Genetic and Physiological Analysis of Juvenility in Plants

by

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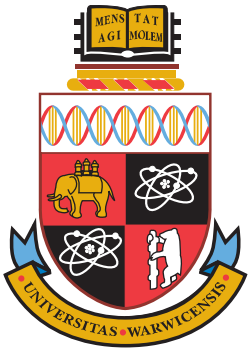

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In loving memory of my father
George K. Matsoukas

Declaration

I hereby declare that this PhD thesis is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at University of Warwick, or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the project by others, with whom I have worked at University of Warwick, is explicitly acknowledged in the thesis.

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Abstract

One of the distinguishable plant developmental events is the transition from the vegetative to reproductive phase (RP) of development. This stage is preceded by the juvenile to adult transition within the vegetative phase. During the juvenile vegetative phase (JVP) plants are incompetent to initiate reproductive development and are effectively insensitive to photoperiod. With the change to the adult vegetative phase (AVP), plants attain competence to respond to floral inducers, which is required for the transition to the RP.

This study exploits *Antirrhinum* and *Arabidopsis* as model systems to understand the genetic and environmental factors that regulate floral incompetence during the JVP. Determinants such as irradiance and [CO_2] were found to be key modifiers of the JVP. A relationship between photosynthetic assimilate levels and vegetative phase transition was revealed by analysis of carbohydrates in plants at defined developmental stages. Experimental data suggest that carbohydrate levels may be required to reach a specific threshold before plants undergo the transition from a juvenile to an adult phase of vegetative growth. This may be necessary in order to sustain a steady supply of sugars for sufficient bulk flow from the leaves to the shoot apical meristem (SAM), via the phloem, to enable delivery of florigen, which thus renders the SAM competent to flower. Determination of the JVP in *Arabidopsis* mutants impaired in different genetic pathways has shown that multiple inputs influence the timing of the vegetative phase transition. Carbohydrates have been demonstrated to be involved possibly through their function as nutrients or signals or by their interaction with hormones. Physiological analysis of flowering time mutants has shown that a variety of signals act to promote and enable the juvenile to adult phase transition that involves both floral activators and repressors.

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The following nomenclature will be used in this thesis:

Names of genes are written in italicised upper-case letters, e.g. *BAM-3*.

Names of proteins are written in non-italicised upper-case letters, e.g. BAM-3.

Names of mutants are written in italicised lower-case letters, e.g. *bam-3*.

Abbreviations and Acronyms

ACN Acetonitrile

ATP Adenosine-5'-triphosphate

APS ADP-glucose pyrophosphorylase

AVP Adult vegetative phase

AGP Alpha-D-glucose-1-phosphate adenyl transferase

Ara Arabinose

Arb Arabitol

bHLH basic helix loop helix

CO2 Carbon dioxide

CIN Cell wall invertase

Col-0 Columbia-0

cDNA complementary Deoxyribonucleic acid

CE Controlled environment

DNP Day neutral plant

DBE Debranching enzyme

DNA Deoxyribonucleic acid

DEB Diepoxybutane

Dragon DB Dragon database

DW Dry weight

Ery Erythritol

EtOH Ethanol

EMS Ethylmethanesulfonate

EMBL European molecular biology laboratory

EST Expressed sequence tag

FI Facultative irradiance response

FLDP Facultative long day plant

FSDP Facultative short day plant

FMI Floral meristem identity

FPI Floral pathway integrators

fru Fructose

Glt Galactitol

Gal Galactose

GA Gibberellic acid

GH Glasshouse

GWD Glucan water dikinase

Gin Glucose insