalactoside, 0.1 M) and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, 20 mg/ml). The plates were incubated at 37°C overnight followed by screening of appropriate colonies using PCR amplification and reverse and forward M13 primer (Promega, Australia). After screening, each single colony was incubated in 5 ml liquid LB containing ampicillin (100 μg/ml), IPTG (isopropyl/-D-thiogalactoside, 0.1 M) and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, 20 mg/ml) at 37°C with 200 rpm shaking overnight. Plasmid DNA was purified using the QIAprep Spin MiniKit (QIAGEN), following the manufacturer's instructions. After purification, the cloned PCR products were sent to Genomic Resource Centre, School of life sciences, University of Warwick for sequencing (section 2.11).

4.2.2.5 Obtain the sequences of full-length cDNA for each carbohydrate gene

The sequences of 5′ and 3′ fragments of each cDNA (section 4.2.2.4) were combined together to make a complete full-length cDNA using LASERGENE® SeqMan™ (LASERGENE 9). The combined sequences were used for primer design (section 2.6) in order to amplify the full-length DNA as a single product. After obtaining the full-length cDNA by PCR, the products were purified and sequenced.

4.2.3 *Examination of carbohydrate levels at each developmental phase of antirrhinum*

Frozen plant material for biochemical analysis (section 3.2.1) was prepared to generate supernatants and pellets following the instruction in section 2.12.1. Soluble sugar concentrations were determined using the assay kits and the supernatants as described in section 2.12.2. The pellets were used for starch analysis (section 2.12.3).

4.3 Results

4.3.1 *Isolation of genes involved in carbohydrate metabolism from antirrhinum*

4.3.1.1 Isolation of partial cDNAs

The six genes involved in carbohydrate metabolism (*PGM*, *PGI*, *SEX-1*, *BAM3*, *SPS*, and *AMY*) were selected based on their functions in starch synthesis and degradation in plants. Since the sequence of each carbohydrate gene in antirrhinum database was not annotated (http://antirrhinum.net/), the putative sequences of antirrhinum carbohydrate genes (antirrhinum EST sequence) were obtained from *Arabidopsis* carbohydrate genes sequences from the National Centre for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST). In order to confirm the expression of six carbohydrate related genes in antirrhinum, gene identification and isolation were carried out using antirrhinum leaf materials. Starch and sucrose synthesis (*PGM*, *PGI* and *SPS*) and starch degradation (*BAM-3*, *SEX-1*and *AMY*) genes were isolated using the first and second set of materials described in section 4.2.1 respectively. Amplification products for all genes were observed on the gel (figure 4.1) and product sizes were similar to the expected sizes. Products of sizes ~ 451 bp, ~ 405, ~ 403 bp, ~ 432 bp, ~ 417 bp and ~ 504 bp for *AmPGM*, *AmPGI*, *AmSEX-1*, *AmBAM-3*, *AmSPS* and *AmAMY* respectively. Comparison of the sequences of the partial cDNAs amplified with ESTs present in the antirrhinum database proved their identity (table 4.1).

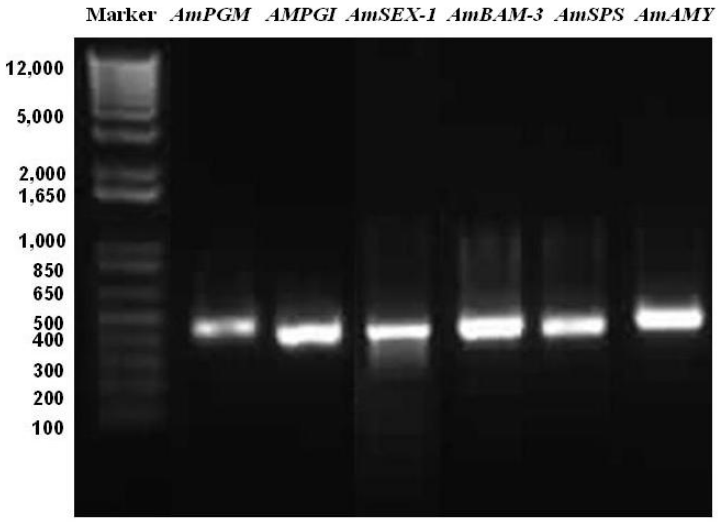


Figure 4.1 PCR fragments representing partial antirrhinum carbohydrate genes

PCR products representing partial cDNA sequences from antirrhinum of the carbohydrate genes indicated, electrophoresis on a 1 % (w/v) Agarose gel, visualised by EtBr staining and UV detection of fluorescence, Marker represent 1kb plus DNA ladder

Gene	Antirrhinum partial cDNA sequence (bp)	Antirrhinum EST sequence from antirrhinum database (bp)	Similarity between antirrhinum EST and antirrhinum partial sequence obtained		Arabidopsis cDNA sequence length	Similarity between antirrhinum partial sequence obtained and the corresponding region in *Arabidopsis* cDNA	
			% nucleotide	% amino acid		% nucleotide	% amino acid
PGM	451	654	99.8	99.3	1872	78.7	90
PGI	405	465	99.5	94	1842	78.5	88.8
SEX-1	403	615	99.3	94	4200	79	91.8
BAM-3	432	461	97.5	94.7	1647	75.1	82.7
SPS	417	595	99.8	100	3132	77.7	82.6
AMY	504	763	99.4	100	2664	49.8	44.4

Table 4.1 Similarity of six antirrhinum partial cDNAs with ESTs representing antirrhinum orthologous and Arabidopsis sequences

The table shows sequence homology between isolated antirrhinum carbohydrate related gene (partial cDNA of AmPGM, AmPGI, AmSEX-1, AmBAM-3, AmSPS and AmAMY) and antirrhinum partial EST sequence and the corresponding region in arabidopsis cDNA.

4.3.1.2 Isolation of full-length cDNAs

After confirming that all six of the target genes were expressed in antirrhinum, GeneRacer™ RACE Ready cDNA Kit were used to attempt to obtain the full-length cDNAs for each gene. In performing both 5′ and 3′ RACE for all cDNAs, there were clear bands for all key genes apart from for the 3′ end of *AmBAM-3*. Only for *AmPGM* and *AmPGI* were products generated of a meaningful size for the 5′ and 3′ reactions (figure 4.2). To obtain sequence information for the 5′ and 3′ end of *AmPGM* and *AmPGI,* the fragments were cloned and sequenced, which confirmed their identity.

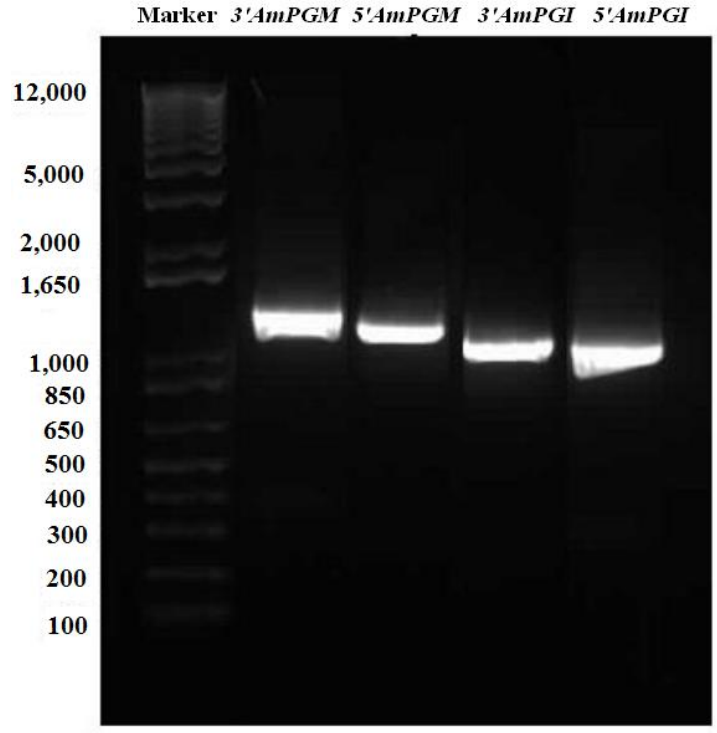


Figure 4.2 PCR fragments representing 5' and 3'ends of AmPGM and AmPGI

PCR products representing 5' and 3'ends of full-legth cDNAs from antirrhinum of the carbohydrate genes indicated, electrophoresis on a 1 % (w/v) Agarose gel, visualised by EtBr staining and UV detection of fluorescence, Marker represent 1kb plus DNA ladder

Sequence information for 5′ and 3′ end of *AmPGI* and *AmPGM* were obtained using RACE and then primers were designed and used to amplify the full-length cDNA sequences. The single full-length product of *AmPGI* and *AmPGM* were amplified using PCR (section 2.7). There were two clear bands with expected sizes for *AmPGI* and *AmPGM* (figure 4.3). These were sequenced and their identity confirmed. The sequences of full-length *AmPGI* and *AmPGM* are presented in appendix, figure A.3 and A.4.

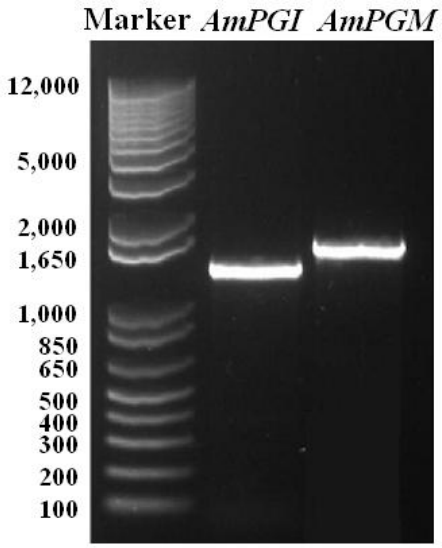


Figure 4.3 PCR fragments representing full length of AmPGM and AmPGI

PCR products representing full-legth cDNAs from antirrhinum of the carbohydrate genes indicated, electrophoresis on a 1 % (w/v) Agarose gel, visualised by EtBr staining and UV detection of fluorescence, Marker represent 1kb plus DNA ladder

4.3.2 *Effect of light integral on the expression of carbohydrate related genes*

The expression of six carbohydrate related genes were analysed throughout the developmental phases in antirrhinum leaf material grown under low light (LL) and high light (HL) conditions (section 3.2.1) using quantitative Real-Time PCR (section 4.2.3). Figure 4.4 and 4.5 represent the expression of carbohydrate related genes in HL and LL grown plant respectively.

The patterns of expression were similar for all six genes. At the end of JP, the level of expression increased and peaked in the AVP. During the JP, the levels of expression of all key genes apart from *AmAMY* and *AmSPS* were higher in HL than

in LL. With *AmPGM*, *AmPGI*, *AmSEX-1*, and *AmBAM-3* which play an important role in starch synthesis and degradation, the level of expression during the JP was influenced by light integral.

During the AVP, the levels of expression of *AmPGM* and *AmSEX-1* were comparable in HL and LL grown plant. Thus, light integral affects the expression level of *AmPGM* and *AmSEX-1* during JP but not in the AVP. The levels of *AmPGI* and *AmBAM-3* expression in HL were higher than that in LL grown plants. This illustrates that light integral affected the expression of *AmPGI* and *AmBAM-3* during both the JP and AVP. The expression levels of *AmSPS* and *AmAMY* during the AVP in LL were higher than in HL grown plants. Therefore, light integral affects the expression of *AmSPS* and *AmAMY* in the AVP but not in the JP.

The expression of these carbohydrate related genes support the results in section 3.3.1 show that late flowering in plants grown under LL conditions was related to extended JP and AVP. The late flowering observed in LL grown plants might be related to the lower levels of expression of *AmPGM*, *AmPGI*, *AmSEX-1* and *AmBAM-3* during the JP that is associated with the extended JP. In LL grown plants, it seems that the genes took longer to reach an amount which may be necessary for promotion of the flowering process in the AVP. Hence, the LL grown plants are slower to reach the AVP compared to the HL grown plants.

Interestingly, under LL conditions, expression of all target genes was very high when plants were in the cotyledon stage. *AmBam-3*, *AmSPS*, and *AmAMY* expression levels in HL grown plant were also very high when the plant started to produce only cotyledons.

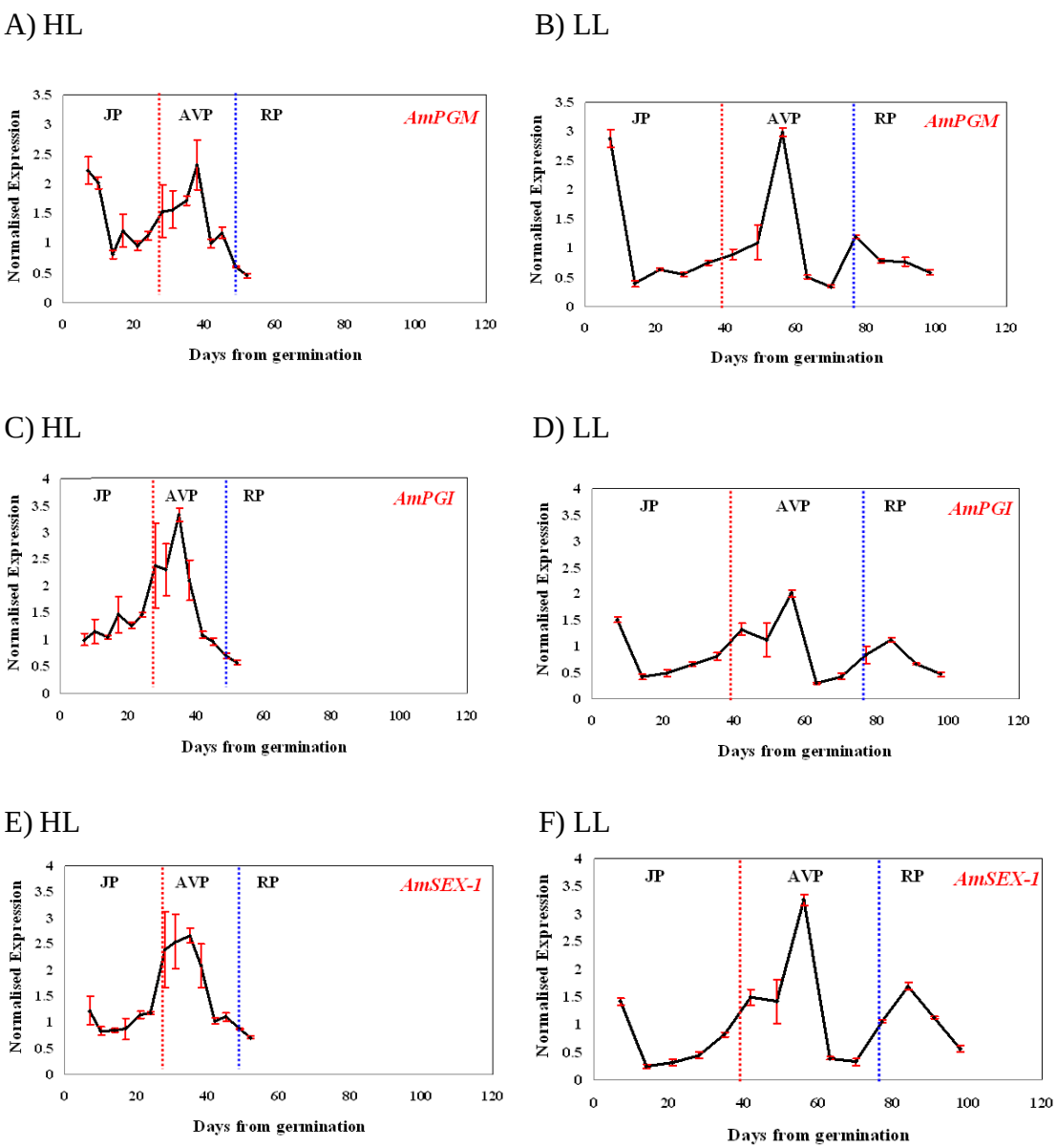


Figure 4.4 Developmental expression of AmPGM, AmPGI, and AmSEX-1 in antirrhinum plants grown under different light integrals

Plants were grown under SD at HL (A, C and E) or LL (B, D and F) and transferred to LDHL conditions. Expression of each gene in leaves harvested at ZT14 in the first LD experienced after transfer was normalised against the geometric mean of Actin and Elongation factor 1 α expression levels .Vertical error bars denote the propagated standard error of the mean of the gene expression obtained using three technical replicates of each gene at each data point. Red and blue dotted lines indicate the end of juvenility and adult vegetative phase respectively. Abbreviations: HL, high light integral; LL, low light integral; JP, Juvenile Phase; AVP, Adult Vegetative Phase; RP, Reproductive Phase

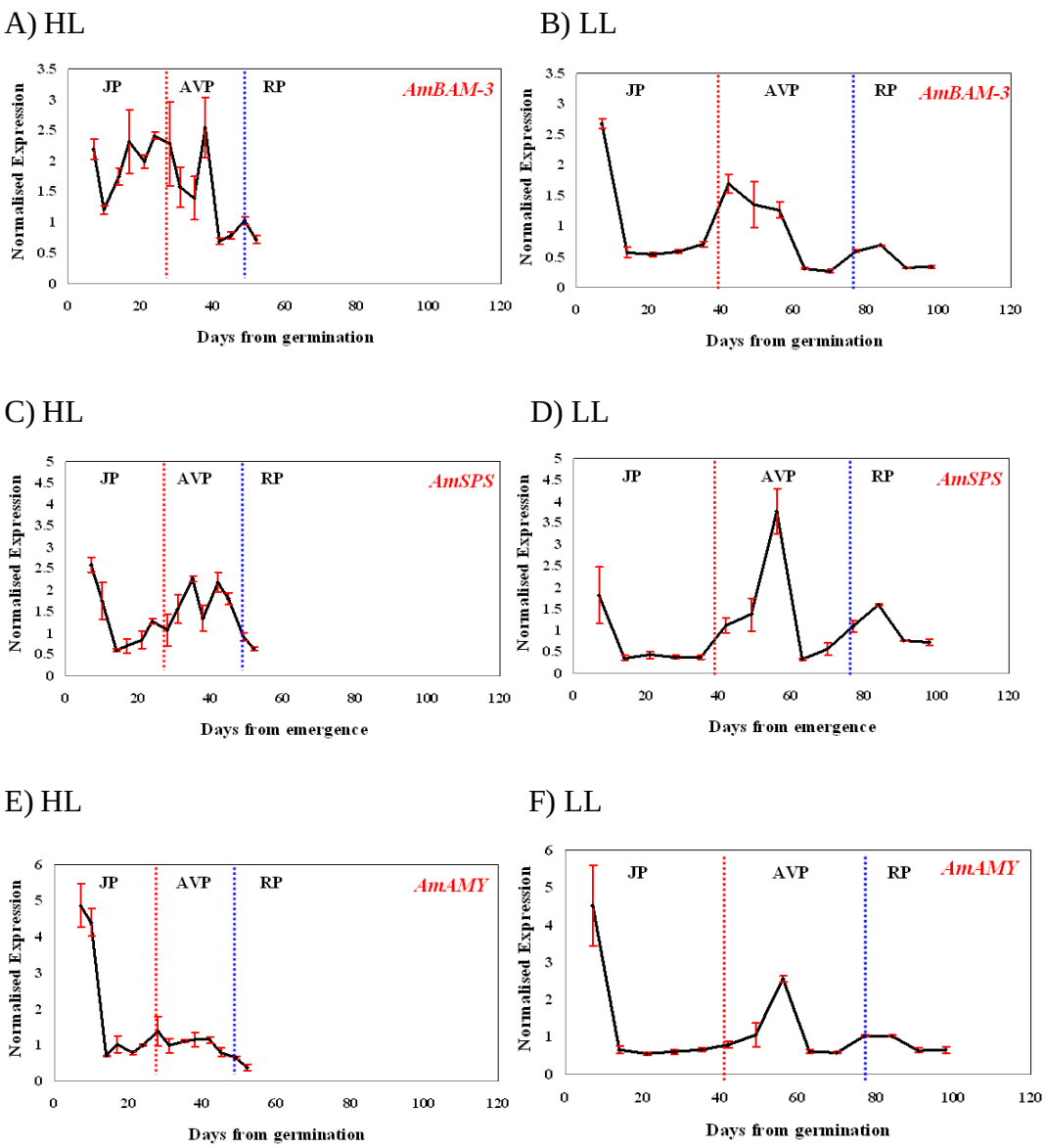


Figure 4.5 Developmental expression of AmBAM-3, AmSPS, and AmAMY in antirrhinum plants grown under different light integrals

Plants were grown under SD at HL (A, C and E) or LL (B, D and F) and transferred to LDHL conditions. Expression of each gene in leaves harvested at ZT14 in the first LD experienced after transfer was normalised against the geometric mean of Actin and Elongation factor 1 α expression levels .Vertical error bars denote the propagated standard error of the mean of the gene expression obtained using three technical replicates of each gene at each data point. Red and blue dotted lines indicate the end of juvenility and adult vegetative phase respectively. Abbreviations: HL, high light integral; LL, low light integral; JP, Juvenile Phase; AVP, Adult Vegetative Phase; RP, Reproductive Phase

4.3.3 *Impact of light integral on carbohydrate content throughout developmental growth phases in antirrhinum*

In additional to the effect of LI on the expression of carbohydrate related genes (section 4.3.2), the impact of LI on carbohydrate content was also needed to determine the correlation between carbohydrate gene expression and carbohydrate levels and clarify the relationship between LI, JP and carbohydrate metabolism. The levels of carbohydrate throughout development in antirrhinum are presented in figure 4.6. In HL and LL grown plants, the pattern of all soluble sugars and starch level during JP to AVP phase transition were similar. The levels were low throughout the JP and generally increased just prior to entering the AVP. Although the amount of carbohydrate throughout the developmental phases were not different between plants grown under LL and HL condition, the levels of all carbohydrates in plants grown under HL started to increase earlier and more rapidly than those grown under LL. HL grown plants transitioned to the AVP quicker than LL grown plants which meant that the JP of HL grown plants was shorter compared to that of LL grown plants.

In HL grown plants, the pattern of glucose and starch level were similar. They were low during JP and then increased continuously until they reached a peak at the middle of the AVP. At the end of the AVP, the levels decreased. It was a little bit different in the pattern of sucrose level in which the level remained high at the end of the AVP. For fructose levels, the peak occurred immediately after JP ended and then the levels dropped at the middle of the AVP. In HL grown plants, the levels of carbohydrate content could not be shown after the end of the AVP since samples were not harvested there.

In LL grown plants, the pattern of glucose and starch levels were similar. The levels were low throughout JP and increased towards the end of the JP. The highest level of glucose and starch were at the middle of AVP and dropped before the plant entered the RP. In the RP, the levels of both carbohydrates were lower than those in AVP but still higher than those in JP.

The low levels of carbohydrate during the JP followed by high levels in the AVP correlated with the pattern of expression of all carbohydrate genes studied in section 4.3.2.

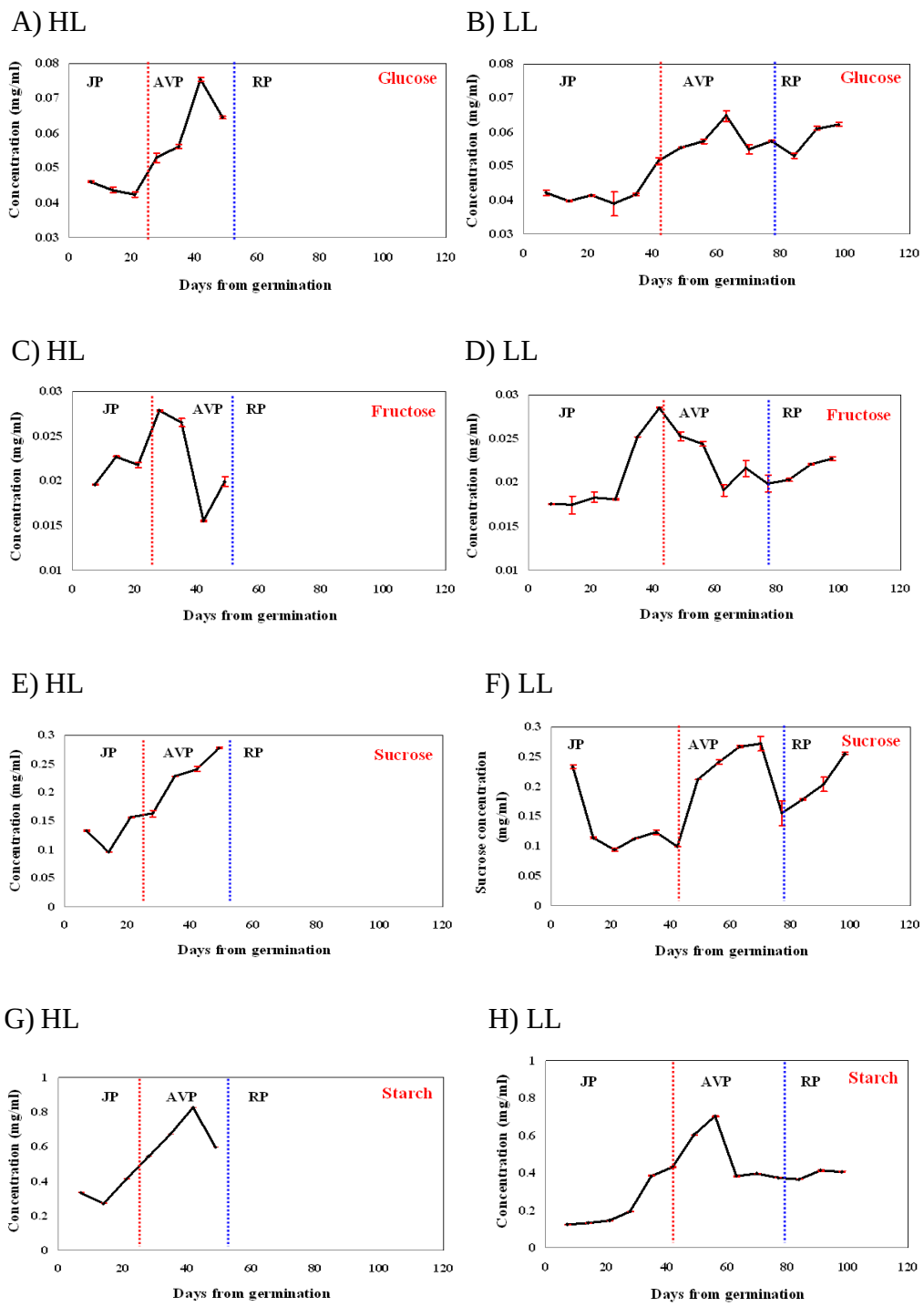


Figure 4.6 Developmental levels of soluble sugars and starch in antirrhinum plants grown under different light integrals *Plants were grown under SD at HL (A, C and E) or LL (B, D and F) and transferred to LDHL conditions. At ZT14 in the first LD experienced after transfer, the leaf materials were harvested to analyse carbohydrate content. Vertical error bars denote the standard error of the mean of the gene expression obtained using three technical replicates of each gene at each data point. Red and blue dotted lines indicate the end of juvenility and adult vegetative phase respectively. Abbreviations: HL, high light integral; LL, low light integral; JP, Juvenile Phase; AVP, Adult Vegetative Phase; RP, Reproductive Phase*

4.4 Discussion

Throughout the developmental phases of antirrhinum from germination to reproduction, the pattern of expression of all six genes, *AmPGM*, *AmPGI*, *AmSEX-1*, *AmBAM-3*, *AmSPS*, and *AmAMY*, were similar. The low and high expressions during JP and AVP respectively indicate that high carbohydrate status is required during the AVP in order to obtain the competence to flower. This result is consistent with many studies that reveal the importance of carbohydrate status for achievement of flowering as stated in the introduction.

During the JP, expression levels of all six carbohydrate related genes varied between HL and LL grown plants. *AmPGM*, *AmPGI*, *AmSEX-1*, and *AmBAM-3* are involved with carbon fixation or photosynthesis which are necessary for plants to produce carbohydrate from inorganic compounds to support their metabolism. Light integral had a bigger effect on the rate of carbon fixation in leaves compared to photoperiod (Chapter3). The greater the light intensity the plant received, the greater the rate of carbon fixation (Obbins and Pharr, 1987). This can support the effect observed of light integral on the expression level of *AmPGM*, *AmPGI*, *AmSEX-1*, and *AmBAM-3* during the JP. *AMY* is not essential for starch degradation since α-amylase mutants are not defective in starch degradation under the condition that supported growth (Zeeman *et al*., 2007; Fulton *et al*., 2008) and *AMY* is more likely to be associated with minor starch breakdown, which possibly supports chloroplast function (Zeeman *et al*., 2007). *SPS* plays a role in sucrose synthesis which is not directly involved with starch synthesis and degradation. These characteristics of the two genes might be the reason why light integral did not have an effect on the level of expression of *AmAMY* and *AmSPS* during the JP.

During the AVP, LI had an effect on some of carbohydrate gene expression. In HL grown plants, the expression of *AmSPS* and *AmAMY* were decreased and the expression of *AmPGI*, *AmBAM-3*were increased compared to those in LL grown. However, LI did not have an impact on the expression levels of *AmPGM* and *AmSEX-1*. Irrespective of differences in JP and AVP due to differences in light integral, the pattern of expression of all carbohydrate genes throughout developmental phases were similar which was low during the JP and high during the

AVP.

Expression of all the carbohydrate genes, apart from *AmPGI* in HL grown plants were high when the plants were at the cotyledon stage. This might because the cotyledons play a role in photosynthesis and carbohydrate storage to support seeding development (Sasaki and Kozlowski, 1968; Lovell and Moore, 1971; Ampofo *et al*., 1976; Kabeya and Sakai, 2003), which require the levels of carbohydrate genes to be high in cotyledon materials.

Throughout the developmental phases of the plant, the pattern of starch and soluble sugar level were similar to those of the carbohydrate related gene expression levels. This indicates that the elevated levels of carbohydrate are needed in the AVP. Since carbohydrate levels in each developmental phase were comparable between HL and LL grown plants, light integral affects how quickly the plant accumulates an amount of sugar that is enough to change the phase but does not affect the level of sugar in each developmental phase.

The effect of light integral on carbohydrate related gene expression and carbohydrate status, which is related to the length of JP and flowering time indicates that carbohydrate metabolism plays a role in controlling the JP and flowering time in plants. The reason why the LL grown plants produce flowers when they have more leaves compared to the HL grown plants might be that when plants obtain lower light integrals, they need more leaves to perceive the light and produce the florigens. Then, plants take longer time to accumulate the photosynthate to produce more leaves and support the metabolism including flowering.High energy is needed for growth and developmental processes of plants.

Plants obtain energy from light which is then involved with the diurnal regulation to balance the energy and nutrient they require with different environmental conditions. The molecular network leading to plant growth and development depends on the availability of carbohydrate content and energy for plants to use. Different light conditions lead to different amounts of carbohydrates plants can produce and high light intensities increases the carbohydrate status in plant. However, the mechanisms involved with regulation of flowering by sugar metabolism and sensing is quite complex. As stated in the introduction, there are many factors such as HXK, miR156, TPS1, and T6P that are associated with the mechanisms. Studies involving the relationship between an environmental cue like

light integral and those factors could also be the key to clarify the mechanism of environmental control of juvenility by carbohydrate metabolism.

Interestingly, the studies from chapter 3 and this chapter reveal that light integral has an influence on both carbohydrate metabolism and flowering gene expression. In order to make it complete, an investigation into the effect of specific carbohydrate related genes on juvenility was needed and this study will be covered in chapter 5.

CHAPTER5. INFLUENCE OF CARBOHYDRATE METABOLISM AND MIR156 ON FLOWERING TIME IN *ARABIDOPSIS*

5.1 Introduction

5.1.1 *The effect of carbohydrate gene mutations on flowering time in Arabidopsis*

When grown under SD conditions, *Arabidopsis* starch deficient mutants, *plastidal phosphoglucomutase* (*pgm*) (Caspar *et al*., 1985; Corbesier *et al*., 1998) and *adp glucose pyrophosphorylase1* (*adg-1*) (Lin *et al*., 1988) and starch excess mutants, *glucan water dikinase* (*sex-1*) (Caspar *et al*., 1991) and *β-amylase3* (*bam-3*) (Lao *et al*., 1999) all display late flowering phenotypes. The enzymes encoded by the defective genes in the mutants play important roles in carbohydrate metabolism. Starch is synthesised in the chloroplast during the day time using fructose 6-phosphate from the Calvin-Benson cycle (Zeeman *et al*., 2007; Geigenberger, 2011). PGM converts Glc6P to Glc1P (Harrison *et al*., 1998; Manjunath *et al*., 1998). Glc1P in the chloroplast is then converted to ADPglucose, which is the component of starch, by ADG1 (Manjunath *et al*., 1998; Harrison *et al*., 1998; Zeeman *et al*., 2007). During the night, starch is degraded using the combination of several enzymes including SEX1 and BAM3.

The mutants, *pgm*, *adg-1*, and *sex-1*, are total knockout T-DNA insertion mutants (http://arabidopsis.info/). Since the *pgm* mutant completely lacks plastidial phosphoglucomutase (Caspar *et al*., 1985), Glc1P can not be converted from Glc6P. Hence starch cannot be synthesized and very little starch is detected in leaves of the mutant compared to wild-type plants at the end of the day (Streb *et al*., 2009). The *adg-1* mutant lacks functional ADG1, which encodes the small subunit of ADP glucose pyrophosphorylase (ADGase). Both small and large subunits of ADGase are absent in this mutant since lacking the small subunit affects the stability of the large one in the chloroplast. Therefore the *adg-1*mutant has no ADGase activity (Wang *et al*., 1998). The *sex-1* mutant is defective in starch-phosphorylating enzyme which caused the inhibition of transitory starch phosphorylation and starch cannot be degraded. As a consequence the concentration of starch remains high at the end of

the dark period (Yu *et al*., 2001; Blennow *et al*., 2002). The *bam-3* mutant has a point mutation at the At4g17090 locus (Lao *et al*., 1999), which causes a reduction in BAM3 activity and high amounts of leaf starch at the end of the night (Fulton *et al*., 2008).

It is interesting that both starch excess and starch deficient mutants exhibit the same phenotype, which is late flowering. In chapter 3 and 4, it was shown that LI has an effect on carbohydrate and flowering gene expression. To a better understand the relationship between LI, carbohydrate metabolism, and flowering time, the relationship between specific carbohydrate genes and flowering genes was investigated in this chapter.

5.1.2 *The effect of miR156 mutation on flowering time in Arabidopsis*

In addition to the *Arabidopsis* carbohydrate mutants, there is another group of mutants whose flowering behaviours differ from the wild type; these are mutants harbouring aberrations in miR156 levels. The 35S:MIM156 mutant has no function of miR156 and has an early flowering phenotype (http://arabidopsis.info/). This mutant is derived from a target mimicry strategy, where the target mimic miR156, MIM156, is used to bind to miR156 to stop its function in the activation of the expression of *SPL* genes (Franco-Zorrilla *et al*., 2007). The *miR156G*, *A*, and *C* mutants derive from the T-DNA insertion method that completely blocks the expression of both genes (http://arabidopsis.info/). miR156A and miR156C have the major effect on the JP to AVP transition as measured by leaf characteristics and their mutations cause early flowering in *Arabidopsis* (Yu *et al*., 2013).

Since miR156 genes are involved with the regulation of the transition from the JP to the AVP by sugar (Proveniers, 2013; Lastdrager *et al*., 2014), it is also very interesting to study the effect of LI on miR156 and flowering time in order to clarify if the regulation of JP by miR156 could be linked to LI.

There were two aims for the studies described in this chapter, (i) to determine the influence of specific carbohydrate related genes on juvenility and flowering time genes using *Arabidopsis* carbohydrate metabolism mutants and (ii) to

determine the relationship between LI, juvenility, and miR156 using miR156 early-flowering mutants.

5.2 Materials and Methods

The descriptions are materials and methods specific for this results chapter. For the common materials and methods in more than one result chapter, the descriptions are in chapter 2. For the transfer experiments, plants were grown under the same environmental conditions at the same time to generate material for flowering time assessment and molecular and biochemical analysis.

5.2.1 *Arabidopsis material*

Arabidopsis thaliana Columbia wild type (Col-0) and starch excess mutants (*bam-3* and *sex-1*) were obtained from the seed stock of previous PhD student in the group, Ioannis G (Matsoukas, 2010). *Arabidopsis thaliana* starch deficient mutants (*pgm-1* and *adg-1*) and miR156 mutants (35S::MIM156, *miR156A*, *miR156G*, and *miR156C*) were obtained from Nottingham Arabidopsis Stock Centre (NASC, UK).

5.2.2 *Plant growth conditions*

Arabidopsis seeds were sown with two seeds per cell into Plantpak P40 trays containing seed and modular compost (Levington F2). After sowing, the seeds were watered heavily and the trays covered with a plastic 634x413 mm clear autoclave bag and then with a black bag (Koehler Technische Produkten). To achieve synchronization of germination, the trays were left at 4°C for two nights before placing in the SDHL growth cabinets (section 2.2).

5.2.3 *Transfer experiment with Arabidopsis carbohydrate mutants*

To generate materials that differed in juvenile phase length with *Arabidopsis* carbohydrate mutants (*bam-3*, *sex-1*, *pgm*, and *adg-1*) and wild type (Col-0), *Arabidopsis* seeds (section 5.2.1) were used for each type of mutant and wild type and were sown following the procedure in section 5.2.2. The Col-0 used as a control with the two experiments were performed, with starch excess mutants was called Col-0(1) and with starch deficient mutants called Col-0(2). At 50% germination, *Arabidopsis* plants were initially grown under SDHL conditions (T_0).

The cabinets used for this experiment were the MLR-351 Versatile plant growth cabinets (SANYO Electric Co., Ltd.) and the settings are described in section 2.2. As *Arabidopsis* has a very short JP, the transfers started 1 day after T_0. Plants were transferred from the SDHL cabinet to the LDHL cabinet daily at ZT7 for six days. Transfers continued at two day intervals for three transfers and the last transfer was at a fourteen day interval with seven plants per transfer. All aerial plant parts were harvested on the same day of transfer at ZT14 of the first long day experienced with 30 plants for the first to sixth transfers and 7 plants for the seventh to tenth transfers. The harvesting method was stated in section 2.4.1. This material was used for developmental expression analysis of flowering time genes (section 5.2.5) and carbohydrate content throughout all developmental growth phases (section 5.2.6).

To assess the flowering times for evaluation of the different growth phases, seeds (section 5.2.1) were sown with two seeds per cell following the procedure in section 5.2.2. After plants reached T_0, the excess seedlings in each cell were removed. Seedlings were put in each SDHL and LDHL Sanyo cabinet and kept there without transfer for the controls. Then the other seedlings were initially put in SDHL. Transfers from SDHL to LDHL conditions were carried out at ZT7 with the number of plants per transfer being seven for all transfers. Flowering times were recorded as the number of rosette leaves at 1 cm bolt length and different phases of development determined as described in section 2.4.2. To determine leaf production rates, the number of rosette leaves (more than 1.5 mm length) not including cotyledons, was recorded daily in all plants up until the end of juvenility.

5.2.4 *Arabidopsis miR156 mutants transfer experiment*

To assess the flowering time of each mutant (35S:MIM156, *miR156G*, *miR156A*, and *miR156C*) and the wild type (Col-0), seeds (section 5.2.1) were sown with one seed per cell following the procedure in section 5.2.2. After plants reached T_0, five seedlings were placed and kept in each SDHL, SDLL, and LDHL cabinet for the controls. Then other seedlings were divided into two groups to put in SDHL and SDLL cabinets equally and were from each SD cabinet to LDHL at the interval described in section 5.2.3. Transfers were carried out at ZT7 with five plants per transfer. Leaf production rates were determined as described in section 5.2.3.

5.2.5 *Determination of carbohydrate levels during growth in Arabidopsis mutants*

The levels of glucose, fructose, sucrose, and starch were examined in rosette leaf materials derived from the experiment detailed in sections 5.2.3 of this chapter using Glucose (HK) Assay Kit (Sigma-Aldrich Co. LLC., UK), Fructose Assay Kit (Sigma-Aldrich Co. LLC., UK), Sucrose Assay kit (Sigma-Aldrich Co. LLC., UK), and Starch (HK) Assay Kit (Sigma-Aldrich Co. LLC., UK) respectively. The procedures including sample preparation and enzymatic assay were described in section 2.12.

5.3 Results

5.3.1 *Impact of knock out of carbohydrate genes on juvenile phase length and flowering time gene expression in Arabidopsis*

5.3.1.1 Effect of carbohydrate genes on juvenile phase length

The transfer experiments from SDHL condition were performed using *Arabidopsis* Columbia wild type (Col-0), starch excess (*bam-3* and *sex-1*), and starch deficient (*pgm* and *adg-1*) mutants in order to estimate the impact of carbohydrate genes on juvenile phase (JP) length (section 5.2.3).The graphs show the effect of knock out of starch degradation genes and starch synthesis genes on the different phases of growth in *Arabidopsis* (figure 5.1 and 5.2). The JP lengths of Col-0(1), Col-0(2), *bam-3, sex-1*, *pgm*, and *adg-1* are presented in table 5.1. The extended JP length and delayed flowering time in carbohydrate mutants compared to Col-0 shows that carbohydrate metabolism effects JP length. Not only was the JP altered, but also the length of the AVP. The AVP of all carbohydrate mutants was extended compared to Col-0.

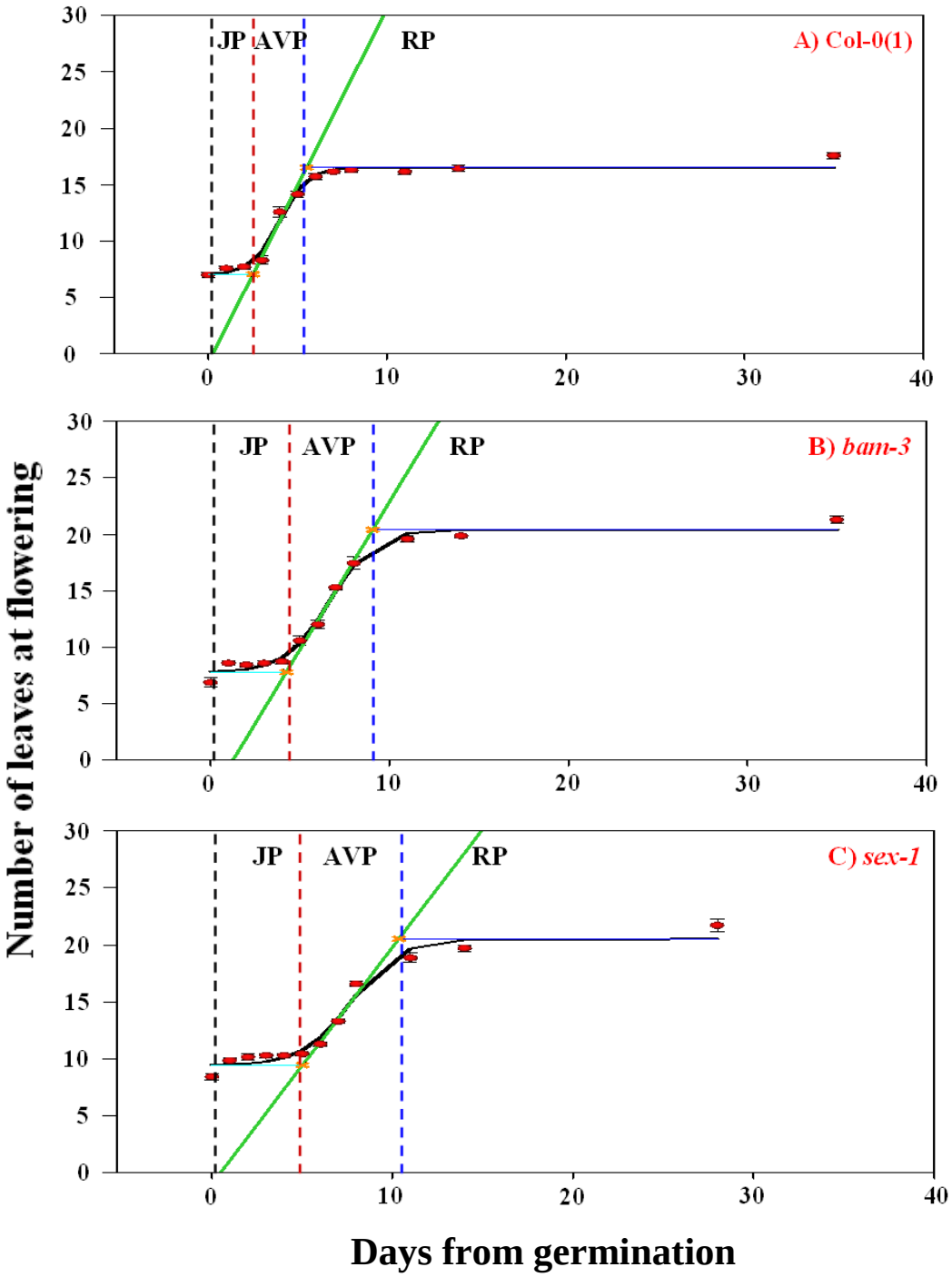


Figure 5.1 Effect of defect in starch degradation on the phases of photoperiod sensitivity in Arabidopsis

Flowering time of Arabidopsis Col-0(1) (A), bam-3 (B), and sex-1 (C) plants transferred from SDHL to LDHL at regular intervals from germination. Vertical error bars indicate the standard error of the mean of the leaf number. Horizontal error bars show the standard error of the mean of the phase length. The black curve, green line, light blue line, dark blue line, and purple dotted line represent the logistic curve, maximum slope, lag time, stationary phase, and boundaries of the different phases respectively. Abbreviations: JP, Juvenile Phase; AVP, Adult Vegetative Phase; RP, Reproductive Phase.

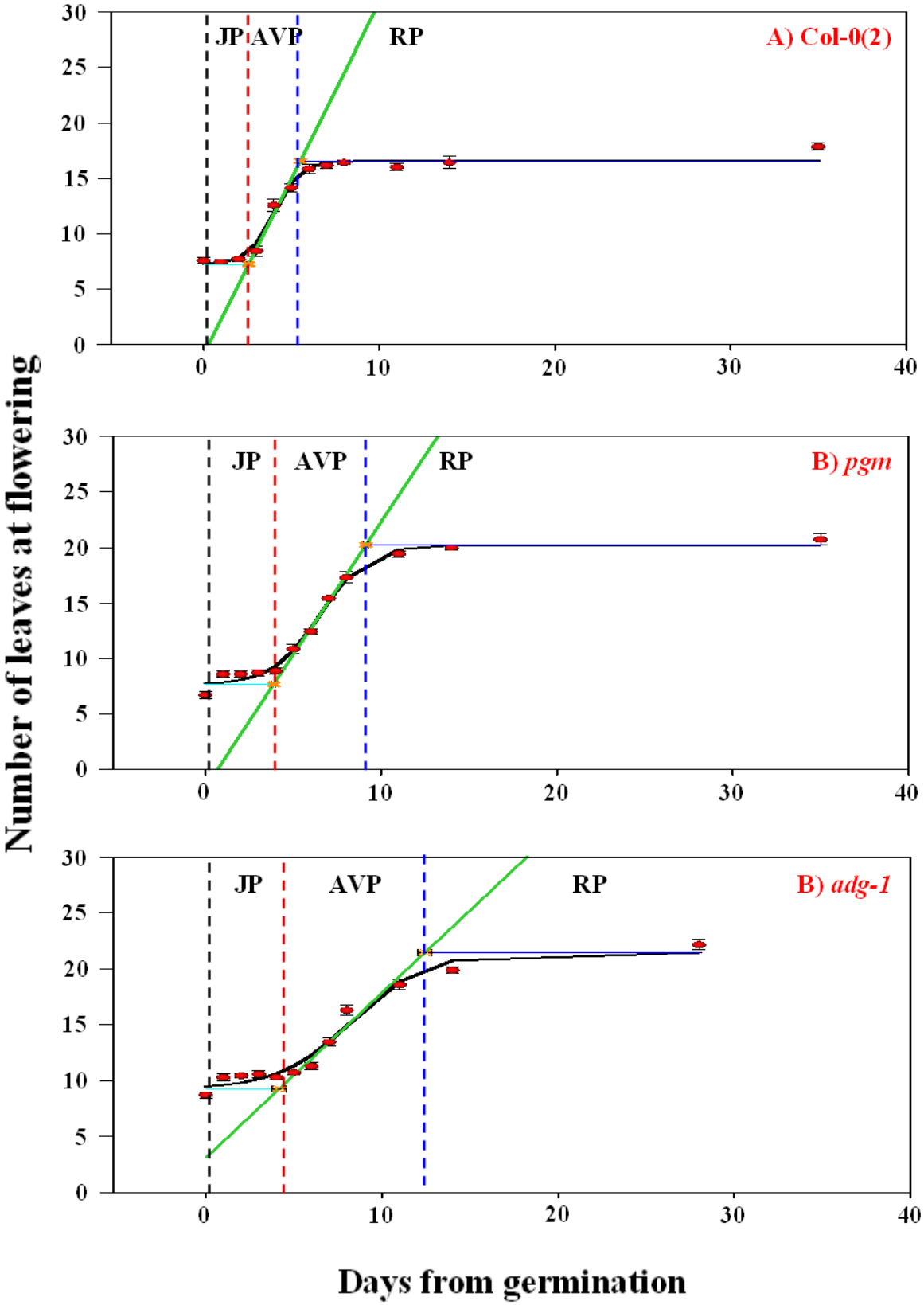


Figure 5.2 Effect of defect in starch synthesis on the phases of photoperiod sensitivity in Arabidopsis

Flowering time of Arabidopsis Col-0(2) (A), pgm (B), and adg-1 (C) transferred from SDHL to LDHL at regular intervals from germination. Figure legend as stated in figure 5.1.

Arabidopsis	Total flowering time (day)	JP length (day)	AVP length (day)	Significantly different compared to wild-type	
				JP	AVP
Col-0(1)	5.5+/-0.09	2.49+/-0.09	3.01+/-0.09	N/A	N/A
bam-3	9.06+/-0.12	4.23+/-0.12	4.83+/-0.12	P<0.05	P<0.05
sex-1	9.94+/-0.16	5.45+/-0.16	4.49+/-0.16	P<0.05	P<0.05
Col-0(2)	5.5 +/- 0.1	2.56 +/- 0.1	2.94+/-0.1	N/A	N/A
pgm	9.12 +/- 0.1	3.87 +/- 0.1	5.25+/-0.1	P<0.05	P<0.05
adg-1	12.43 +/- 0.3	4.16 +/- 0.3	8.27+/-0.3	P<0.05	P<0.05

Table 5.1 JP and AVP lengths in the Arabidopsis carbohydrate mutants and wild type
Flowering time of plants transferred from SDHL to LDHL at regular intervals from germination. Abbreviations: JP, Juvenile Phase; AVP, Adult Vegetative Phase; +/-, Standard error of the mean of the phase length

5.3.1.2 Impact of defect in carbohydrate genes on leaf production rate during juvenility

To determine whether observed differences in JP length are not due to differences in the rate of plant development, leaf production rates of continuous SDHL grown plants were compared between the Col-0 wild type and the mutants. Although the JP length, AVP length, and flowering time were different between Col-0, *bam-3*, *pgm*, and *adg-1*, the leaf production rates during JP (over the first 6 days) of all plants were similar (figure 5.3). This was not the case with the *sex-1*mutant since its leaf production rate was lower and the flowering time was delayed compared to the other plants in this study. The differences in *sex-1* leaf production rate occurred early in development during the JP; later on the leaf production rate of this mutant was similar to that of Col-0. The results show that the extended JP that occurred in *bam-3, pgm, and adg-1* were not because of a reduced rate of development with leaf production rate.

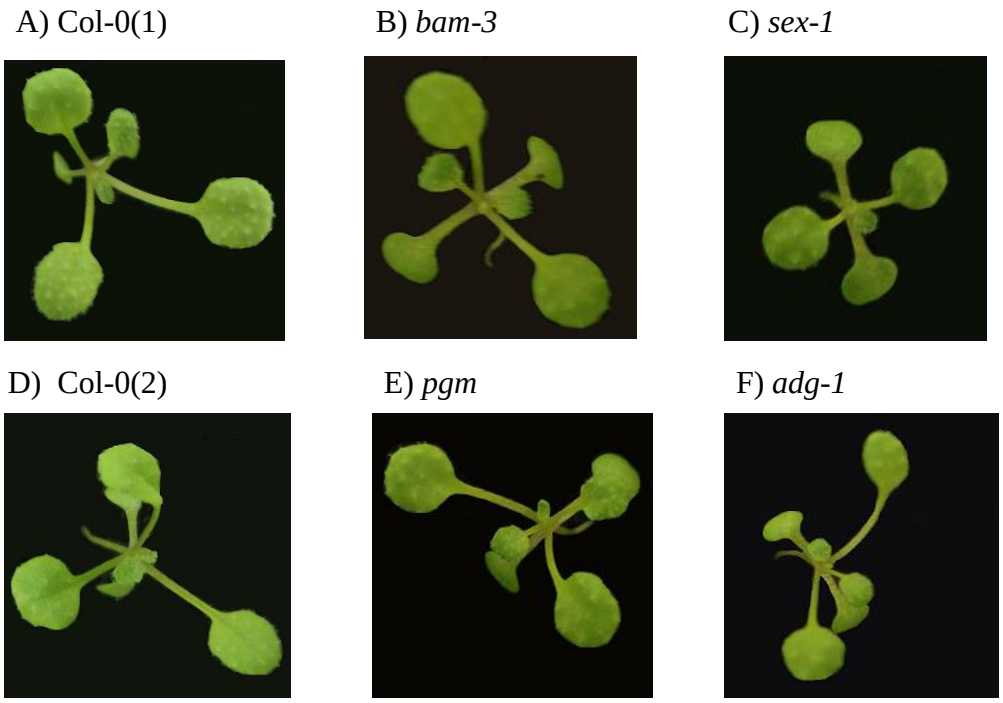


Figure 5.3 Comparison of plant developmental rate during juvenility of Arabidosis wild type and carbohydrate mutants

The pictures show leaf production rates of 6-day old Col-0(1) (A), bam-3 (B), sex-1 (C), Col-0(2) (D), pgm (E) and adg-1 (F) are displayed. Plants were kept continuously under SDHL growth condition. The numbers of rosette leaves greater than 1.5 mm produced were counted at daily intervals from germination.

5.3.1.3 Influence of defect in carbohydrate gene expression on flowering time gene expression

In order to determine the effect of defects in carbohydrate gene expression on the expression of flowering time genes, developmental expression of *CO*, *FT*, and *TEM* were analysed in the material harvested from the transfer experiment described in section 5.2.3. Developmental expressions of *CO* in Col-0(1), Col-0(2), *bam-3*, *sex-1*, *adg-1*, and *pgm* are presented in figure 5.4. In Col-0 and all four carbohydrate mutants that have an extended JP, *CO* expression remained low during the JP and started to increase around the end of the phase transition. The levels of *CO* during

the AVP in all mutants were lower than in Col-0. For all mutants apart from *sex-1*, the general pattern of the curves were similar to Col-0 which was the low expression level during the JP, increase and peak of expression level during the AVP, except that the initial levels during the JP of all mutants were low for longer than Col-0. The low level of CO expression in *sex-1* and the distinguishable pattern of expression from the other plants in this study coincide with the leaf production rate in *sex-1* that was lower compared to the others as well.

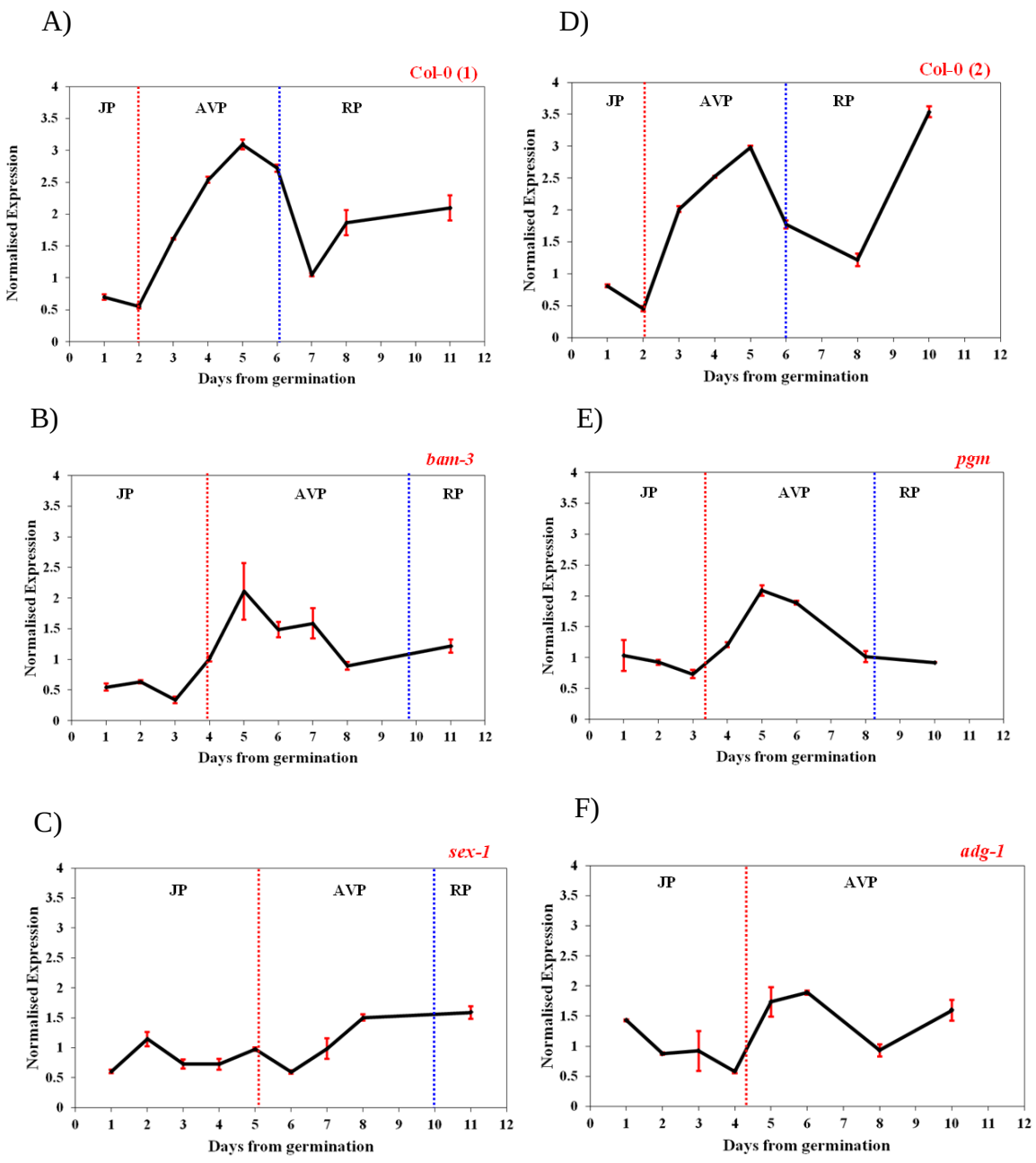


Figure 5.4 Developmental expression of CO in Arabidopsis wild type and carbohydrate mutants
Col-0(1), bam-3(B), sex-1 (C), Col-0(2) pgm (E), and adg-1(F) were grown under SDHL and transferred to LDHL conditions. Expression of CO in rosette leaves harvested at ZT14 in the first LD experienced was normalised against the geometric mean of actin and β tubulin expression levels .Vertical error bars denote the propagated standard error of the mean of the gene expression obtained using three technical replicates of each gene at each data point. Red and blue dotted lines indicate the end of juvenility and adult vegetative phase respectively. Abbreviations: SDHL, short day high light; LDHL, long day high light; JP, Juvenile Phase; AVP, Adult Vegetative Phase; RP, Reproductive Phase. Note the AVP ended after 12.43 days in adg1 (see table 5.1)

Developmental expression of *FT* in Col-0(1), Col-0(2), *bam-3*, *sex-1*, *adg-1*, and *pgm* is shown in figure 5.5. Delay in induction of *FT* in all carbohydrate mutants compared to wild-type coincides with extended JP and correspondence with *CO* expression. In Col-0, *bam-3* and *pgm*, *FT* expression was low during the JP before the induction of *FT* started at the end of the JP and the peak was presented at the AVP. The induction of *FT* was delayed in *bam-3* and *pgm* which linked to the extended in JP of both mutants. Interestingly, in both mutants, there was no peak of *FT* in the RP like Col-0. In *sex-1* and *adg-1*, *FT* was hardly induced throughout the developmental phases. For all mutants in all phases apart from the AVP of *bam-3*, the level of *FT* expression was lower compared to wild type.

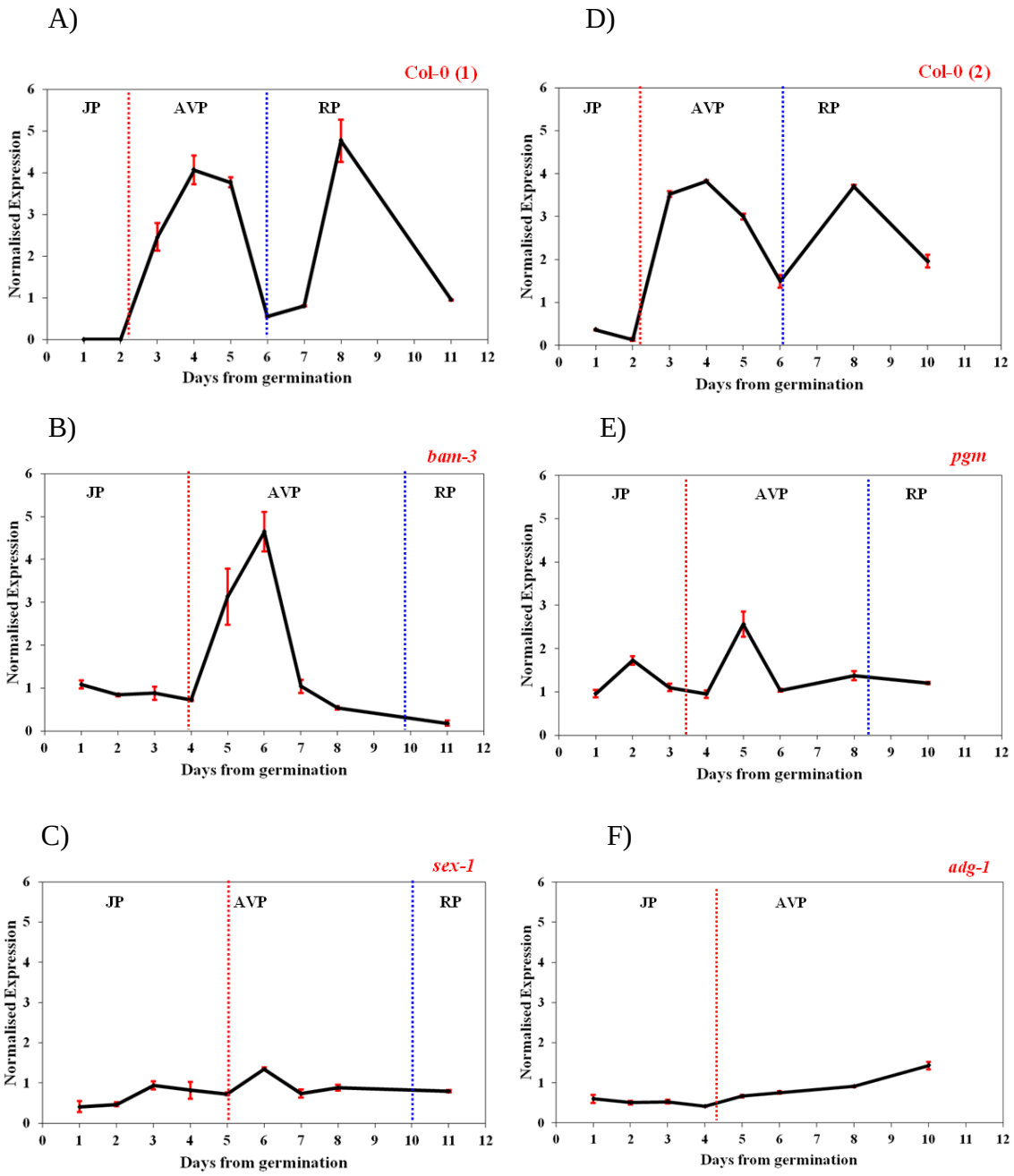


Figure 5.5 Developmental expression of FT in Arabidopsis wild type and carbohydrate mutants

Col-0(1) (A), bam-3(B), sex-1 (C), Col-0(2) (D) pgm (E), and adg-1(F) were grown under SDHL and transferred to LDHL conditions. Figure legend as stated in figure 5.4.

Developmental expression of *TEM* in Col-0(1), Col-0(2), *bam-3*, *sex-1*, *adg-1*, and *pgm* is presented in figure 5.6. For all mutants apart from *sex-1*, the developmental pattern and the expression level of *TEM* were very similar to Col-0 although JP was extended in the mutants. Therefore, *TEM* expression was unaffected by carbohydrate status defects. Interestingly, the level of *TEM* was decreased dramatically after a day from germination in all mutants, except sex-1, where the *FT* levels were not affected. This was different in Col-0 where *FT* levels were increased when the level of *TEM* dropped. In *sex-1*, the level of *TEM* did not really fluctuate. It was high throughout the developmental phases, which coincided with the low level of *FT*.

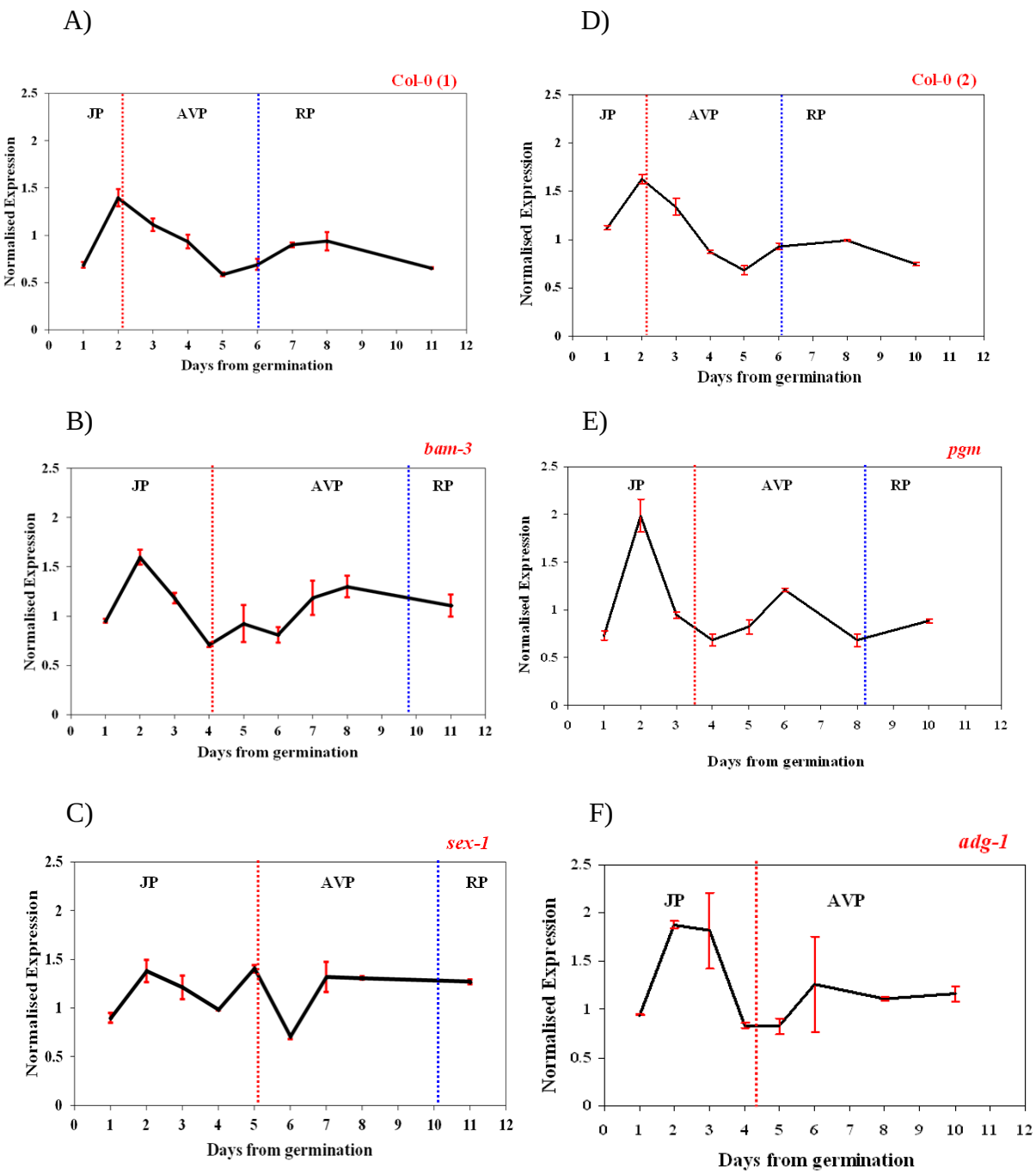


Figure 5.6 Developmental expression of TEM in Arabidopsis wild type and carbohydrate mutants

Col-0(1) (A), bam-3 (B), sex-1 (C), Col-0(2) (D) pgm (E), and adg-1(F) were grown under SDHL and transferred to LDHL conditions. Figure legend as stated in figure 5.4.

5.3.2 *Impact of defect in carbohydrate genes on carbohydrate content throughout all developmental growth phases in Arabidopsis*

To ensure that all the mutants were defective in carbohydrate metabolism, the levels of carbohydrates in each mutant were compared to the Col-0 wild type in materials harvested from the transfer experiment described in section 5.2.3. The levels of starch in Col-0(1), Col-0(2), *bam-3*, *sex-1*, *adg-1*, and *pgm* are presented in figure 5.7. In Col-0 wild type, the starch level was lowest during the JP and started to increase during the AVP. For the starch excess mutants, *bam-3* and *sex-1*, the level of starch throughout the developmental phases was higher compared to Col-0; levels of starch in *sex-1* were higher than *bam-3* levels. In these mutants starch levels started to rise directly from germination.

In starch deficient mutants, *pgm* and adg-1, the level of starch was very low throughout the developmental phases compared to Col-0 and starch excess mutants.

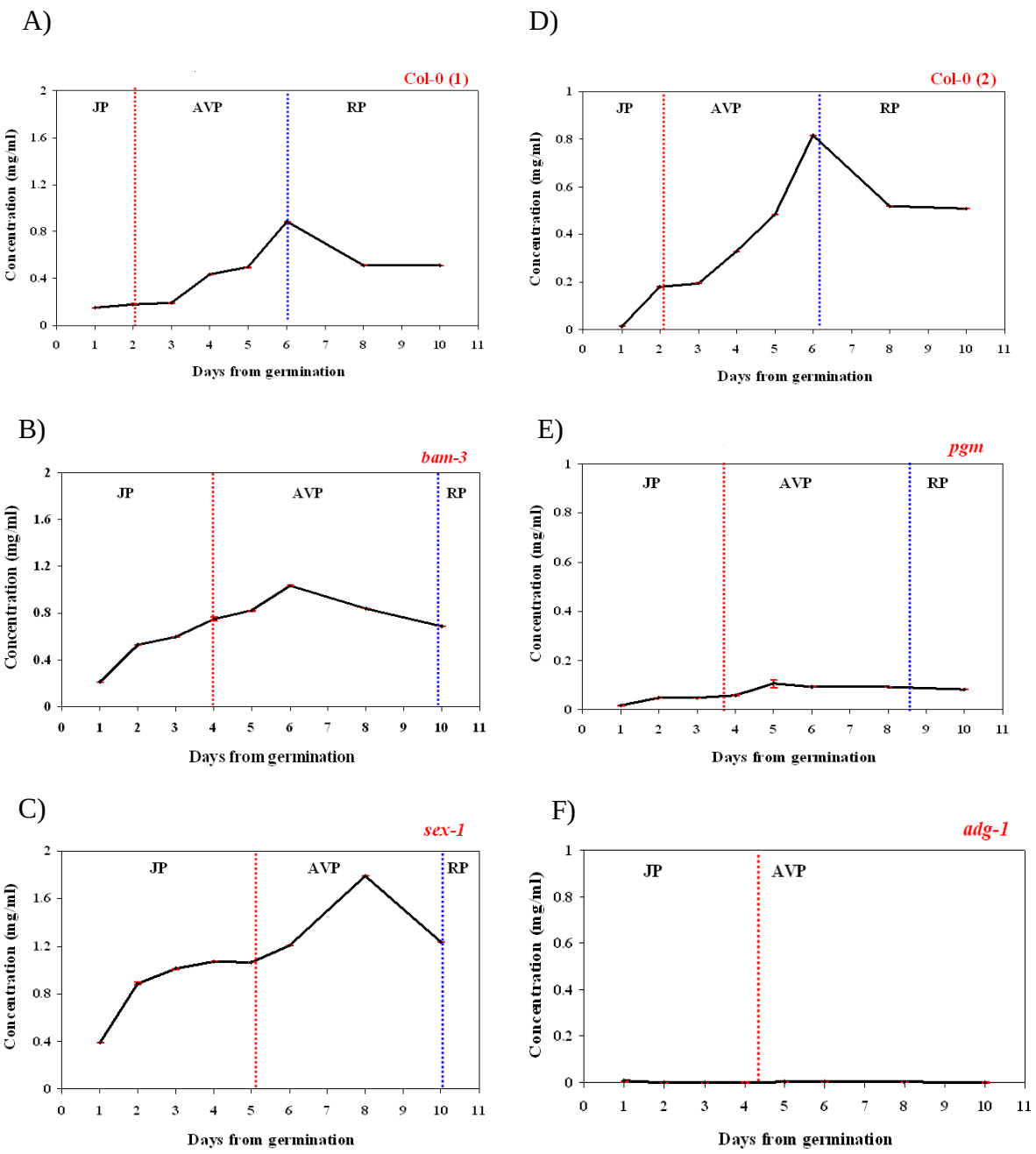


Figure 5.7 Developmental level of starch in Arabidopsis wild type and carbohydrate mutants

Col-0(1) (A), bam-3(B), sex-1 (C), Col-0(2) (D) pgm (E), and adg-1(F) were grown under SDHL and transferred to LDHL conditions. At ZT14, the first LD experience, rosette leaf material was harvested to analyse starch content. Vertical error bars denote the standard error of the mean of the starch level obtained using three technical replicates at each data point. Red and blue dotted lines indicate the end of juvenility and adult vegetative phase respectively. Abbreviations: SDHL, short day high light; LDHL, long day high light; JP, Juvenile Phase; AVP, Adult Vegetative Phase; RP, Reproductive Phase

The levels of glucose, fructose and sucrose in Col-0(1), Col-0(2), *bam-3*, *sex-1*, *adg-1*, and *pgm* are presented in figure 5.8, 5.9, and 5.10 respectively. For the level of all soluble sugars, the general trend was the same for all mutants and Col-0, which was lowest during JP and the rise occurred around the end of JP. When the JP was delayed, the rise was also delayed.

The levels of glucose and fructose peaked during the AVP and they were higher in all mutants compared to Col-0. It was different with sucrose since the level of sucrose was similar between Col-0 and all mutants.

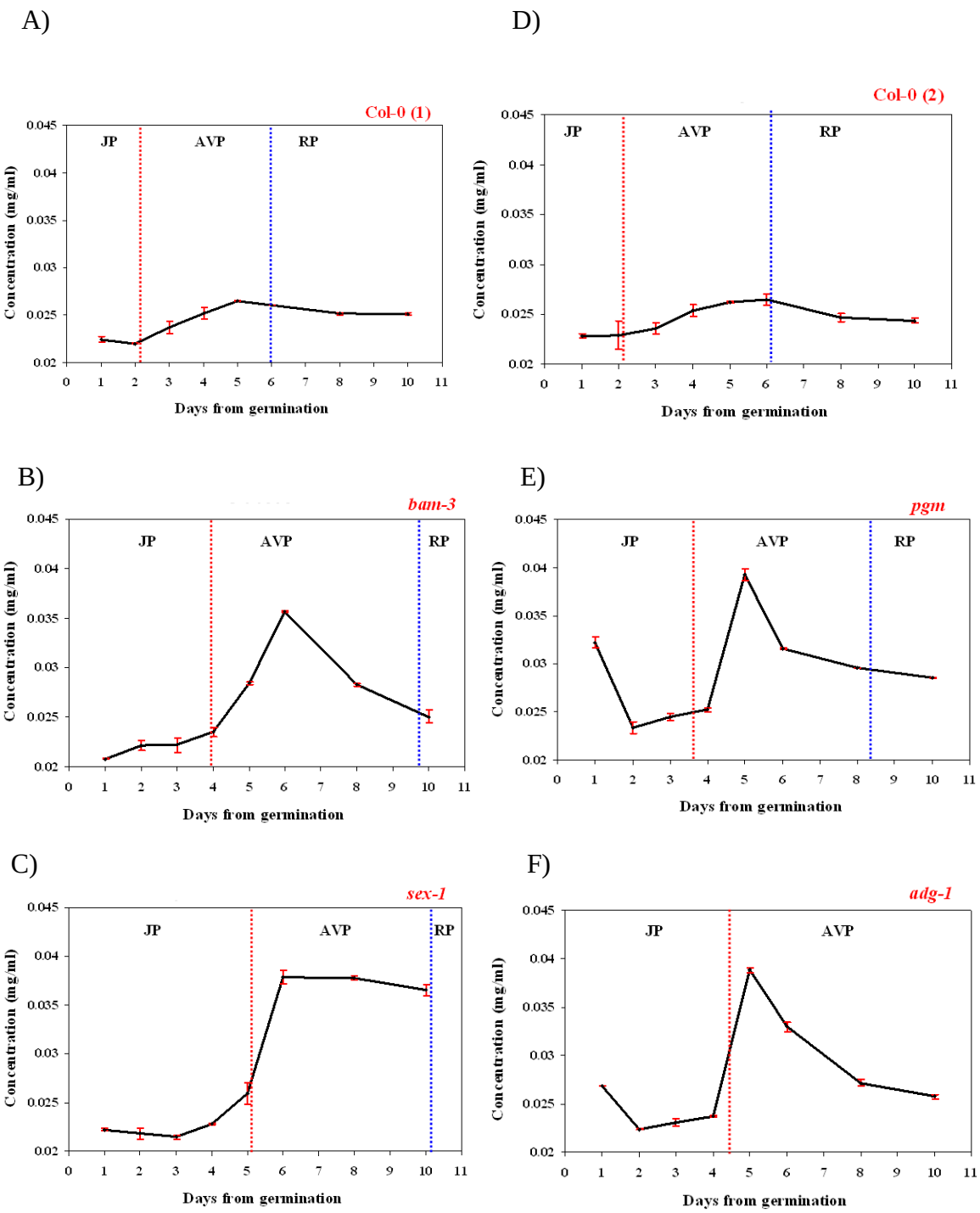


Figure 5.8 Developmental level of glucose in Arabidopsis wild type and carbohydrate mutants
Col-0(1) (A), bam-3(B), sex-1 (C), Col-0(2) (D) pgm (E), and adg-1(F) were grown under SDHL and transferred to LDHL conditions. At ZT14, the first LD experienced, rosette leaf material was harvested to analyse glucose content. Figure legend as stated in figure 5.7.

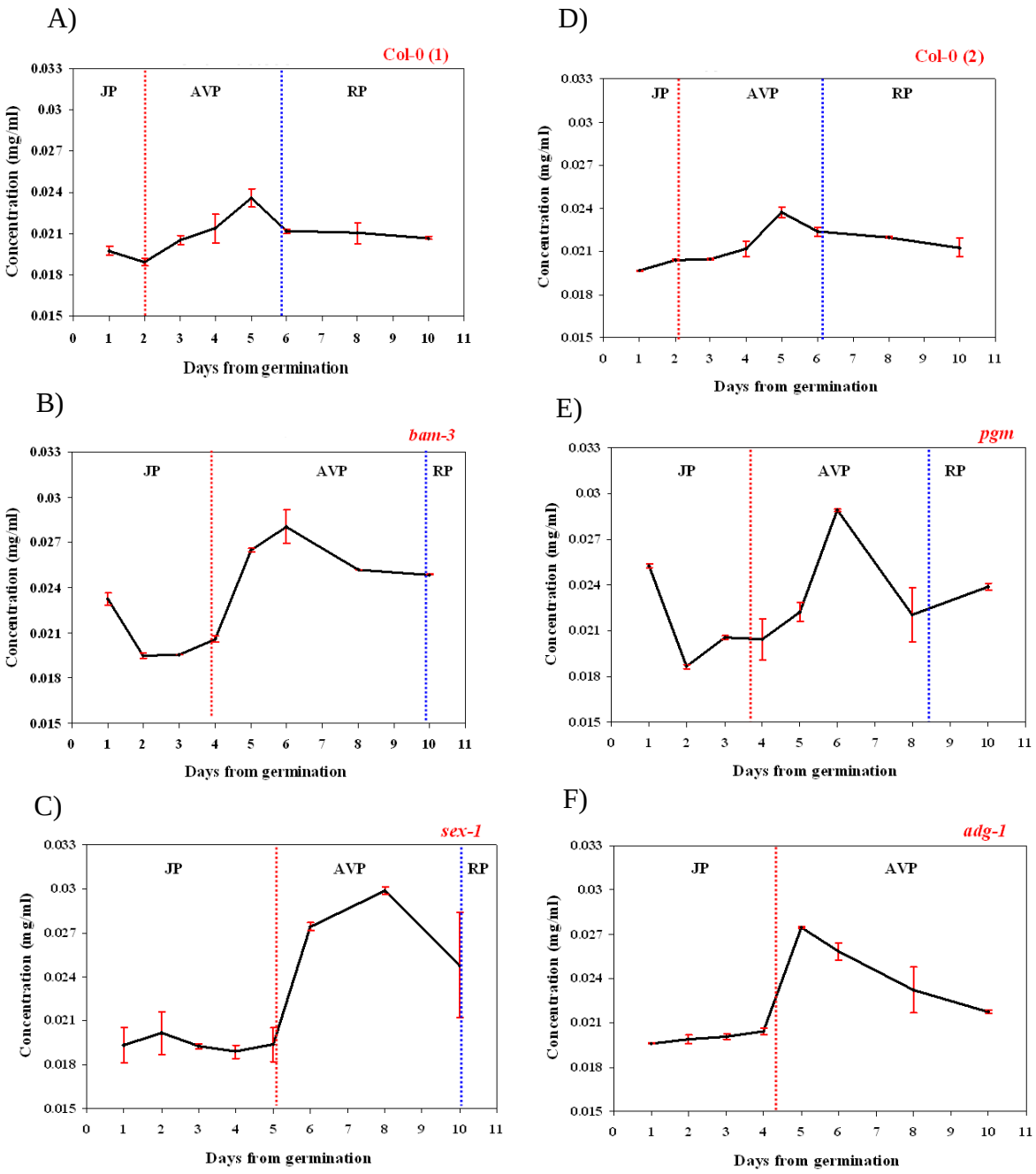


Figure 5.9 Developmental level of fructose in Arabidopsis wild type and carbohydrate mutants
Col-0(1) (A), bam-3(B), sex-1 (C), Col-0(2) (D) pgm (E), and adg-1(F) were grown under SDHL and transferred to LDHL conditions. At ZT14, the first LD experience, rosette leaf material was harvested to analyse fructose content. Figure legend as stated in figure 5.7.

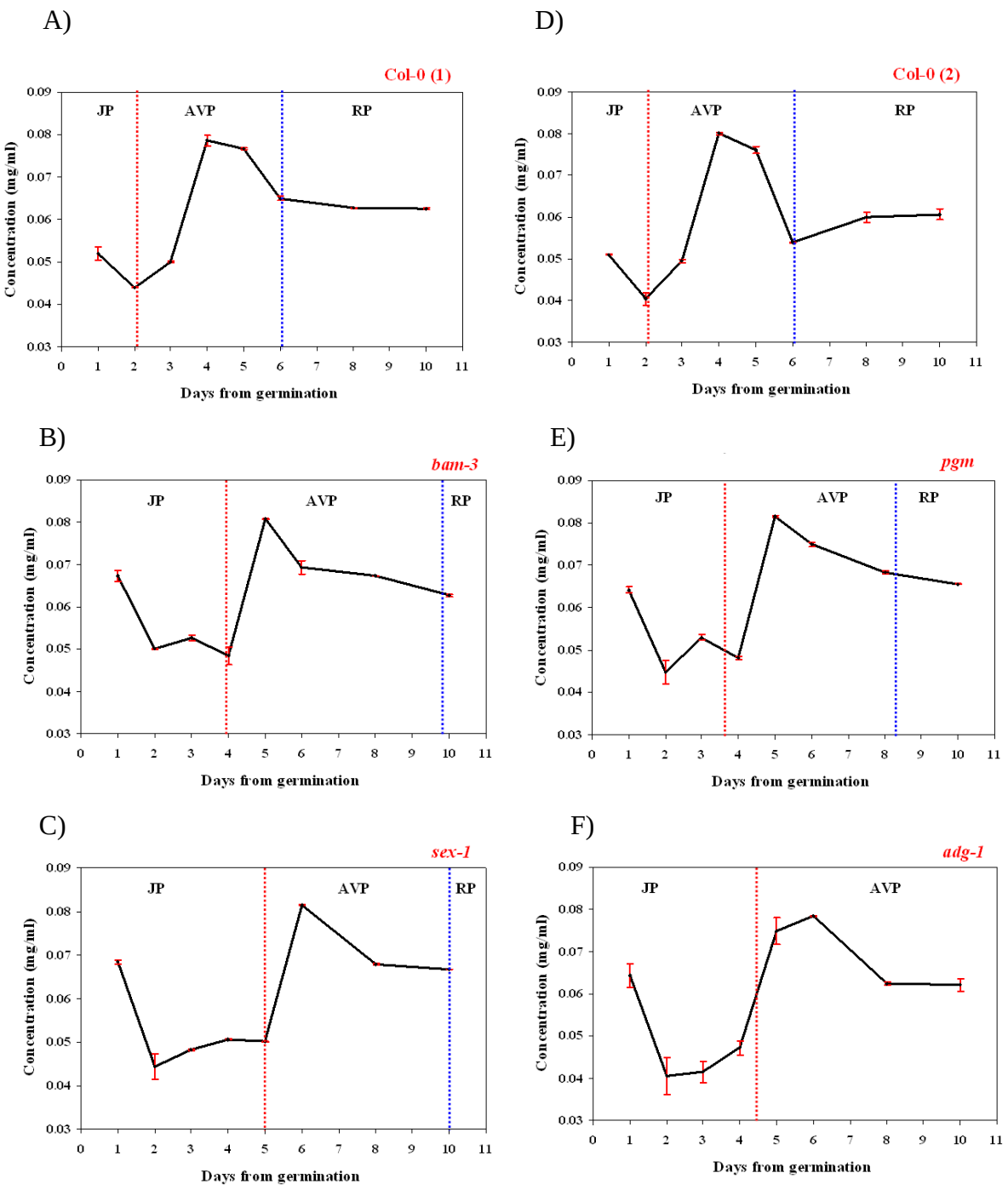


Figure 5.10 Developmental level of sucrose in Arabidopsis wild type and carbohydrate mutants
Col-0(1) (A), bam-3(B), sex-1 (C), Col-0(2) (D) pgm (E), and adg-1(F) were grown under SDHL and transferred to LDHL conditions. At ZT14, the first LD experience, rosette leaf material was harvested to analyse sucrose content. Figure legend as stated in figure 5.7.

5.3.3 *Involvement of miR156 in regulation of juvenile phase length in Arabidopsis*

In antirrhinum, the level of miR156 was high in young plants and reduced when the plants aged. The reduction in leaves was delayed in SDLL grown plants compared to SDHL grown plants (section 3.3.1.3). This suggested that the reduction of miR156 level could contribute towards the phase transition in antirrhinum and that LI might also be involved. In *Arabidopsis*, miR156 is encoded from eight genes, miR156 A, B, C, D, E, F, G, and H and the transcriptions of two genes, miR156C and miR156A, play the dominant role in phase transition in plant (Wu and Poethig, 2006; Wu *et al*., 2009).MiR156 species were shown to be involved with the regulation of the transition from the JP to AVP by sugar as measured by the phase length by the characteristic of the leaf margins and leaf trichomes on the abaxial side (Yang *et al*., 2013; Yu *et al*., 2013). The purpose of the experiments in this study were (i) to determine the involvement of different miR156 species in regulating the JP using incompetence to flower as measure of juvenility and (ii) to determine the effect of LI on expression of the different miR156 species.

The transfer experiments were performed to estimate the length of JP in different miR156 mutants (35S:MIM156, *miR156G*, *miR156A*, and *miR156C*) and the wild type (Col-0) under different LIs, SDHL (8 h of PAR 7.2 $m^{-2}d^{-1}$) and SDLL (8 h of PAR 1 $m^{-2}d^{-1}$. The defects in all miR156 mutants was shown to affect both the JP and AVP under both HL and LL in relation to the wild type (figure 5.11). The JP and AVP lengths of all plants in this experiment are shown in table 5.2.

In Col-0, the length of the JP and AVP were extended in SDLL compared to SDHL grown plants (table 5.2). This was similar to what was observed in antirrhinum grown under different LI where SDLL grown plants had delayed JP and AVP compared to SDHL. LI also has an effect on both the JP and AVP length in all mutants. The JP length of all mutants apart from *miR156G* was delayed in LL compared to HL grown plants. Interestingly with all mutants apart from *miR156C*, under both HL and LL conditions, the JP lengths were shorter whereas the AVP lengths were longer than Col-0. Although the lengths of the AVP were longer, the total flowering time in miR156 mutants were still shorter than that in Col-0.

In 35S:MIM156, where all miR156 genes are completely knocked out, there was no JP when grown under HL condition. This was also the case with *miR156C*,

which only lacked the miR156C gene. Therefore, miR156C seems to have the greatest influence on JP compared to miR156G and miR156A.

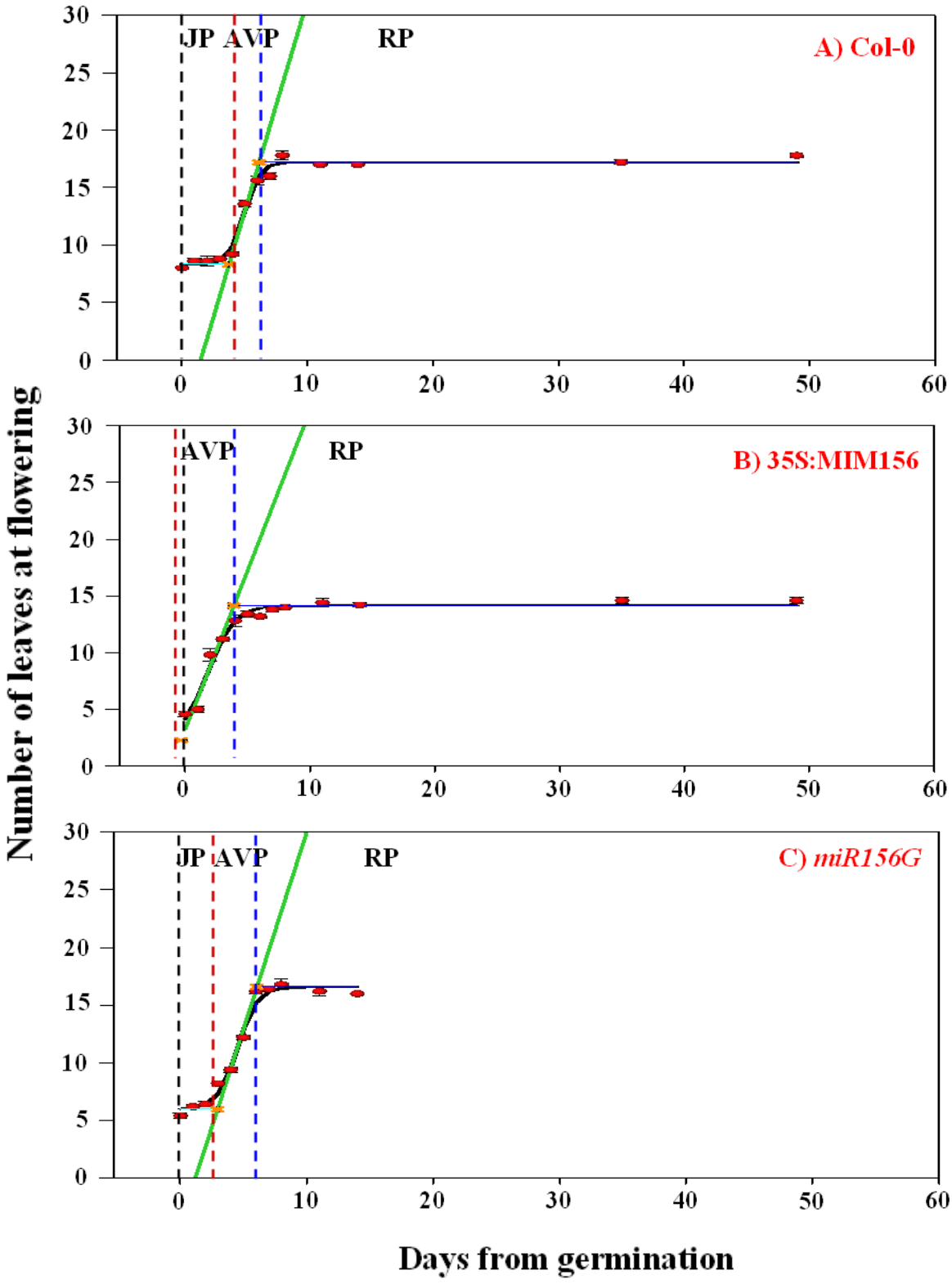


Figure 5.11 Effect of light integral and miR156 on the phases of photoperiod sensitivity in Arabidopsis

Flowering time of Arabidopsis wild type and miR156 mutants under SDHL transferred to LDHL at regular intervals from germination. Figure legend as stated in figure 5.1.

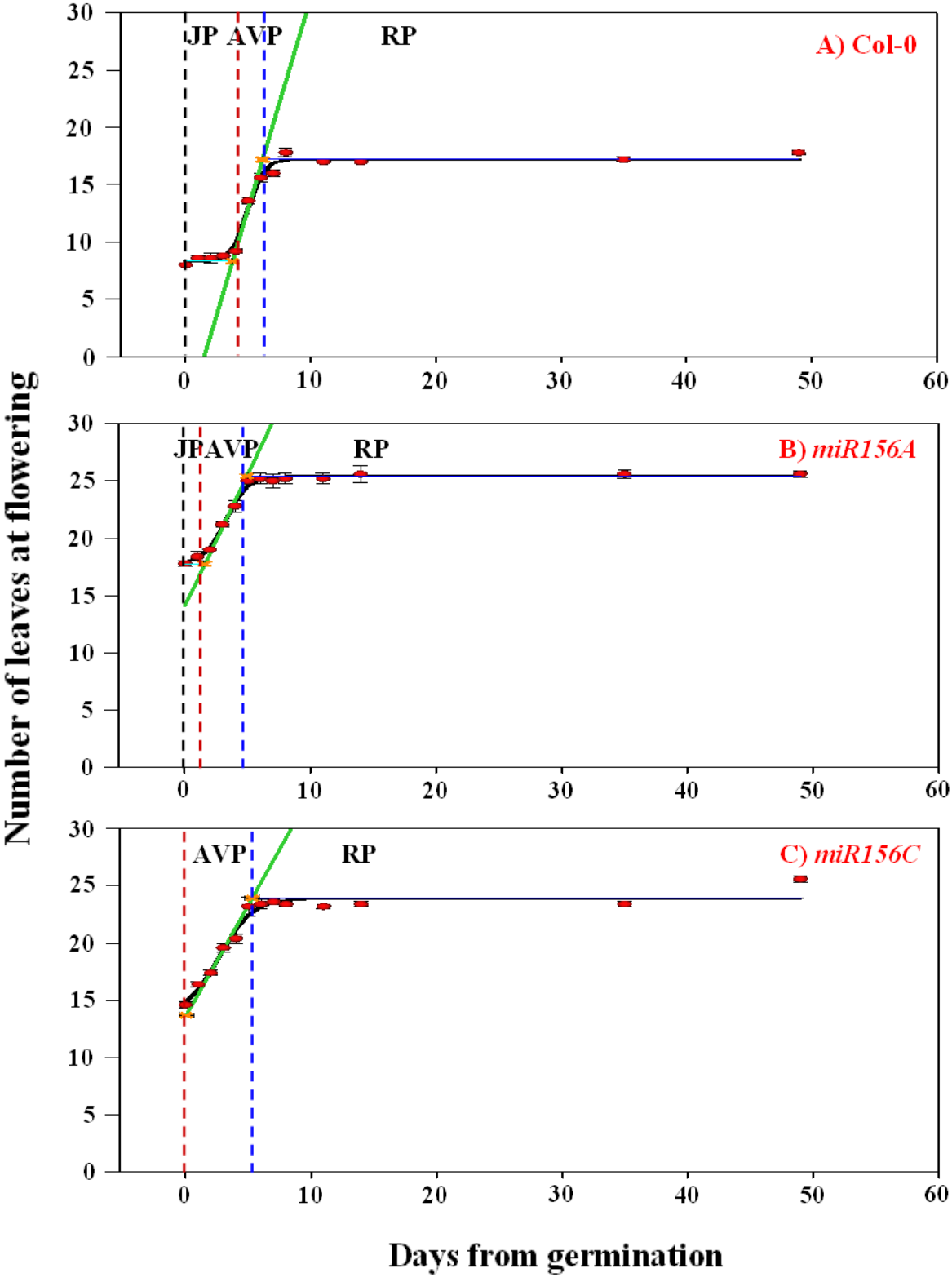


Figure 5.11 Continued

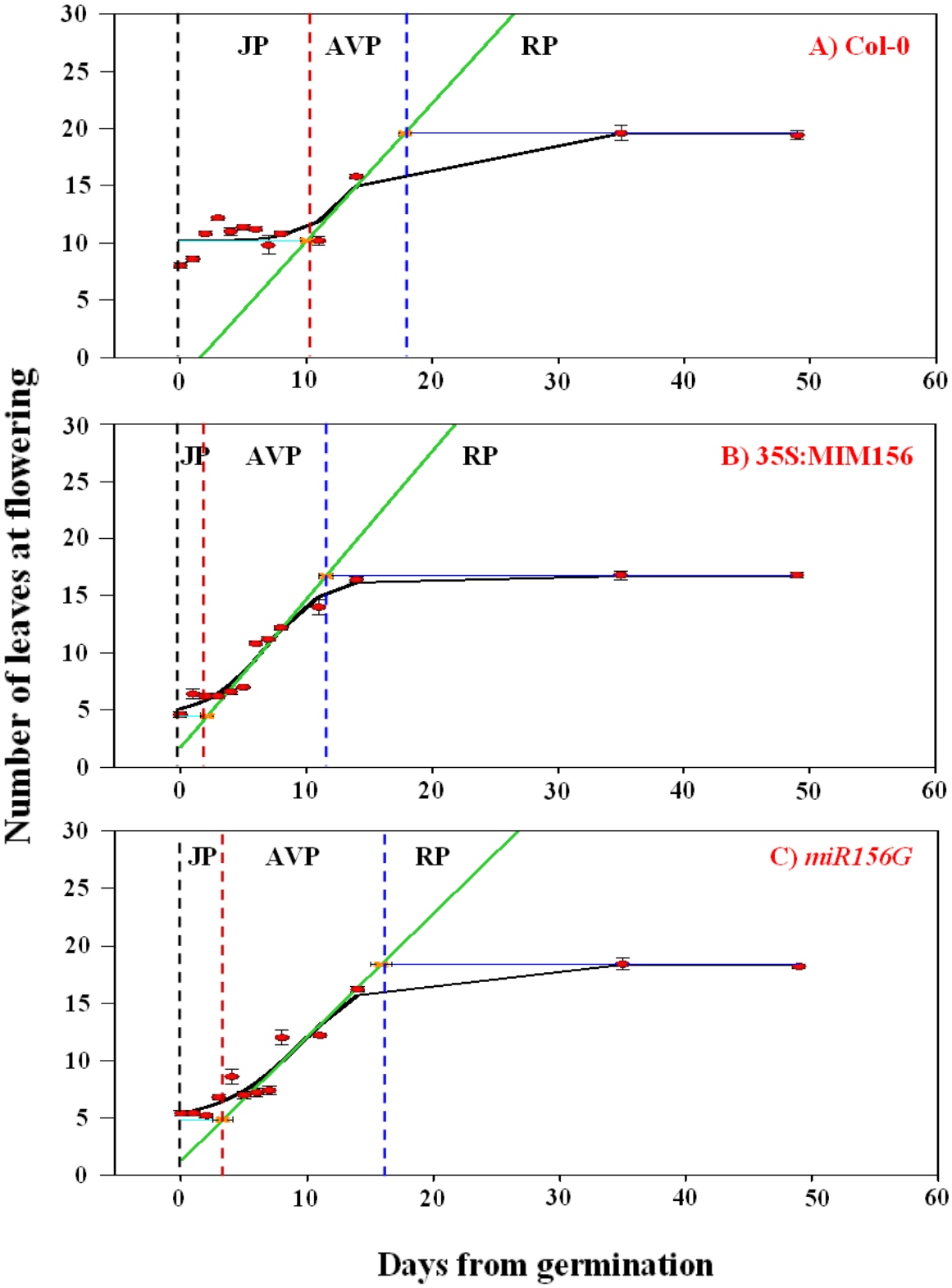


Figure 5.11 Continued

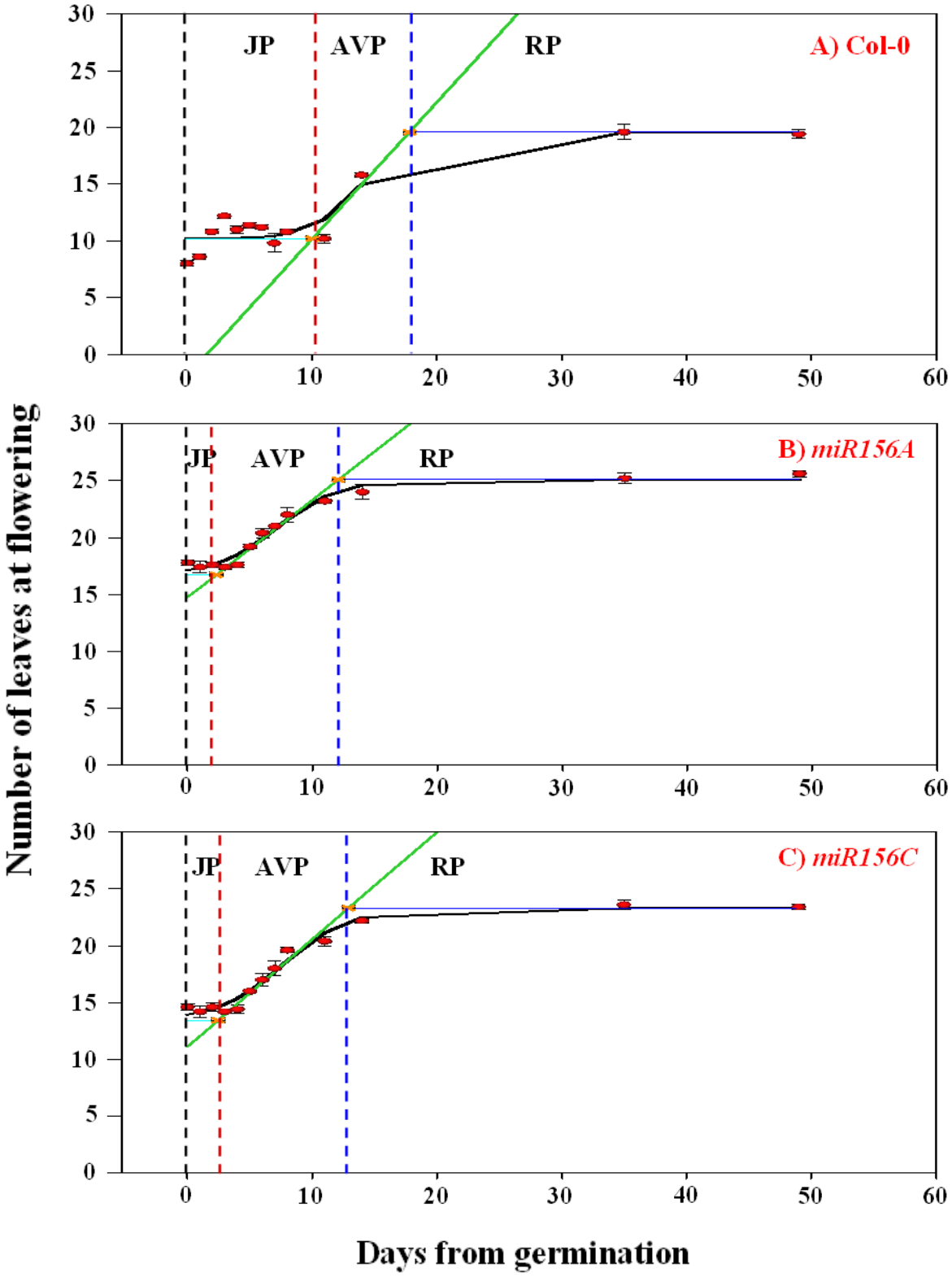


Figure 5.11 Continued

Genotype	Total flowering time (day)		JP length (day)		AVP length (day)		Significantly different compared to wild-type			
	LL	HL	LL	HL	LL	HL	LL		HL	
							JP	AVP	JP	AVP
Col-0	17.83 +/- 0.48	6.14 +/- 0.06	10.05 +/- 0.48	3.73 +/- 0.06	7.78 +/- 0.48	2.41 +/- 0.06	N/A	N/A	N/A	N/A
35S:MIM156	11.58 +/- 0.54	3.88 +/- 0.43	2.13 +/- 0.54	-0.34+/- 0.43	9.45 +/- 0.54	4.22 +/- 0.43	P< 0.05	P< 0.05	P< 0.05	P< 0.05
miR156G	15.87 +/- 0.81	6.06 +/- 0.1	3.29 +/- 0.81	2.94 +/- 0.1	12.58 +/- 0.81	3.12 +/- 0.1	P< 0.05	P< 0.05	P< 0.05	P< 0.05
miR156A	12.08 +/- 0.54	4.93 +/- 0.08	2.29 +/- 0.54	1.59 +/- 0.08	9.79 +/- 0.54	3.34 +/- 0.08	P< 0.05	P< 0.05	P< 0.05	P< 0.05
miR156C	12.89 +/- 0.52	5.29 +/- 0.57	2.46 +/- 0.52	0.04 +/- 0.57	10.43 +/- 0.52	5.25 +/- 0.57	P< 0.05	P< 0.05	P< 0.05	P< 0.05

Table 5.2 JP and AVP lengths in the Arabidopsis miR156 mutants and wild type

Abbreviations: JP, Juvenile Phase; AVP, Adult Vegetative Phase; HL, Plants transferred from SDHL to LDHL; LL, Plants transferred from SDLL to LDHL; +/-, Standard error of the mean of the phase length

5.4 Discussion

The difference between JP length, AVP length and flowering time between wild type and carbohydrate mutants shows that carbohydrate metabolism has an influence on phase change and flowering time in plants. From the results, the apparent extended JP length in all mutants apart from *sex-1* is true since plants developed at similar rates to Col-0 during the JP. *SEX-1* encodes the glucan water dikinase (GWD) which plays an important role in starch phosphorylation (Ritte *et al*., 2000; Zeeman *et al*., 2007) before the degradation of starch can be catalysed by β-amylase encoded by *BAM-3* (Zeeman *et al*., 2007). Therefore *SEX-1* is needed in order to prepare the products for *Bam-3* to degrade. This might be the reason why leaf production rates are slower in *sex-1* compared to the other mutants. Therefore,

the effect of the *SEX-1* mutation on JP length may be a consequence of slower leaf production rate.

The lower expression of *CO* and *FT* in all carbohydrate mutants compared to Col-0 and the delayed induction of these genes in *bam-3* and *pgm* showed that *CO* and *FT* might be controlled or mediated by carbohydrate metabolism. Interestingly, *TEM* expression was not affected by carbohydrate. Moreover, in the carbohydrate mutants, the *FT* level was not affected by the level of that *FT* might be regulated by other factors during JP rather than *TEM or TEM* activity is reliant on carbohydrate status. In Col-0 the drop in *TEM* expression around the end of JP associates with an increase in *FT* expression. In the *sex-1* mutant, high levels of *TEM* are maintained across development and these correlate with low levels of *FT* across development. These finding are consistent with the role of *TEM* as a regulator of JP acting through *FT* (Sgamma, 2014). However, in the *bam-3, pgm* and *adg-1* mutants, the timing of the drop in *TEM* does not correlate with the end of JP, which occurs later, and it does not coincide with an increase in *FT* expression, which occurs around the end of JP. Even where *TEM* levels are low during JP, *FT* levels do not rise. This indicates that in these mutants although repression of *FT* by *TEM* is lifted, the carbohydrate status affects *CO* expression and thus *FT* levels remain low following lifting of repression by *TEM* because *CO* levels are also low during the extended JP in the mutants.

The defect in starch level in all mutants was confirmed since there was more starch in *bam-3* and *sex-1* and less starch in *pgm* and *adg-1* compared to Col-0. Although the level of starch contrasts between starch excess and starch deficient mutants, the levels of glucose and fructose in both types of mutants were higher than in Col-0. In starch excess mutants, *sex-1* and *bam-3*, the starch cannot be broken down at night, which results in high levels of starch accumulation and this is fed back to cause inhibition of starch synthesis. Therefore, the levels of soluble sugars, the precursor of starch synthesis, are increased especially at the end of the light period when the materials were harvested for analysis in this study. Similar finding were also observed in *sex-1* in an earlier study (Caspar *et al*., 1991). Starch deficient mutants, *pgm* and *adg-1*, had higher levels of soluble sugars compared to the wild type. This is because the mutants cannot convert the sugars to starch which results in excess amounts of sugar during the day. These results correlate with previous

studies, which showed that under SD, the levels of leaf sugars in *pgm* during the day were higher than in the wild type (Corbesier *et al*., 1998; Gonzali *et al*., 2006).

Unlike the other soluble sugars, the level of sucrose was comparable between the carbohydrate mutants and wild type. In *sex-1* and *bam-3*, the sucrose level was unaffected by the lack of starch degradation enzymes, unlike glucose and fructose, since sucrose is not a direct precursor of starch synthesis. In this study, leaf material for analysis was harvested during the light period. Zeeman *et al*. (1999) showed that although levels of sucrose in a starch excess mutant, *sex-4*, and wild type were similar during the day, at night sucrose levels in the mutant dropped quickly to lower levels compared to wild type. This might be because lack of starch degradation results in a lack of maltose which is the product of starch breakdown (Fulton *et al*., 2008; Stitt and Zeeman, 2012). Not enough maltose causes the reduction of sucrose levels since maltose is the substrate for sucrose synthesis (Geigenberger, 2011).

For the starch deficient mutants, the leaf materials were also harvested at the end of the light, which is the period where the levels of sucrose are similarly high as the wild type. In the light period, sucrose is synthesised in the cytoplasm using GAP obtained from the chloroplast (Zeeman *et al*., 2007). GAP is the upstream product before PGM and ADG-1 get involved, so there is no interruption of sucrose synthesis from GAP of *pgm* and *adg-1* in the daytime. Levels of sucrose in the starch deficient mutants should drop to lower levels compared to wild type at night. This is because sucrose synthesis is supported by Glu-6P from starch degradation at night (Zeeman *et al*., 2007). Lacking starch, which is the case in the mutants leads to insufficient levels of Glu-6P to support sucrose synthesis.

Although the levels of starch in leaves of starch excess and starch deficient mutant are different, both types of mutant still exhibit late flowering phenotype. Since both mutants are defective in starch metabolism, the flux of carbohydrates to the starch pool is possibly interrupted and then there is the accumulation of essential metabolites which could be the floral signal in the chloroplast that cannot be transported outside. This indicates that the delay in flowering is not because of the amount of starch contained in leaf but it might be due to starch mobilization and the ability of the plant to export the sugars or flowering signal from the leaf to the phloem, which then could be used by plant. Therefore, the level of starch in the leaf might not be necessary to indicate whether the plant will flower normally or not. The

important thing might be that carbohydrate metabolism is needed to be a complete cycle, from sugar being converted to starch and starch being degraded to soluble sugars and these sugars being sent out to the cytoplasm to support plant development.

These results are supported by studies that propose that starch mobilization, rather than the level of leaf starch, influences plant development. A study with *pgm* showed that the mutant contains more soluble sugars in the leaf but less sugars in the phloem during the light period compared to the wild type, which indicated that there is a restriction of the transportation of soluble sugars from the leaf to the phloem and it shows that high amounts of sugar in the leaf is not always necessary for the transition to flowering (Corbesier *et al*., 1998). Also, it has been suggested that the late flowering phenotype in the *sex-1* mutant is not due to excess levels of starch in the leaf or the reduction in growth rate, but that it is more likely to do with the ability to mobilize the stored starch for use (Koornneef *et al*., 1998). Moreover, there are several late flowering double mutants, *carbohydrate accumulation mutant1* and *ADP-glucose pyrophosphorylase* (*cam1 adg1*), *carbohydrate accumulation mutant1 and phosphoglucomutase* (*cam1 pgm*), and *gigantea and phosphoglucomutase* (*gi pgm*), which display a late flowering phenotype without abnormal levels of starch in the leaf (Eimert *et al*., 1995).

The study in section 5.3.3 indicates that all types of miR156 species analysed in this study are involved with the phase transition and that miR156C plays the dominant role in this compared to the others. Although using a different method of measuring the JP length, this study shows similar results to Yang *et al*. (2013), which showed that miR156A and miR156C have the major effect on the JP to AVP transition and that miR156 genes are involved with the regulation of the transition from the JP to the AVP by sugar (Yu *et al*., 2013). High levels of miR156 prevent the plant from undergoing the JP to AVP transition, especially when the plants are young and have not enough leaves to produce sugar from photosynthesis (Proveniers, 2013). Sugars play a role in signalling that is transported from leaf to the shoot apex to control the phase transition. Sucrose is transported from source tissues to the sink tissues such as young leaf and the SAM (Zeeman *et al*., 2007). Sucrose is then converted to glucose, which will be able to promote the degradation of primary miR156 A and C transcripts and then cause the reduction of miR156

levels (Proveniers, 2013; Yu *et al*., 2013). The involvement of miR156 in the phase transition and sucrose metabolism emphasises the important of carbohydrate metabolism and sugar transportation in flowering time regulation.

When miR156 early flowering mutants were grown under different LI, the JP, AVP, and flowering time of the plants were altered. This means that LI can still show the effect on the length of JP even though the miR156 gene is defective. The results indicated that miR156 could act downstream of LI in order to control flowering time.

CHAPTER6. RNA SEQUENCING THROUGHTOUT THE DEVELOPMENTAL PHASES BETWEEN HL AND LL IN ANTIRRHINUM

6.1 Introduction

From the previous chapters, the genetic network that controls the transition from JP to AVP is still unclear and it was found that LI has an effect of the length of JP which was greater than that of the photoperiod. Antirrhinum plants grown under reduced LI had an extended JP. In this chapter transcriptome sequencing is introduced in order to obtain a better understanding of the genes being expressed expression during JP under different LIs.

The transcriptome is an entire collection of transcripts present in the cell. It is encoded by genome and transcribed into RNA. Several types of RNA, such as protein-coding mRNAs, small RNAs and non-coding RNAs are produced from one gene, which makes the transcriptome more complex compared to the genome (Wang *et al*., 2009; Bräutigam and Gowik, 2010). Analysing and sequencing the entire RNAs (RNASeq) rather than genomic DNA can provide information about genetic switches, the position and time at which each gene is turned on or off, and the level of expression of RNAs in the cells. This can be linked to differences of the transcriptome during different developmental phases and with different environmental conditions.

In the study of this chapter, Illumina RNASeq was carried out to characterise the transcriptome of antirrhinum grown under different LI to provide the genomic information to support the effect on LI on JP and flowering in plants. Illumina RNASeq (Bennett, 2004; Bentley, 2006; Wang *et al*., 2010) is a sequencing technique involving a process that contains three parts, RNA sequence library preparation, cluster generation, and sequencing. RNA sequence library preparation is the process of preparing the library of single strand cDNA from RNA sample to be amplified in the flowcell by introducing an adapter sequence. Cluster generation is the process of multiplying the DNA copies by loading the prepared library into the flow cell, which is a hollow glass slide that contains the lawn of complementary

primers to the adapter sequence. Cycles of extension, denaturation, and annealing are then performed in the flowcell producing the cluster, each of which contains ~1,000 strands. The reverse strands of DNA are washed away and only the forward strands, which are identical to original DNA template remain. After that the sequencing is performed.

6.2 Materials and Methods

The descriptions are materials and methods specific for this results chapter. For the common materials and methods in more than one result chapter, the descriptions are in chapter 2.

6.2.1 Antirrhinum material

6.2.1.1 *Materials for reference transcriptome sequence (type1)*

Antirrhinum material type1 consisted of three types of materials, cotyledon, AVP, and RP material (figure 6.1). To generate cotyledon material, antirrhinum seeds (section 2.1) were sown cell following the procedure in section 2.2 under SDHL condition (section 2.2). After germination, the above ground cotyledon materials were harvested at ZT14 (light harvest) and ZT20 (dark harvest). These cotelydon materials were used for library1 preparation later.To generate AVP materials, antirrhinum seeds (section 2.1) were sown and wait after plants reached T_0. The seedlings were divided equally to put in SDHL and SDLL cabinets (section 2.2). In both cabinets, after 42 days from germination, 1^{st} leaves and apex were harvested from plants at ZT14. The leaves and apex were kept separate when harvested. A biological replication of each harvest was performed. Materials from SDHL cabinet were used for library 5 and 6 preparation and materials from SDLL cabinet were used for library 3 and 4 preparation.

To generate RP materials, antirrhinum seeds (section 2.1) were sown following the procedure in section 2.2 under SDHL cabinet. After 56 days from germination, 1st leaves and apex were harvested at ZT14 and leaves and apex were kept separate. A biological replication of the harvest was performed.These RP materials were used for library 2 preparation.

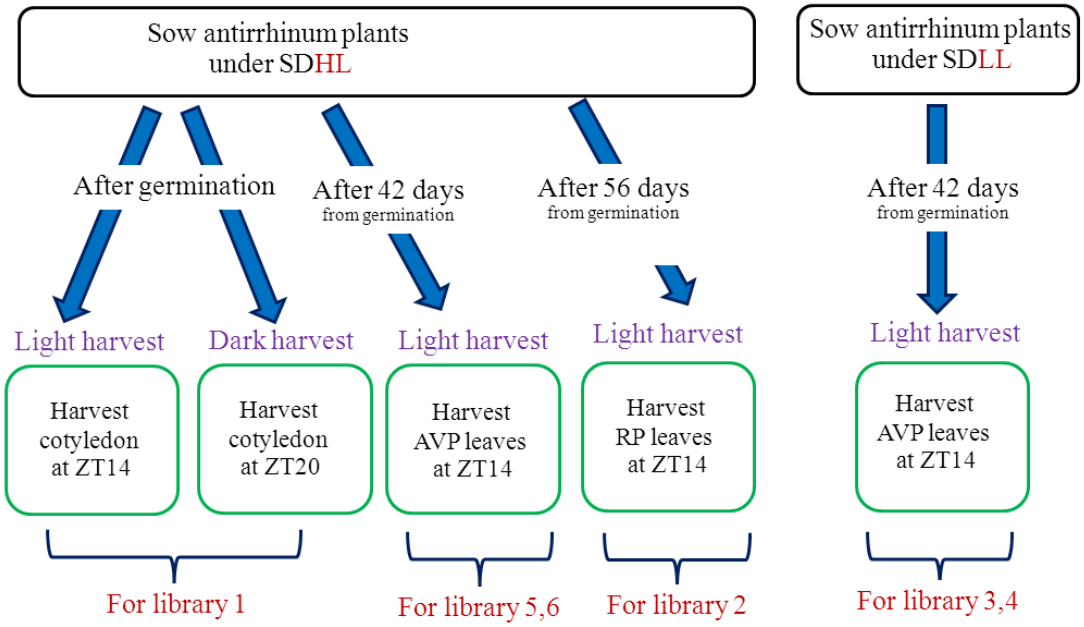


Figure6.1 Schematic representation of the antirrhinum harvesting plan (type1) under different light integrals, developmental phases, and time point to generate leaf materials for RNASeq

Abbreviations: SDHL, short day high light; SDLL, short day low light; AVP, adult vegetative phase; RP, reproductive phase

6.2.1.2 *Materials under different LI (type2)*

Antirrhinum material type2 consists of JP materials grown under LDLL and JP materials grown under LDHL (figure 6.2). To generate both types of materials, antirrhinum seeds (section 2.1) were sown following the procedure in section 2.2. The seedlings were divided equally between SDLL and SDHL cabinets (section 2.2). In both cabinets, after 20 days from germination, 1st leaves and apex were harvested separately at ZT3, ZT14 and ZT20. A biological replication of the harvest was performed. The library preparation plan was shown in figure 6.2.

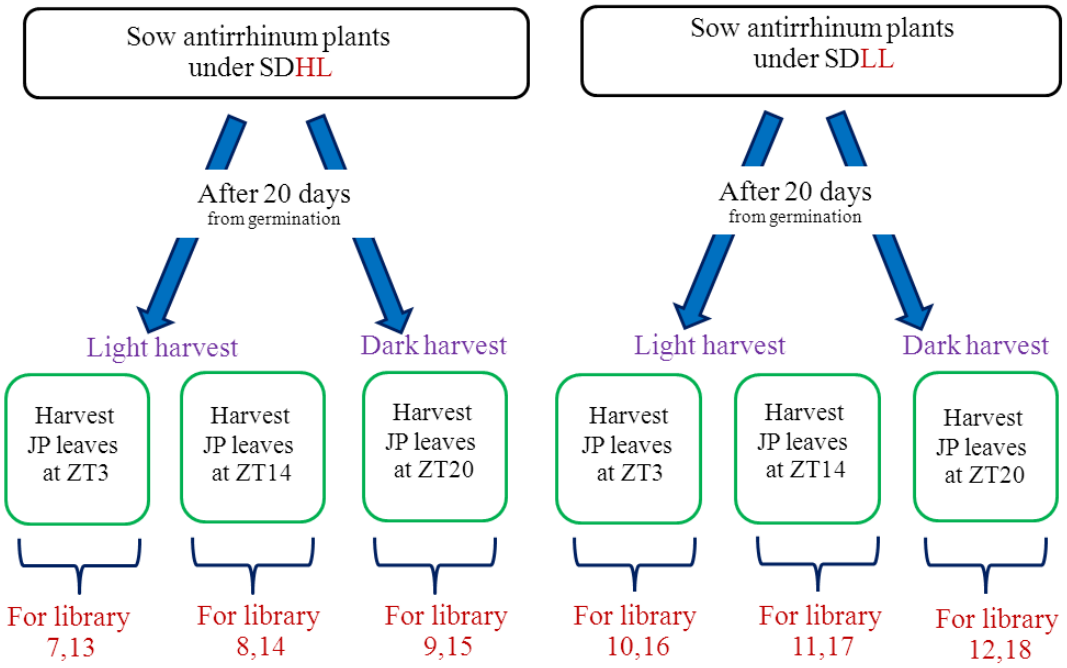


Figure6.2 Schematic representation of the antirrhinum harvesting plan (type2) under different light integrals and time point to generate leaf materials for RNASeq

Abbreviations: SDHL, short day high light; SDLL, short day low light; JP, juvenile phase

6.2.2 RNA Extraction

Total RNA was extracted from all harvested materials in separate tubes as described in section 2.5. After extraction, equal amounts of RNA from leaves and apex of the same sample type was pooled together.

6.2.3 Library preparation, cluster generation, and sequencing

All RNA samples were sent to the Genomics Facility Centre, School of Life Sciences, University of Warwick for library preparation, cluster generation, and sequencing. Different plant materials were generated for the different library (figure 6.1 and 6.2).The summary of library name and the position of all RNA samples loaded in the flowcell is provided in the table 6.1 and figure 6.3.

Lane in flow cell	Library name	Library Detail
lane1	1	Cotyledon of HL plant
	2	RP materials of HL plant
	3	AVP materials of LL plant
	4	Biological replicate of sample in library 3
	5	AVP materials of HL plant
	6	Biological replicate of sample in library 5
lane2	7	JP materials of HL plant at ZT3
	8	JP materials of HL plant at ZT14
	9	JP materials of HL plant at ZT20
	10	JP materials of LL plant at ZT3
	11	JP materials of LL plant at ZT14
	12	JP materials of LL plant at ZT20
Lane3	13	Biological replicate of sample in library 7
	14	Biological replicate of sample in library 8
	15	Biological replicate of sample in library 9
	16	Biological replicate of sample in library 10
	17	Biological replicate of sample in library 11
	18	Biological replicate of sample1 in library 2

Table 6.1 The loading position of all RNA samples in the flowcell.

There were 3 lanes used in flowcell for this study. Each lane consists of 6 libraries. Sequence information from both lanes 1 and 2 were used to generate the reference sequence.Lane 2 contained materials that multiplex LL and HL in JP. Lane3 contained biological replicate of lane2.

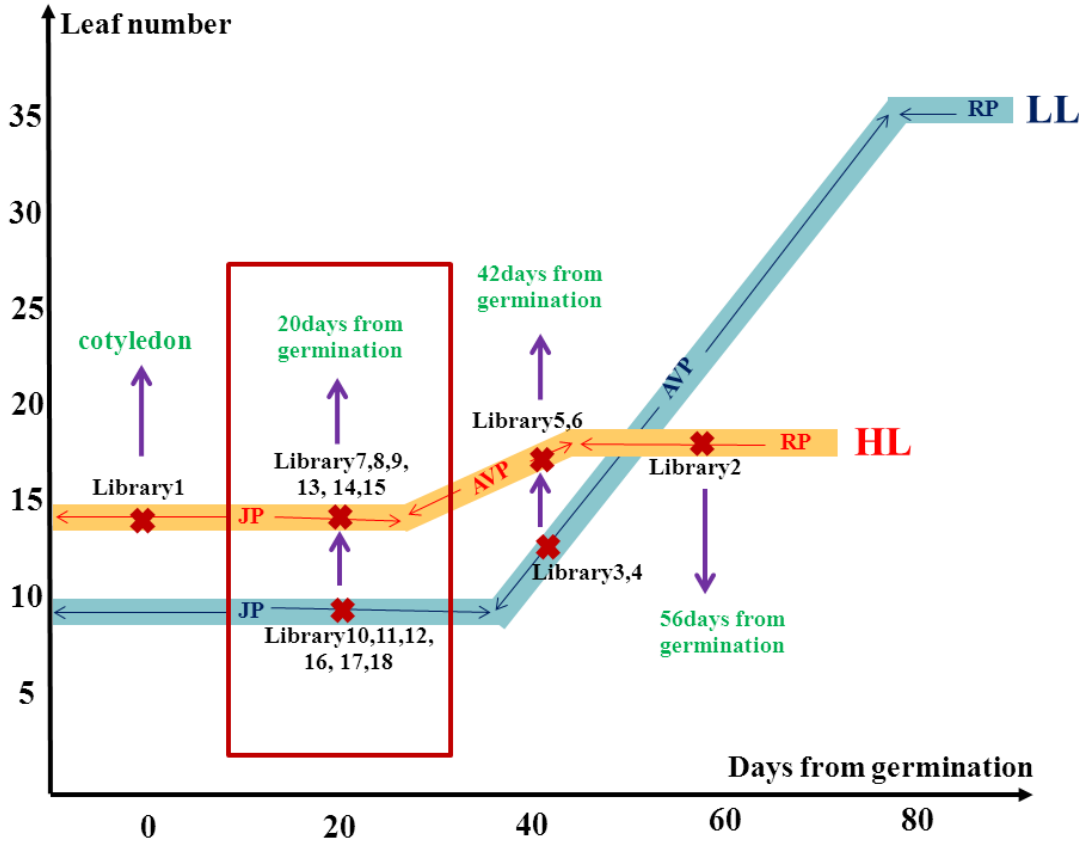


Figure 6.3 The harvesting plan explaining the antirrhinum material used to generate RNA and prepped libraries

The graph shows the developmental phases between LL (blue line) and HL (orange line) grown plants. Red crosses represent the position of each sample harvested.Plants were harvested at the different growth phase and time between high and low light grown plant in order to generate materials for RNAseq. To compare the expression of genes between two light conditions, there were 18 libraries in total (table 6.1).Plants for library1 were HL grown plant in the cotelydon stage.Plants for library2 were HL grown plant in RP (56 days from germination).Plants for library3 to 6 were HL and LL grown plant in AVP (42 days from germination). Plants for library 7 to18 were HL and LL grown plant in JP (20 days from germination).The harvesting time for each library are shown in table 6.1. The data analysis presenting in this chapter is focusing on the difference of gene expressions between HL and LL grown plants when they were in JP (red box).Abbreviations: JP, Juvenile Phase; AVP, Adult Vegetative Phase; HL, high light condition; LL, low light condition

6.2.4 Data analysis

The RNASeq reference transcriptome generation and expression analysis was carried out by Siva Samavedam, School of Life Sciences, University of Warwick using Galaxy Biotinformatics Platform (http://galaxyproject.org/). The plants for RNASeq were harvested at many time points and many developmental phases. This means the comparison could be made between any developmatanl phases and time points. For this chapter, the analysis is only focused on the comparison of the gene expression when plants from both SDHL and SDLL grown plants are at 20 days after germination (figure 6.3). This was the stage when plants from both conditions were in JP. The reason is to support the study of this thesis that emphasising the mechanism that control JP which is related to LI.

6.3 Results

After obtaining the raw sequencing reads, the reads were checked for the quality and cleaned to eliminate the contaminants and low quality read.There are 78,008,906 sequencing reads after cleaning for analysis. The differentially expressed transcripts in JP materials between HL and LL conditions are 6,517 (table 6.2).

Plant materials	**Differentially expressed transcripts between 2 conditions**			
	Over all	**Function in flowering time control**	**Function in carbohydrate metabolism**	**Other functions**
JP HL vs LL at ZT13	2712	9*	12*	2691
JP HL vs LL at ZT14	2769	13*	5*	2751
JP HL vs LL at ZT20	2286	10*	5*	2271

Table 6.2 the summary of number of differentially expressed transcripts detected in each plant condition and time point. *Abbreviations: JP, Juvenile Phase; HL, Plants transferred from SDHL to LDHL; LL, Plants transferred from SDLL to LDHL. The red star indicates the transcript data that is shown in table 6.3.*

The genes that were significantly different between LL and HL condition in JP material are not all listed in the tables below since the perpose of this chapter is only to confirm the study in this thesis which focusing on the involvement of carbohydrate metablisim, light integral, and flowering time. The genes listed are selected based on their function that can be linked to phase transition and flowering. The total numbers of genes that only based on these selected functions are shown in table 6.2 with the red stars behind. The material harvested at early light (ZT3), late light (ZT14), and dark (ZT20) are presented in the table 6.3, 6.4, and 6.5 respectively. Genes involved in carbohydrate metabolism, growth and development,

and other pathways that can be linked to flowering have the significantly different levels in JP plants grown under SDHL or SDLL.

Table 6.3 Differential gene expression in antirrhinum between plants grown under SDHL and SDLL during the JP. Plants were harvested at ZT3. All differences are highly significant (P value < 0.01). Genes involved with carbohydrate metabolism are presented in yellow. Other genes that can be linked to flowering time are presented in pink.

Genbank id	Annotation	Percent Identity	e value	Expression level		PValue
				JPHL	JPLL	
gi|225444357| ref|XP_002266056.1|	PREDICTED: glucose-6-phosphate/phosphate translocator 2, chloroplastic [Vitis vinifera]	82.42	1.00E-174	35.328	0.16	1.51E-38
gi|255582745| ref|XP_002532149.1|	Ribulose bisphosphate carboxylase small chain, chloroplast precursor, putative [*Ricinus communis*]	78.75	3.00E-90	1336.936	296.308	6.69E-15
gi|460377578| ref|XP_004234568.1|	PREDICTED: ribulose bisphosphate carboxylase small chain 2A, chloroplastic [*Solanum lycopersicum*]	77.5	3.00E-86	1336.936	296.308	6.69E-15

Table 6.3 *Continued*

Genbank id	Annotation	Percent Identity	e value	Expression level		PValue
				JPHL	JPLL	
gi|255558824| ref|XP_002520435.1|	glycogen phosphorylase, putative [*Ricinus communis*]	84.87	0	5.386	0	8.12E-12
gi|460373224| ref|XP_004232421.1|	PREDICTED: putative glucose-6-phosphate 1-epimerase-like [*Solanum lycopersicum*]	78.1	1.00E-175	17.65	0.418	1.87E-11
gi|225427786| ref|XP_002269232.1|	PREDICTED: NADP-dependent D-sorbitol-6-phosphate dehydrogenase [*Vitis vinifera*]	74.18	7.00E-166	7.723	0	8.79E-08
gi|255536759| ref|XP_002509446.1|	Auxin-repressed 12.5 kDa protein, putative [*Ricinus communis*]	70.59	7.00E-43	11.13	167.177	8.43E-41
gi|225435571| ref|XP_002285582.1|	PREDICTED: beta-glucosidase 12-like [*Vitis vinifera*]	49.9	6.00E-177	767.466	68.145	7.45E-48
gi|225427786| ref|XP_002269232.1|	PREDICTED: NADP-dependent D-sorbitol-6-phosphate dehydrogenase [*Vitis vinifera*]	74.18	7.00E-166	19.713	1.537	2.09E-12
gi|255556097| ref|XP_002519083.1|	sugar transporter, putative [*Ricinus communis*]	69.63	0	20.363	4.063	4.79E-10
gi|470126259| ref|XP_004299107.1|	PREDICTED: polyol transporter 5-like [*Fragaria vesca* subsp. vesca]	70.26	0	20.363	4.063	4.79E-10

Table 6.3 *Continued*

Genbank id	Annotation	Percent Identity	e value	Expression level		PValue
				JPHL	JPLL	
gi|359486863| ref|XP_002271820.2|	PREDICTED: pullulanase 1, chloroplastic-like [*Vitis vinifera*]	74.02	0	0	3.189	5.01E-07
gi|359490767| ref|XP_003634163.1|	PREDICTED: nascent polypeptide-associated complex subunit alpha-like protein 2-like [*Vitis vinifera*]	84.17	2.00E-62	30.451	0	6.29E-25
gi|334182773| ref|NP_001185066.1|	gibberellin-regulated protein 9 [*Arabidopsis thaliana*]	62.63	2.00E-22	383.294	82.535	1.05E-23
gi|225440230| ref|XP_002283808.1|	PREDICTED: dihydrofolate reductase [*Vitis vinifera*]	79.39	4.00E-131	57.788	7.42	1.58E-20
gi|225435571| ref|XP_002285582.1|	PREDICTED: beta-glucosidase 12-like [*Vitis vinifera*]	58.92	0	54.353	8.2	4.48E-16
gi|460397354| ref|XP_004244232.1|	PREDICTED: protein SPA1-RELATED 3-like [*Solanum lycopersicum*]	55.94	4.00E-75	2.141	14.067	1E-11

Table 6.3 *Continued*

Genbank_id	Annotation	Percent Identity	e value	Expression level		PValue
				JPHL	JPLL	
gi|460369548| ref|XP_004230624.1|	PREDICTED: MADS-box protein SVP-like [*Solanum lycopersicum*]	56.56	8.00E-70	29.086	7.015	1.35E-08
gi|460401057| ref|XP_004246046.1|	PREDICTED: F-box/FBD/LRR-repeat protein At1g13570-like [*Solanum lycopersicum*]	38.22	1.00E-74	1.025	10.495	4.9E-08
gi|460383759| ref|XP_004237585.1|	PREDICTED: probable protein phosphatase 2C 33-like [*Solanum lycopersicum*]	67.62	1.00E-123	0	5.872	6.14E-08
gi|255547560| ref|XP_002514837.1|	protein phosphatase-2c, putative [*Ricinus communis*]	75.29	3.00E-119	0	5.872	6.14E-08

Table 6.4 Differential gene expression in antirrhinum between plants grown under SDHL and SDLL during the JP. Plants were harvested at ZT14. All differences are highly significant (P value < 0.01). Genes involved with carbohydrate metabolism are presented in yellow. Other genes that can be linked to flowering time are presented in pink.

Genbank_id	Annotation	Percent Identity	e value	Expression level		PValue
				JPHL	JPLL	
gi|460397086| ref|XP_004244099.1|	PREDICTED: 1,4-alpha-glucan-branching enzyme 3, chloroplastic/amyloplastic-like [*Solanum lycopersicum*]	75.26	0	1.869	7.546	1.14E-05
gi|225427786| ref|XP_002269232.1|	PREDICTED: NADP-dependent D-sorbitol-6-phosphate dehydrogenase [*Vitis vinifera*]	74.18	7.00E-166	15.021	166.261	1.23E-27
gi|460365453| ref|XP_004228616.1|	PREDICTED: sucrose-phosphatase 1 [*Solanum lycopersicum*]	79.31	0	9.057	0	3.12E-09
gi|460397086| ref|XP_004244099.1|	PREDICTED: 1,4-alpha-glucan-branching enzyme 3, chloroplastic/amyloplastic-like [*Solanum lycopersicum*]	75.26	0	0	4.272	8.61E-08
gi|460373063| ref|XP_004232342.1|	PREDICTED: plastidic glucose transporter 4-like isoform 1 [*Solanum lycopersicum*]	75.64	0	22.326	0.868	2E-07
gi|225461361| ref|XP_002284668.1|	PREDICTED: NAC domain-containing protein 72 [*Vitis vinifera*]	70.75	5.00E-154	18.021	1.238	4.84E-12
gi|350536069| ref|NP_001233972.1|	jasmonic acid 2 [*Solanum lycopersicum*]	67.71	1.00E-150	18.021	1.238	4.84E-12

Table 6.4 *Continued*

Genbank_id	Annotation	Percent Identity	e value	Expression level		PValue
				JPHL	JPLL	
gi|255538956| ref|XP_002510543.1|	cyclophilin, putative [*Ricinus communis*]	80.59	3.00E-86	144.127	27.56	1.05E-11
gi|460407715| ref|XP_004249296.1|	PREDICTED: F-box/kelch-repeat protein At2g44130-like isoform 1 [*Solanum lycopersicum*]	65.33	1.00E-170	19.767	1.779	2.39E-09
gi|470120866| ref|XP_004296508.1|	PREDICTED: protein SPA1-RELATED 3-like [*Fragaria vesca* subsp. vesca]	61.84	0	0	5.223	5.72E-09
gi|470126259| ref|XP_004299107.1|	PREDICTED: polyol transporter 5-like [*Fragaria vesca* subsp. vesca]	70.26	0	19.211	3.902	1.21E-07
gi|460369548| ref|XP_004230624.1|	PREDICTED: MADS-box protein SVP-like [*Solanum lycopersicum*]	56.56	8.00E-70	45.054	8.12	4.18E-13
gi|255550187| ref|XP_002516144.1|	erecta, putative [*Ricinus communis*]	85.36	0	5.193	0	3.4E-12
gi|225429690| ref|XP_002280069.1|	PREDICTED: LRR receptor-like serine/threonine-protein kinase ERECTA [*Vitis vinifera*]	85.65	0	5.193	0	3.4E-12
gi|225470820| ref|XP_002264755.1|	PREDICTED: protein GIGANTEA-like [*Vitis vinifera*]	79.76	0	10.219	1.112	3.41E-12

Table 6.4 *Continued*

Genbank_id	Annotation	Percent Identity	e value	Expression level		PValue
				JPHL	JPLL	
gi|460396369| ref|XP_004243748.1|	PREDICTED: casein kinase I isoform delta-like [*Solanum lycopersicum*]	86.34	0	8.83	0.435	1.29E-06
gi|460406081| ref|XP_004248502.1|	PREDICTED: putative F-box/kelch-repeat protein At1g15680-like [*Solanum lycopersicum*]	33.73	4.00E-29	4.495	0	1.23E-05
gi|255556243| ref|XP_002519156.1|	Gibberellin 20 oxidase, putative [*Ricinus communis*]	72.68	0	13.194	1.878	1.3E-05

Table 6.5 Differential gene expression in antirrhinum between plants grown under SDHL and SDLL during the JP. Plants were harvested at ZT20. All differences are highly significant (P value < 0.01). Genes involved with carbohydrate metabolism are presented in yellow. Other genes that can be linked to flowering time are presented in pink.

Genbank id	Annotation	Percent Identity	e value	Expression level		PValue
				JPHL	JPLL	
gi|502080521| ref|XP_004486591.1|	PREDICTED: ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic-like [*Cicer arietinum*]	75.65	1.00E-160	4.369	0	4.3E-07
gi|358346465| ref|XP_003637288.1|	Ribulose bisphosphate carboxylase/oxygenase activase [*Medicago truncatula*]	81.85	2.00E-160	4.369	0	4.3E-07
gi|460391575| ref|XP_004241396.1|	PREDICTED: inositol transporter 1-like [*Solanum lycopersicum*]	87.68	6.00E-57	4.836	0.049	4.79E-07
gi|225433847| ref|XP_002263742.1|	PREDICTED: probable inositol transporter 1 [*Vitis vinifera*]	86.43	1.00E-56	4.836	0.049	4.79E-07
gi|225435571| ref|XP_002285582.1|	PREDICTED: beta-glucosidase 12-like [*Vitis vinifera*]	52.32	2.00E-162	107.222	10.41	1.86E-25
gi|225453650| ref|XP_002267720.1|	PREDICTED: protein LHY-like [*Vitis vinifera*]	52.68	0	15.15	0	1.07E-24
gi|449493693| ref|XP_004159415.1|	PREDICTED: nitrate transporter 1.4-like [*Cucumis sativus*]	76.48	0	22.203	3.246	4.89E-11
gi|255538956| ref|XP_002510543.1|	cyclophilin, putative [*Ricinus communis*]	80.59	3.00E-86	111.785	22.397	2.59E-07

Table 6.5 *Continued*

Genbank id	Annotation	Percent Identity	e value	Expression level		PValue
				JPHL	JPLL	
gi|460383231| ref|XP_004237334.1|	PREDICTED: putative methyltransferase DDB_G0268948-like [*Solanum lycopersicum*]	73.86	3.00E-148	1.425	17.889	4.88E-07
gi|225459099| ref|XP_002285687.1|	PREDICTED: MADS-box protein SVP [*Vitis vinifera*]	50.67	3.00E-61	68.114	8.44	5.84E-15
gi|460369548| ref|XP_004230624.1|	PREDICTED: MADS-box protein SVP-like [*Solanum lycopersicum*]	56.56	6.00E-70	71.518	9.816	1.86E-13
gi|460407715| ref|XP_004249296.1|	PREDICTED: F-box/kelch-repeat protein At2g44130-like isoform 1 [*Solanum lycopersicum*]	65.33	1.00E-170	16.68	2.22	7.96E-07
gi|356538883| ref|XP_003537930.1|	PREDICTED: flowering time control protein FPA-like [*Glycine max*]	48.06	1.00E-59	3.555	0	1.27E-06
gi|225431962| ref|XP_002278263.1|	PREDICTED: phytochrome B-like [*Vitis vinifera*]	84.83	0	0	2.923	2.4E-06
gi|225453650| ref|XP_002267720.1|	PREDICTED: protein LHY-like [*Vitis vinifera*]	52.68	0	3.791	0	7.32E-06

6.4 Discussion

Genes that have a difference in expression level during JP between HL and LL grown plants are the focus of this study in order to investigate how LI affects JP length. The expression level of the genes listed in the results section that differ between HL and LL grown plants can provide a clearer understanding of the relationship between LI and JP.

6.4.1 *Genes expressed more highly in HL than in LL plants.*

Several genes that encode carbohydrate related proteins were highly expressed in HL compared to LL. The transcriptome sequencing data emphasises that growing plants under LL caused reduced expression of the genes encoding starch synthesis and starch degradation enzymes. These include glucose-6-phosphate/phosphate translocator 2 (Glc6P translocator2) that helps to transport Glc6P into plastids to support starch biosynthesis (Kunz *et al*., 2010) and is necessary for photosynthetic assimilation (Athanasiou *et al*., 2010), Ribulose bisphosphate carboxylase (RuBisCO) which is the enzyme in the Calvin cycle that fixes CO_2 to make glucose using energy generated by the the light reaction (Bassham and Calvin, 2008; Farazdaghi, 2009), 1,4-alpha-glucan-branching enzyme-like isoform 2 (from light harvested plants) involved in starch synthesis (Dumez, 2006), and glycogen phosphorylase and alpha-glucan phosphorylase which are both essential for starch degredation (Rathore *et al*., 2009). Sucrose-phosphatase 1 has SPS as a functional partner and is involved in sucrose synthesis (Huber and Huber, 1996). Although *AmSPS* expression during JP was not different between HL and LL grown plants (section 4.3.2), the expression of sucrose-phosphatase1 indicates that sucrose also plays a role in regulating JP relating to LI. The SPS expression is similar to the results in chapter 4 that shows the effect of LI on starch synthesis and starch breakdown genes.

In addition to starch synthesis and starch degradation, genes that encode enzymes involved in synthesising other photosynthesis products were more highly

expressed in HL than LL grown plants. An example is NADP-dependent D-sorbitol-6-phosphate dehydrogenase, which is the enzyme that synthesizes sorbitol-6-phosphate, an intermediate in the synthesis of sorbitol, which is a major photosynthetic product that can be induced by sucrose (Suzuki and Dandekar, 2014).

The transportation of sugars and thus the levels of sugar transporters, which are the proteins supporting the distribution of sugars throughout the plant may also be important for floral transition (Williams *et al*., 2000). Plastidic glucose transporter, a protein that involved in glucose transportation to cytosol (Weber *et al*., 2000), and polyol transporter 5-like, a protein involved with the transportation of hexoses, pentoses and sugar alcohols (Klepek *et al*., 2005; Reinders *et al*., 2005), were also expressed more highly in HL compared to LL. Other transporter molecules such as the nitrate transporter may also be associated with floral transition like since nitrate can induce flowering (Marín *et al*., 2011). Converting sugar to the energy form that can be used by plants also seems to be essential for phase transition as the gene for glucose-6-phosphate 1-epimerase that encodes an enzyme involved in glycolysis / gluconeogenesis (Wurster and Hess, 1974) has higher expression in HL than LL grown plants.

Interestingly there were high levels of expression of genes that encode hormones or proteins related with hormones, such as beta-glucosidase 12 that provides beta-D-glucose which can activate phytohormones and also is involved in vegetative growth (Kristoffersen *et al*., 2000; Lee *et al*., 2006), jasmonic acid 2, involved in growth and development including flowering (Hsieh *et al*. 2000), gibberellin-regulated protein 9 and Gibberellin 20 oxidase which plays a role in GA biosynthesis (Rieu *et al*., 2008) and gibberellins support floral transition (Corbesier and Coupland, 2005).

Moreover LI affected the expression of several genes reported to be involved with flowering including genes involved directly with the phase transition in plants, such as dihydrofolate reductase which stimulates phase transition in plants (Trovato *et al*., 2001) and cyclophilins that can regulate vegetative phase change (Berardini *et al*., 2001). There were also genes associated with the circadian clock that control photoperiodic flowering (Alabadi *et al*., 2001), including GIGANTEA and F-box/kelch-repeat protein. The At2g44130-like gene mediates the ubiquitination and proteasomal degradation of target proteins and proteins in the F-box family such as

FKF1 that activates CO expression (Imaizumi *et al*., 2005). LHY-like protein is associated with with the regulation of circadian clock which regulates *CO* expression (Mizoguchi *et al*., 2002) and casein kinase I isoform delta-like interacts with CCA1 in the circadian clock and promote early flowering (Ogiso *et al*., 2010).

Erecta, which plays a role in floral development (Torii *et al*., 1996; Douglas *et al*., 2002) and NAC domain-containing protein 72: involved with flower organ formation (Cheng *et al*., 2012) were more highly expressed in HL, which indicates that floral development proteins can be produced during the JP and might contribute to other factors necessary for the phase transition. LI effects on floral formation and developmental- related proteins might possibly lead to the slower development of competence to flower in LL than HL grown plants. Finally, the MADS-box protein SVP-like that downregulates the floral transition in young plant (Gregis *et al*., 2006) is expressed differentially between HL and LL. This is a gene that acts independently of the light and is normally expressed when plants are in JP. The lower level of this gene expression in LL grown plants might imply that LL interrupts the normal autonomous pathway in plants.

6.4.2 *Genes expressed more highly in LL than in HL plants.*

There were also several genes that encode proteins that were expressed more highly in LL compared to HL grown plants. Interestingly, these included genes associated with starch synthesis, e.g. 1,4-alpha-glucan-branching enzyme-like isoform 2 (in dark harvested plants) (Dumez *et al*., 2006) and starch degradation, pullulanase 1(Delatte *et al*., 2006; Streb *et al*., 2008). The mixture of starch synthesis and degradation genes that can be high and low under LL condition supports the idea in chapter 5 suggests that the normal JP length and flowering time is not depend on the level of starch from the starch synthesis and degradation but might dependent on how the carbohydrate cycle is completed.

Genes involved with late flowering proteins were also found to be expressed more highly in LL than HL grown plants. These genes were putative methyltransferase DDB_G0268948-like associated with late flowering phenotype

(Soppe, 2000) and phytochrome B which is the photoreceptor that repress *FT* expression and inhibits flowering (Endo *et al*., 2005). This indicates that LL condition provides proteins that delay flowering in unfavourable conditions. The SPA1 protein is an important factor that forms a complex with COP1 to degrade CO in the dark (Suarez-Lopez *et al*., 2001). More SPA1 protein in LL grown plants might be linked to higher degradation of CO than in HL grown plants and lead to the extension of the JP in LL

In addition, certain genes encoding proteins associated with hormones were more highly expressed in LL grown plants. Auxin-repressed 12.5 kDa is, as its name indicates, a protein that is repressed by auxin. Higher amount of this protein in LL grown plants implies that there is less auxin. Auxin-regulated proteins are involved with initiation of flower primordia at the shoot apex (Yamaguchi *et al*., 2013). Lack of auxin can cause the delay of flower initiation which may contribute to why LL grown plants have a longer JP. Probable protein phosphatase 2C 33-like (PP2C) is involved in Abscisic acid (ABA) signalling (Fuchs *et al*., 2013). ABA inhibits floral transition (Wang *et al*., 2013) which may provide a link between a higher amount of PP2C in LL grown plants and the repression of flowering, contributing to a longer JP.

The transcriptome results indicate that the control of JP via LI is very complex. Regulation may involve not only starch synthesis and starch degradation but also cellular respiration, glycolysis, molecular transport, circadian clock, autonomous pathway, flowering time proteins, and plant hormones. All of these factors are integrated to mediate a response to the environment for the benefit of the plant.

CHAPTER7. GENERAL CONCLUSIONS

7.1 The transition from the juvenile to adult phase in antirrhinum is affected by light integral

The onset of flowering involves the transition from the vegetative to reproductive phase. Plants stop producing leaves and the shoot apical meristem is modified to become the flowering organ. Light is one of the environmental cues that plays a role in regulating floral induction by its duration, quality, and quantity (Thomas, 2006). Light duration is necessary for the photoperiodic response in plants, resulting from the integration of the light signalling and the circadian system. Light quality is involved with light perception which is an important key to regulate flowering time. In *Arabidopsis*, there are two types of critical photoreceptor that coordinate flowering, phytochromes and crytochromes.

The mechanism by which light quantity controls flowering is still not clear. Therefore, the investigation of the effect of LI on JP length and flowering time genes using antirrhinum was carried out in this study in order to illustrate the mechanism by which LI controls JP and flowering time. Antirrhinum is a facultative photoperiodic plant in which flowering time and number of leaves at flowering is reduced when plants are grown under LD (Cremer *et al*., 1998). The life cycle of this plant is long enough to see the effect of environmental factors on the phase transition. Hence, the transfer experiments from SDHL and SDLL to LDHL in antirrhinum was used to generate plant materials that were different in JP length in order to investigate the expression of flowering time genes. The results in section 3.3.1.1 show that the JP length but not early plant development is affected by LI. The JP length and AVP length are longer and flowering time is delayed in plants grown under low LI. Since LI is the amount of PAR received by the plants, the JP length is possibly correlated with photosynthesis and carbohydrate assimilation in plants.

7.2 Light integral affects the expression of flowering genes in antirrhinum which is linked to juvenility.

Antirrhinum materials that differed in JP length were generated from the transfer experiments as mentioned above. The expression of the flowering genes *AmFT* and *AmTEM* was measured in generated antirrhinum materials using Real-time PCR quantification. In the photoperiod control flowering time pathway, FT protein is the florigen that promotes flowering (Corbesier *et al*., 2007). It can be repressed by *TEM* at the transcription level (Castillejo and Pelaz, 2008). Induction of *AmFT* was delayed and the expression level was reduced when plants were grown under SDLL (Section 3.3.1.3). Moreover the decline of *AmTEM* expression was delayed in SDLL grown plants, which indicates that the repression of *AmFT* by *AmTEM* was longer and resulted in the extended JP. These results supports the proposal that *TEM* that regulates JP by the suppression of *FT* (Sgamma *et al*., 2014).

Apart from *AmFT* and *AmTEM*, miR156 is one of the genetic factors that regulates flowering time in plants. Studies of the association between miR156 expression during JP and LI can be linked to the relationship between JP, LI, and carbohydrate metabolism since miR156 level can be repressed by sugar to promote the phase transition from JP to AVP (Yang *et al*., 2013). Normally miR156 level is reduced as the plant ages to allow phase change. The reduction was delayed when plants are grown under SDLL (Alison Jackson, School of Life Sciences, University of Warwick). The extension of JP length together with the delay in *AmFT* induction, delayed *AmTEM* reduction and delayed in miR156 reduction in SDLL grown plants provide evidence to support the relationship between LI, JP, and carbohydrate metabolism.

7.3 Light integral has a greater effect on *AmFT* induction and phase transition than photoperiod.

Under SDLL condition, plants took longer to induce *AmFT* and the level of *AmFT* expression was lower compared to plants grown under SDHL conditions. These results applied to SDLL grown plants that were exposed to one LD before harvesting for molecular analysis. However, only one LD might not be enough to stimulate the full induction and expression of *AmFT*. To clarify how LI affects *AmFT* induction and expression in relation to the number of LDs plants received before transfer from SDs to LDs, six transfer groups, SDLL to LDHL, SDLL to LDLL, SDLL to SDHL, and SDHL to LDHL, SDHL to LDLL and continuous SDHL as controls were carried out. In each group, the first expanded leaves were harvested after each consecutive LD for seven days and the *AmFT* expression was analysed using Real-time PCR from these harvested materials. *AmFT* expression in plants from each transfer group was different, depending on LI that plants were exposed to prior and after transfer. When SDLL grown plants were transferred to either SDHL or LDHL or for plants grown in continuous SDHL, *AmFT* increases as the plants get older. In contrast when SDLL and SDHL grown plants were transferred to LDLL, there was no induction of *AmFT* and for the SDHL treated palnts the expression of *AmFT* was reduced after several LDs. Although *FT* is is normally upregulated at the molecular level by CO protein in LD (Ma, 1998; Reeves and Coupland, 2000), the results from the current study suggested that in antirrhinum, LI has a greater effect on *AmFT* expression than day length.

To confirm the stronger effect of LI on *AmFT* expression compared to photoperiod and to link this with phase transition and flowering time, three transfer experiments, SDLL to LDLL, SDHL to LDLL, and SDLL to SDHL were carried out in order to measure each developmental phase of the antirrhinum plants. The results showed that the JP and AVP length were extended in plants transferred from SDHL to LDLL and SDLL to LDLL compared to SDLL to SDHL. Therefore, these flowering time data are consistent with and emphasise the influence of LI on *AmFT* expression and flowering time.

7.4 High light integral promotes the induction of *AmFT* under short-day condition.

From the experiments discussed above, flowering was induced under SD by HL in antirrhinum. In *Arabidopsis,* which is also a facultative LD plant, there is no induction of *FT* under SDs. To gain a better understanding about how LI promotes flowering under SDHL, quantitative RT- PCR analysis of the diurnal expression of *AmFT* under, LDHL, LDLL, and SDHL was carried out. The study found that in contrast to *Arabidopsis*, in antirrhinum there was some induction of *AmFT* in the photoperiod under SDHL. Also the level of diurnal *AmFT* expression throughout in SDHL grown plants was higher than in the LDLL grown plants. This also supports the stronger effect of LI on *AmFT* expression compared to photoperiod. Moreover, *AmFT* expression for all conditions peaked at the same time in each day which is around ZT14 to ZT16. This confirms that the harvesting used for the *AmFT* analysis in the current study is appropriate.

7.5 Light integral affects the expression of carbohydrate related genes during juvenility

Since HL advances the JP to AVP phase transition and directly affects *AmFT* expression, it was proposed the expression of carbohydrate genes could be linked to a mechanism by which LI controls JP via carbohydrate metabolism. Carbohydrate genes selected for this study were the starch synthesis genes (*PGM* and *PGI*), the starch degradation gene**s** (*SEX-1BAM-3,* and *AMY*), and the sucrose synthesis gene (*SPS*). All of these genes were isolated and cDNA sequences confirmed identified from antirrhinum to enable expression analysis. Full-length sequences of *AmPGI* and *AmPGM* and partial sequences of *AmSEX-1*, *AmSPS,* and *AmAMY* were used to design primers for all carbohydrate gene expression analysis.

Plant materials used for carbohydrate gene expression analysis were in the same batch with those materials used for flowering gene expression analysis as discussed in section 7.2. The expression of all carbohydrate genes was low during

the JP and increased during the AVP. This indicates the importance of carbohydrate metabolism on competence to flower. The expression levels of *AmPGM*, *AmPGI*, *AmSEX-1*, and *AmBAM-3* during the JP were lower in SDLL compared to SDHL grown plants, indicating that LI has an effect on carbohydrate metabolism. The greater the LI received by a leaf, the greater the rate of photosynthesis and the more carbohydrate assimilation is generated. However, the photosynthesis rate can be eventually limited after plants receive excess amount of LI (Nickelsen, 2007). In the current study, SDHL (PAR = 3.6 mol s^{-2} d^{-1}) condition in the growth cabinet was set in a range suitable for plant growth in order to see the effect of higher and lower LI on flowering time. Therefore, even the SDHL condition in this study is still lower than natural sunlight (PAR varies from 5 to 60 mol m^{-2} d^{-1}) (Korczynski *et al*., 2002). Hence, the rate of photosynthesis is not limited when LI is increased because HL condition in the study is not excess. And the results in this study shows that a sufficient and not excess amount of LI acerelates flowering time which relevant to the natural environment and correlate with the study of Dr.Steve Adams, 2007. Dr Adams did the experiment under natural light in green house and found that LL grown plants had longer flowering time compared to that of HL grown plants (personal communication)

LI does not have an effect on the expression of *AmAMY* and *AmSPS* during the JP. This is might be because *AmAMY* and *AmSPS* are not primarily involved in starch synthesis and starch degradation. *AmAMY* is essential for supporting the chloroplast (Zeeman *et al*., 2007) and *AmSPS* is necessary for sucrose synthesis (Geigenberger, 2011).

7.6 Light integral affects the time to accumulate sufficient carbohydrate to support phase transition

To illustrate the relationship between LI, JP, and carbohydrate metabolism, the effect of LI on carbohydrate levels is also needed. The plant materials used for carbohydrate analysis were in the same batch with materials used for flowering gene

and carbohydrate gene expression analysis. The pattern of starch, glucose, fructose, and sucrose levels were similar to the pattern of carbohydrate gene expression mentioned above, being low during the JP and high during the AVP. This emphasized the importance of increased carbohydrate level in the AVP related to flowering. Although the levels of carbohydrate were comparable with SDHL grown plants, plants grown under SDLL took longer to accumulate the same amount of carbohydrate, correlating with later phase change. It is concluded that as LI has an influence on phase transition, the expression of flowering time genes, the expression of important carbohydrate genes and the time to accumulate carbohydrate level to threshold level, carbohydrate metabolism has a role in regulating the length of the JP and flowering time.

7.7 Defective in carbohydrate gene expression in *Arabidopsis thaliana* affects JP length and flowering time gene expression

Using antirrhinum in the study shows that LI has an influence on flowering time and carbohydrate metabolism. To clarify the mechanism of this relationship, the effect of specific carbohydrate genes on phase change and flowering was investigated using *Arabidosis*. The carbohydrate mutants in *Arabidopsis* used to support this study included the starch excess mutants (*sex-1* and *bam-3*) and starch deficient mutants (*pgm* and *adg-1*). Interestingly, both types of mutants have a late flowering phenotype. Transfer experiments from SDHL (8 h of PAR 2.7 mol m^{-2} d^{-1}) to LDHL (8 h of PAR 2.9 mol m^{-2} $d^{-1}$and 8 h of PAR 0.4 mol m^{-2} d^{-1}) condition were carried out using *Arabidopsis* Columbia wild type (Col-0) and carbohydrate mutants in order to estimate the influence of carbohydrate genes on JP length and to generate plant material for molecular and biochemical analysis. The lengths of JP and AVP in carbohydrate mutants were extended compared to wild type, which leads to the late, flowering phenotype. Apart from *sex-1*, all mutants developed at the same rate during JP compared to wild type. The *sex-1* mutant lacks GWD which is an important enzyme that acts upstream of the other starch break down enzymes, which may explain its slower early rate of development. Results from the other three

mutants, however confirm that carbohydrate metabolism affects the phase transition and flowering time in plants.

The effect of defects in carbohydrate gene expression on flowering time gene expression was investigated using the materials from transfer experiment discussed above. In all mutants, the levels of expression of *CO* and *FT* were lower compared to wild type. Moreover, in *bam-3* and *pgm*, the induction of *CO* and *FT* was delayed. Therefore carbohydrate metabolism might regulate the expression and induction of *CO* and *FT*. Unlike *CO* and *FT*, the expression of *TEM* was not affected in the carbohydrate gene mutants. In all of the mutants apart from *sex-1*, low level of *TEM* expression did not cause the elevated *FT* expression level. This may be because the defects in carbohydrate gene expression repressed the expression of *CO*, which is the activator of *FT*. Therefore *FT* expression level remained low and the flowering time delayed in carbohydrate mutants. Therefore carbohydrate metabolism controlled flowering time by affecting *CO* and *FT* expression.

From the current study, the level of starch in the leaf does not seem to be a key factor for the JP to AVP phase transition and flowering time control. Either excess or lack of leaf starch can extend JP or delay flowering. Because both types of mutants are defective in starch metabolism, the transportation of carbohydrates and metabolites needed for flowering process from chloroplast might be obstructed.

7.8 Light integral affects juvenility in all miR156 mutants.

As discussed in section 7.2, LI affected the level of miR156 expression. Based on changes in leaf characteristics during phase transition it has been reported that miR156 species, acting through sugars, regulated the length of plant developmental phases and the JP to AVP transition (Yu *et al*., 2013). The regulation of JP by LI and carbohydrate metabolism can be linked to the relationship between miR156, LI, and flowering time. A set of transfer experiments was performed using different miR156 mutants to investigate the effect of miR156 species on the JP length using the competence to flower rather than leaf characteristics. The experiment also looked at the effect of LI on the expression of different miR156 species. miR156 mutants (35S:MIM156, *miR156G*, *miR156A*, and *miR156C*) and the

wild type (Col-0) were transferred from and SDLL to LDHL. Mutants lacking in miR156 expression exhibited shorter JP, leading to earlier flowering compared to wild type. Among the miR156 species used in the current study, miR156C had the greatest effect on JP. LI affected the JP length in both wild type and all miR156 mutants. The effectiveness of LI on JP in WT with functional miR156 indicates that miR156 might act at downstream of LI in the flowering time control process.

7.9 Future work

Studies on JP are important to the economics and scientific side. JP is necessary for crop scheduling in commercial horticulture in order to manage plant products to meet market requirements. Also JP is involved with the correct timing of the transition to flowering, which is important to plants as it has a strong impact on fitness allowing successful seed set. LI is the main factor used in the current study to investigate the mechanism by which carbohydrate metabolism controls the JP in photoperiod partway. The current study shows that LI has an effect on JP length, flowering genes, miR156 and expression of some of the carbohydrate genes JP. Under low LI, the response of plants to inductive condition seems to be changed and the length of JP and AVP are extended. From the carbohydrate levels and plant growth rate during JP results of this study, this change might be to balance metabolic changes arising from from lack of enough light without disturbing growth resulting in JP, AVP, and flowering time being prolonged. Further study should aim to clarify what is exactly changed or how the way of the response to inductive conditions is applied under low LI. Trehalose phosphate could be introduced in the study to since it is involved with regulating flowering time and real-time PCR can still be the useful method to see the expression of interesting genes under different LI.

Interestingly, LI can have a stronger effect on flowering and the length of the JP than photoperiod. This suggests that LI could be linked to the photoreceptors and proteins in circadian clock-controlled flowering pathway. Further study of the association between LI, and circadian clock proteins such as phytochrome, crytochrome, FKF1 and GI could clarify how LI involves with photoperiod control

flowering time. Real–time PCR can be used to see the difference in gene expression.

This study also emphasises the importance of carbohydrate mobilisation from source to sink tissue on flowering time, rather than the amount of carbohydrate in leaf. Flowering time can be delayed if the appropriate metabolites for the phase transition are not sufficient to supply the flowering meristem. However, more information or study is required to support the importance of carbohydrate mobilisation from source to sink tissue on JP and flowering time in relation to LI. Since more energy and metabolites must be required to generate proteins necessary for flower induction, the demands on carbohydrate mobilisation from source to sink tissue must change during the transition to flowering. Future work could be done to investigate how the source to sink properties of carbohydrate change is associated with JP and flowering time. The effect of LI on the expression of carbohydrate transporter genes could be one of the future experiments.

Moreover there might be several genes related to carbohydrate and flowering time that are expressed differently under different LI condition. Therefore the RNA sequencing or transcriptome study can indicate which genes could be linked to the mechanism. From the RNASeq results, there is a range of candidate genes that would merit further study. These could include Glc6P translocator2, RuBisCO, 1,4-alpha-glucan-branching enzyme-like isoform 2, glycogen phosphorylase, alpha-glucan phosphorylase, Sucrose-phosphatase1, NADP-dependent D-sorbitol-6-phosphate dehydrogenase, sugar transporters, nitrate transporter, enzyme involved in glycolysis / gluconeogenesis, some hormones or proteins related with hormones, dihydrofolate reductase, and cyclophilins. Approaches could involve gene espression studies in Antirrhinum and genetic studies in the Arabidopsis model system.

MicroRNAs especially miR156 and miR172are important factors for JP and flowering time. However there is no clear explanation about the roles of miRNAs that associate with the effect of environmental factors on JP. This study provided a better understanding of how the LI environmental is associated with miR156. It seems that miR156 acts at downstream of LI for flowering time control. Future molecular and genetic studies to investigate the mechanism should be carried out.

7.10 Overall conclusion

The conclusions related to the main project objectives (Section 1.6) are listed below.

7.10.1 *In A. majus*

• To establish a relevant experimental system to investigate the effect of LI on phase development

A repeatable experiment based on transfer of antirrhinum plants from SDHL and SDLL to LDHL was developed. The results showed that LI had an effect on both JP and AVP length in antirrhinum by extending these growth phases.

• To determine the effect of light integral on photosynthetic assimilation in relation to juvenility

The SDHL and SDLL grown antirrhinum leaf materials specific in each growth phase were used for genetics and biochemical analysis. The expressions of carbohydrate genes (*AmPGM*, *AmPGI*, *AmSEX-1*, *AmSPS*, *AmBAM-3*, and *AmAMY*) were different in each growth phase. They were low during JP and high during AVP which then emphasised the importance of carbohydrate metabolism for floral competence. In relation to developmental phases of plants, the expression of all carbohydrate genes were low during the JP and high during the AVP. LI had an effect on the expression levels of all carbohydrate genes during JP apart from *AmSPS* and *AmAMY*. LI did not affect the level of carbohydrates throughout the developmental phases but it affected the time plant used to reach the appropriate carbohydrate levels needed for phase change. SDLL grown plants took longer time to reach these levels compared to SDHL grown plants.

• To determine the effect of light integral on the expression of key genes in relation to juvenility

Using the antirrhinum transfer system, LI was found to affect the expression of flowering genes (*AmFT* and *AmTEM*) and genetic factor associated with flowering time (miR156) in antirrhinum. The level of *AmFT* was lower in SDLL compared to

SDHL grown plants. Lower LI also caused the delay of AmFT induction of, and the decreasing of *AmTEM* and miR156. Moreover, LI had a greater effect on *AmFT* induction, phase transition, and flowering time than photoperiod.

All the evidence in the antirrhinum study supported the impact of LI on JP and carbohydrate metabolism.

7.10.2 *In A. thaliana*

• To determine the influence of specific carbohydrate related genes on JP

From the transfer experiment from SDHL to LDHL using carbohydrate mutants (*pgm*, *agd-1*, *sex-1*, and *bam-3*) and Col-0, JP and AVP were delayed in all mutants compared to wild type. Apart from *SEX-1*, the lack of all carbohydrate gene expression affected JP, AVP, and flowering time is true since all mutants excluding *sex-1* had the same developmental rate compared to wild type during JP. The expression levels of *CO* and *FT* were also affected by the lack of specific carbohydrate genes and the induction of *CO* and *FT* were affected by the mutations in *BAM-3* and *PGM*. In contrast, the expression of *TEM* was not affected by the loss of theses carbohydrate genes, indicating that low expression of *FT* was a consequence of low *CO* expression in the carbohydrate gene mutants. The late flowering phenotype in both starch excess and starch deficient mutants indicates that the level of starch in leaf is not a decisive factor that regulates JP and flowering time. The key factor controlling JP and flowering time might be the ability of carbohydrate to be transported from source to sink tissue to support flowering.

• To determine the effect of miR156 on flowering time

Transfer experiments with *Arabidopsis* miR156 mutants showed that JP length and time to flowering were reduced when the expression of miR156 was impaired. Among the miR156 mutants used for this study, miR165C was the most effective in regulating the JP.

• To determine the effect of miR156 expression of JP as affected by LI

Mutants defective in miR156 expression did not lose the control of JP by LI. The result suggests that LI controls flowering time at a position upstream of miR156.

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Figure A.1 Transfer experiments show the effect of photoperiod and LI on flowering time. *The longer plants are kept in SDs, the more time plants need to produce flower. Also plants grown under SDLL take longer time to flower compared to SDHL. T2 to T14 refer to transfer number. For transfer experiment from SDLL to LDHL (A), each trnasfer was performed once a week. For transfer experiment from SDHL to LDHL (B), each transfer was performed twice a week.*

Gene	Primer Name	Sequence (5'- 3')	Annealing temperature (°C)	Product size (bp)
PGM (AJ80283)	PGM1F PGM1R	GCCTATGCTAAACCAATCTTCGTC TTCCAAAACTTTCTTCCCCACAA	59	451
PGI (AJ806687)	PGI1F PGI1R	CTCCGCTCAAGGTAAGATTCATT TACTGTGGTCCTATTTGCTTCGTC	61	405
SEX-1 (AJ800856)	SEX1F SEX1R	ACAAGCTGGCTGGCCTAAAGAAGT ATCATAAAGGCCAGCACCAGCATA	65	403
BAM (AJ807611)	BAM1F BAM1R	GCACGAGGAGAAAGATGGACCT GGGAATCTCCAAGAACCATTGC	61	411
SPS (AJ802173)	SPS1F SPS1R	CCTTTTGGTCTTACTCTGATTGA TATSTTTTCTCGGCTCTCGCTCTT	59	417
AMY (AJ87150)	AMY1F AMY1R	CAAGGTGAGGAGGCGTAATGAGTG GATGTTCCAAAAAGTAAGTTCC	59	504

Table A.1 Primers used for gene isolation and identification in antirrhinum
All primers were used for standard PCR with the final concentration of 0.5µM.

Gene	Primer Name	Sequence (5'- 3')	Annealing temperature (°C)	Product size (bp)
antirrhinum Elongation factor α (AJ805055)	ANT elf F ANT elf R	GAGTACCCACCTCTTGGACGTT CTGGGGTCTTTCTTCTCAACAC	60	92
Arabidopsis β Tubulin (AY040074)	TUBBY F TUBBY R	TGGCAAGATGAGCACAAAAG AGACCTCGGGGAGCTATG	60	129

Table A.2 Housekeeping gene primers used in the study
All primers were used for standard PCR with the final concentration of 0.5µM.

Primer Name	Sequence (5'-3')	Product size (bp)
3'PGM GSP1 5'PGM GSP1	CTTCGTCGACAAGCTGGGAGGAAGC GCCCAGATCCCGTCTTTCTCACGA	~ 1300 ~1500
3'PGI GSP1 5'PGI GSP1	TCCTGATAATCCTCCGCTCAAGGT A CAATTCCCTGAAGAGCCGCAGCA	~ 1500 ~ 100
3'SEX GSP1 5'SEX GSP1	TGGAACGAGAGGGCTTACTTCAGCA AGAATGGCGGAAGTCGCCATCAA	~ 1000 ~ 4000
3'BAM GSP1 5'BAM GSP1	CACGAGGACACTGACATGGACTGA A CCATTGCCTTGTGGATAGGATGGA	~ 1500 ~ 1000
3'SPS GSP1 5'SPS GSP1	CTACCGTCTGGCGGCAAAGACTAAG TCTCGGCTCTGCCTCTTATCATCA	~ 1600 ~ 2300
3'AMY GSP1 5'AMY GSP1	GGTGAGGAGGCGTAATGAGTGGAAC AGCCTCCTGGGCTGAATGTGCA	~ 2400 ~ 800
3'PGM GSP2 5'PGM GSP2	TTGAACGGGGTGCCTTTAGAAGA ACGCGATCTAAGGCACCACTTGTTGG	~ 1300 ~ 1400
3'PGI GSP2 5'PGI GSP2	AGATCCAGCTGGCATTGATCACC CCTGAAGAGCCGCAGCAAGTAAACCA	~ 1500 ~ 1100
3'SEX GSP2 5'SEX GSP2	TCCAGGTCGTGCGTTGAGTTTTC GGTTTGCTGGGGTAACCCAAAACCTG	~ 700 ~ 4000
3'BAM GSP2 5'BAM GSP2	AAGGAAATCGAATTCGGCACGAG TCGCAACCCAAGGATATGTATTCAGG	~ 1400 ~ 1000
3'SPS GSP2 5'SPS GSP2	CCCATGACGAGCAGTCTGTTGCT CGTCGTCGTCATCATTCCTCAACCAA	~ 1600 ~ 2300
3'AMY GSP2 5'AMY GSP2	GCCTCGCATAGTGCAGAACCTGA GTCGAGGAACCATCCAGGGAAACCTC	~ 2300 ~ 800
GeneRacer™ 3′ GeneRacer™ 5′	GCTGTCAACGATACGCTACGTAACG CGACTGGAGCACGAGGACACTGA	N/A
GeneRacer™ 3′ Nested GeneRacer™ 5′ Nested	CGCTACGTAACGGCATGACAGTG GGACACTGACATGGACTGAAGGAGTA	N/A

Table A.3 Primers used for RACE

The gene specific primers and nested primers contain GSP1 and GSP2 in their name respectively. The annealing temperature for all primers is 70 °C.

Gene	Primer Name	Sequence (5'- 3')	Annealing temperature (°C)	Product size (bp)
AmPGM	PGMFULL_F PGMFULL_R	AATCTTGGTTGGCAAGGATG GCACGGAATGACTCCAAAAT	55	~1672
AmPGI	PGIFULL_F PGIFULL_R	GGCTTTACCAGCACAAGGAG CAGCAAGAACACGCTTTTGA	55	~1379

Table A.4 Primers used for generating full length AmPGM and AmPGI cDNA
All primers were used for standard PCR with the final concentration of 0.5μM.

Organism and Gene	Primer Name	Sequence (5'- 3')	Annealing temperature (°C)	Product size (bp)	Concentration (µM)
antirrhinum *AmPGM* (from gene isolation)	QPGMF QPGMR	AGGGAGCAGCCGACAGTCATC ACGTGCCCCAGCTGTTATTTATCC	60	89	0.4
antirrhinum *AmPGI* (from gene isolation)	QPGIF QPGIR	GGAAACAAAGGGAGCACTGATCAAC CGAAGAACTTCAATGAACGTC ACGA	60	92	0.2
antirrhinum *AmSEX -1* (AJ800856 (EST))	QSEXF QSEXR	TCAAAGTGGAACGAGAGGGCATA GGACAAGGACAGCCATGCAAA	60	82	0.2
antirrhinum *AmBAM-3* (AJ807611 (EST))	QBAMF QBAMR	TGTTTTTAGAGGAAGAACGCCAAT TCCCATGTTTTCCTTGAATCTTCC	60	82	0.1
antirrhinum *AmSPS* (AJ802173 (EST))	QSPSF QSPSR	ACGAAAAATGGTGGCCCAGTCGAT GACTGCTCGTCATGGGGATCAACAA	67	77	0.1
antirrhinum *AmAMY* (AJ787150 (EST))	QSPSF QSPSR	TTTTTCCCACCAAGATGGCCATTGTAA TCCAAAAAGTAAGTTCCGGGCCGTTT	67	86	0.4
antirrhinum *AmFT* (AJ803471 (EST))	QFT ant F QFT ant R	GCCAGAATTTCAACACGAGAGAC GGCAATTGAAGTAGACAGCA	63	78	0.2

Table A.5 Primers used for Real-time PCR quantification

Organism and Gene ***T***	Primer Name	Sequence (5'- 3')	Annealing temperature (°C)	Product size (bp)	Concentration (µM)
antirrhinum *AmTEM* (TEM-LIKE)	AntTEM F AntTEM R	AATCTGAAAGCGGGCGATGTTGTA CCGACCCATTACCATTACTCCTCA	65	100	0.2
antirrhinum Elongation factor α (AJ805055 (EST))	ANT elf F ANT elf R	GAGTACCCACCTCTTGGACGTT CTGGGGTCTTTCTTCTCAACAC	60	92	0.2
antirrhinum Actin (HQ853640)	QACT ant F QACT ant R	TCAGTGGAGGGTCTACCATGTTTCCTG GCTACTGGGAGCCAACGCCGTA	60	92	0.2
Arabidopsis *FT* (NM_105222)	ARAB FT F ARAB FT R	GGCCTTCTCAGGTTCAAAACA TCGGAGGTGAGGGTTGCTA	55	119	0.2
Arabidopsis *CO* (NM_121589)	CO FORq F CO FORq R	GAGAAATCGAAGCCGAGGAGCA TCAGAATGAAGGAACAATCCCATA	61	80	0.2
Arabidopsis *TEM* (NM_102367)	ARAB TEM1 F ARAB TEM1 R	CTGGAACAGCAGTCAAAGTTACGTGT TGATCTCTCGAAACAAACCACATCAC	67	100	0.2
Arabidopsis β Tubulin (AY040074)	TUBBY F TUBBY R	TGGCAAGATGAGCACAAAAG AGACCTCGGGGAGCTATG	60	129	0.4
Arabidopsis Actin2 (BE038458)	ACTINF ACTINR	TGTCGCCATCCAAGCTGTTCTCT GTGAGACACACCATCACCAGAAT	63	85	0.2

Table A.5 Continue

>Antirrhinum majus AmPGM AJ802383 Putative EST
GTGTTTGATTTTTCACTTATCAAGGGCCTTCTTTCAAGACCAGATTTCAGG
TTTATTTTTGATGCGATGCATGCTGTTACTGGTGCCTATGCTAAACCAATC
TTCGTCGACAAGCTGGGAGGAAGCCCAGATTCTATTTTGAACGGGGTGC
CTTTAGAAGATTTTGGACATGGTCATCCAGATCCTAATCTTACATATGCG
AAAGATTTGGTCAATATAATGTATGGAGACAATGGACCGGATTTTGGAG
CTGCAAGTGATGGAGATGGTGATAGAAACATGATTCTTGGGAGAGGGTT
TTTCGTTACACCTTCAGATTCAGTAGCTATCATTGCCGCCAACGCACAAG
AAGCCACCCCATACTTCAGCTCTGGACCAAAGGGTCTAGCTCGATCCATG
CCAACAAGTGGTGCCTTAGATCGCGTGGCTCTCAAGTTAAATCTTCCTTT
TTACGAGGTGCCTACTGGTTGGAAATTCTTTGGTAACTTAATGGATGCAG
GAAAATTGTCAATTTGTGGGGAAGAAAGTTTTGGAACTGGTTCTGACCAC
ATTCGTGAGAAAGACGGGATCTGGGCTGTTTTAGCTTGGTTGTCGATATT
AGCATATCGTAACAAGGACACAAAGCCTGGAGAGAAGCTGGTTTCCGTT
TCAGAT
>Antirrhinum majus AmPGI AJ806687 Putative EST
AGCTTTAGGACCACAGTTTGTTGCGGAGGCATTGGCTCCTGATAATCCTC
CGCTCAAGGTAAGATTCATTGACAACACAGATCCAGCTGGCATTGATCA
CCAAATTGCACAGCTCGGCGACGAGCTGGCAACTACACTTGTCATAGTG
GTTTCCAAGAGTGGAGGTACCCCTGAAACAAGAAATGGTTTACTTGAAG
TTCAGAAAGCCTTCCGTGAAGCTGGCCTGGAGTTTGCTATATCAGGGTGT
TGCTATTACACAAGAAAACTCATTACTTGATAACACCGCACGTATTGAGG
GATGGTTAGCTAGATTTCCTATGTTTGACTGGGTGGGTGGTAGAACATCT
GAAATGTCTGCTGTTGGTTTACTTGCTGCGGCTCTTCAGGGAATTGATAT
AAAAGAAATGCTTGCTGGTGCAGCATTGATGGACGAAGCAAATAGGACC
ACAGTAGTCAGGGATAATC
>Antirrhinum majus Am SEX-1 AJ800856 Putative EST
GGGAACAAGCTGGCTGGCCTAAAGAAGTGTGGGCGTCAAAGTGGAACG
AGAGGGCATACTTCAGCACAAGAAAAGTGAAACTTGATCATGACTGTCT
TTGCATGTCTGTCCTTGTCCAAGAAATAGTAAATGCTGATTATGCATTTG
TTATCCATACCACTAACCCTTCTTCGGGGGATTCATCTGAAATATACGCC
GAGGTGGTCAAGGGACTTGGAGAGACACTGGTGGGAGCATATCCAGGTC
GTGCGTTGAGTTTTCTTTGCAAGAAAAATGACCTCAACTCTCCCCAGGTT
TTGGGTTACCCCAGCAAACCCATAGGCCTTTTTATCAGGCGTTCTATAAT
TTTCAGATCAGATTCAAATGGTGAAGATCTCGAAGGATATGCTGGTGCTG
GCCTTTATGATAGTGTCCCAATGGATGAAGAGGAGGAAGTTGTGCTTGAT
TACACAAATGATGCATTAATTATTGATGGCGACTTCCGCCATTCTGTTCT
CTCTAGTATAGCTCGCGCAGGAATTGCTATTGAGGAACTATATGGATCCG
CACAAGATATCGAAGGTGTGGTTAAGGATGGCAAAATTTATGTTGTCCA
AACTAGACCTCAGGTATGAT

Figure A.2 List of EST sequences of antirrhinum carbohydrate genes

>Antirrhinum majus AmBAM3 AJ807611 Putative EST
CAAAAAGGAAATCGAATTCGGCACGAGCGGCACGAGGAGAAAGATGGA
CCTTTGAAATACAACTGGGAAGGTTATGCTGAGCTCATAAGGATTGTTGA
ATTACTCGGTATGAAGCTTCAAGTTGTCATGTCTTTTCATCAGTGTGGAG
GAAATGTCGGAGACTCTTGCAATATTCCTCTTCCCCCATGGGTGCTTGAA
GAGATTAGCAAAAATCCGGACCTTGTCTATACGGATAAATCAGGTCGGC
GCAACCCTGAATACATATCCTTGGGTTGCGACTCAGTACCTGTTCTTAGA
GGAAGAACGCCAATTCAGGTCTATTCTGACTATATGAGGAGTTTCAGAG
GAAGATTCAAGGAATACATGGGACACGTTATAGTGGACATCCAAGTTGG
GATGGGACCTTGTGGAGAGCTAAGATTTCCATCCTATCCACAAGGCAAT
GGTTCTTGGAGATTCCC
>Antirrhinum majus AmSPS AJ802173 Putative EST
ATGTCCCTGACATCTACCGTCTGGCGGCAAAGACTAAGGGTGTTTTCATA
AATCCAGCTTTCATCGAACCTTTTGGTCTTACTCTGATTGAGGCTGCAGC
ACATGGTTTGCCCATTGTGGCTACGAAAAATGGTGGCCCAGTCGATATAC
ACCGGGTTCTAGACAATGGTCTTCTTGTTGATCCCCATGACGAGCAGTCT
GTTGCTGATGCGCTTCTGAAGCTTGTTGCAGATAAGCATTTGTGGGCGAA
ATGTAGAACAAATGGATTGAAAAATATTCACCTTTTCTCATGGCCAGAGC
ACTGCAAAACCTATCTGTCTAAGATAGCAAGTTGCAAACCCAGACAACC
TCGTTGGTTGAGGAATGATGACGACGACGAAAATTCAGAATCGGATTCA
CCAAGTGACTCCTTGAGAGATATACAAGATATATCTTTGAATTTGAAATT
CTCTTTTGATGATAAGAGCGAGAGCCGAGAAAATATAGAGGGTTCTTTA
GATTCTGAAGACAGAAAAAGTAAGCTAGAAAACGCTATGTTGACATGGT
CTAAAAGTATGGGAAAAAACATACAGAAATCTGTGTCTATCGATAGAGC
>Antirrhinum majus AmAMY AJ806506 Putative EST
CGGCACGAGTTTTTCTATCTTACTATAACTAAGTTGAACTAAGGAAATAG
TTGTCTGCTCTTTGTTTTACCAGAGAGTTCGGAATGTTGCTTTGTGGCAAC
TGAAGTACTAGTTATATGAAATGCACAGATCAAGGTTGCCTTTTTCTCTG
AATTTTCAGGTTCAGCACTTGACAAACGATATCACAGTAATTTTGGAGGC
CTTGAGATATTCAACCGTTGTGGAAGTACAGGGTGACAAGGTGAGGAGG
CGTAATGAGTGGAACAGATGGCTTCCAGCTGCTGATTCGCGAAACAACA
ATTCTGCCTCGCATAGTGCAGAACCTGAAAATTCCCTGGCAACATCTTTA
CAGGAGGTTTCCCTGGATGGTTCCTCGACTATCGCAAATGGCGATATGGA
GATAAAAGAAGATCGCACGGAGTTGGCTACAAATAGCTCCATGCCTGAG
GAGTTGATTGGTCAGTCAAAGTTGGTTAATGGTGAAGACACTGCTGAAG
AAAGTCTTCCATCTGGAGTATGATAATTATATCATGAAGAAAGTTTTGCA
CATTCAGCCCAGGAGGCTGCTGGAGGAATTCAAAGATAATAACTATTGT
AAGTTATCGAAAAAGGAAGATTCTTTGAAGAGAATACAAGAACTGACTT
AAATTTTTTTCCCACCAAGATGGCCATTGTAATTGCTACGTGGACGAACG
GTGGTTTTTTGTATTAAACGGTCCGGAACTTACTTTTTGGAACATCCAGG
GGAA

Figure A.2 Continue

>Antirrhinum majus AmPGM
GTGTCCGCCGTGATTAGAAAGAGAAAGGCTAATGGTGGTTTTGTAATGA
GTGCCAGTCACAACCCTGGTGGTCCTGATTATGATTGGGGTATCAAGTTC
AACTACAGCAGTGGCCAACCTGCACCTGAGTCTATAACTGACAAGATCT
ATGGAAACACTCTATCTATCAATGAAATAAAGATGGCAGATATTCCTGAT
GTTGATCTCGCTAGTGTTGGTGTAACGGACTATGGAACTTTTGCTGTAGA
GGTAGTTGACCCAGTGGCGGATTATTTGGAGCTTATGGAGAGTGTGTTTG
ATTTTTCACTTATCAAGGGCCTTCTTTCAAGACCAGATTTCAGGTTTATTT
TTGATGCGATGCATGCTGTTACTGGTGCCTATGCTAAACCAATCTTCGTC
GACCAAGCTGGGAGGAAGCCCAGATTCTATTTTGAACGGGGTGCCTTTA
GAAGATTTTGGACATGGTCATCCAGATCCTAATCTTACATATGCGAAAGA
TTTGGTCAATATAATGTATGGAGACAATGGACCGGATTTTGGAGCTGCAA
GTGATGGAGATGGTGATAGAAACATGATTCTTGGGAGAGGGTTTTTCGTT
ACACCTTCAGATTCAGTAGCTATCATTGCCGCCAACGCACAAGAAGCCAT
CCCATACTTCAGCTCTGGACCAAAGGGTCTAGCTCGATCCATGCCAACAA
GTGGTGCCTTAGATCGCGTGGCTCTCAAGTTAAATCTTCCTTTTTACGAG
GTGCCTACTGGTTGGAAATTCTTTGGTAACTTAATGGATGCAGGAAAATT
ATCAATTTGTGGGGAAGAAAGTTTTGGAACTGGTTCTGACCACATTCGTG
AGAAAGACGGGATCTGGGCTGTTTTAGCTTGGTTGTCAATATTAGCATAT
CGTAACAAGGACACAAAGCCTGGAGAGAAGCTGGTTTCCGTTTCAGATG
TCGTGACTGAATATTGGGCCACTTATGGGAGGAATTTCTTTTCCAGATAC
GACTATGAGGAGTGTGAATCAGAAGGGGCGAATAAAATGATAGAATATC
TTAGAGATCTAATTTCTAAGAGCAAGGCCGGTGATGCCTATGGAAGCTAT
GTTCTTCAATTTGCCGATGATTTCAACTACACTGACCCAGTGGAATGGGA
ATTGTGGCTTCGAAGCAAGGTGTTCGTTTTGTTTTCACTGATGGATCGAG
GATCATATTTAGATTATCGGGTACGGGTTCTGCTGGTGCAACTGTTCGGA
TATATATCGAACAATTTGAGCCAGATGTAACTAAACACGACGTGGATGC
ACAAATAGCATTAAAACCTCTAATAGATTTGGCACTGTCAACTTCGAGAC
TGAAGGAATTTACCGGAAGGGAGCAGCCGACAGTCATCACATAGAGTCA
TAGACAAAGTTTCCAACCATTAAGTTTGTGACGGGATAAATAACAGCTG
GGGCACGTTCGGCTTTTCATCGTAGAATCCTCTAAGCGCTTTATAATAAT
GTCATTAGTTTAATTTATGCATGAAAACCGATTGCGATTGTTGGAAAGTG
AATAGCGTTTCCCAGTGGTCAAAAAGTCCGGTCATGAATGGACT

Figure A.3 Full-length sequences of AmPGM

>Antirrhinum majus AmPGI
AGTCGGGTCGGGTTCACGGACGAGTTCTTCAAGGCAATGGAGCCCCGAT
TGCAGGACGCTTTTAAGGACATGGAGGAGCTGGAGAAGGGCGCTATTGC
TAACCCTGACGAGGGACGGATGGTGGGTCACTATTGGTTGAGAGCTCCT
CACCGTGCCCCCAAGCCCATCTTGACCAACCAGATTCAGGATACACTGCT
CAGTATCTCTCACTTTGCCCACCAAGTCATCACCGCTAAGATTAAGCCCC
CGCAAAAGGAGCGCTTTACACAAATTCTTTCAATTGGAATTGGTGGTTCA
GCTTTAGGACCACAGTTTGTTGCGGAGGCATTGGCTCCTGATAATCCTCC
GCTCAAGGTAAGATTCATTGACAACACAGATCCAGCTGGCATTGATCAC
CAAATTGCACAGCTCGGCGACGAGCTGGCAACTACACTTGTCATAGTGG
TTTCCAAGAGTGGAGGTACCCCTGAAACAAGAAATGGTTTACTTGAAGTT
CAGAAAGCCTTCCGTGAAGCTGGCCTGGAGTTTGCGAAACAGGGTGTTG
CTATTACACAAGAAAACTCATTACTTGATAACACCGCACGTATTGAGGG
ATGGTTAGCTAGATTTCCTATGTTTGACTGGGTGGGTGGTAGAACATCTG
AAATGTCTGCTGTTGGTTTACTTGCTGCGGCTCTTCAGGGAATTGATATA
AAAGAAATGCTTGCTGGTGCAGCATTGATGGACGAAGCAAATAGGACCA
CAGTAGTCAGGGATAATCCAGCGGCGCTGCTAGCTTTATGTTGGTATTGG
TGCACTGATGGAGTAGGATCAAAGGATATGGTTGTCCTTCCTTACAAGGA
CAGTCTGCTGTTGTTCAGTCGGTACTTGCAGCAGCTAGTCATGGAATCTC
TTGGAAAAGAGTTTGACCTGGATGGTAACAGGGTAAATCAAGGTATAAC
TGTTTATGGAAACAAAGGGAGCACTGATCAACATGCCTACATTCAACAG
CTTAGAGATGGTGTCCATAATTTTTTCGTGACGTTCATTGAAGTTCTTCGA
GATAGGCCACCTGGTCATGATTGGGAGCTTGAGCCAGGTGTTACCAGTG
GTGACTACCTGTTTGGATTTTTACAGGGAACAAGATCCGCTTTGTATTCC
AATGAGCGGGAGTCTATTACAGTGACTGTGCAAGAGGTGACGCCTAGAT
CTGTTGGGGCCTTAGTAGCACTTTACGAGCGGGCAGTAGGGATTTATGCC
TCACTTGTCAATATCAATGCCTATCNATCAACCTGGGGTGGAA

Figure A.4 Full-length sequences of AmPGI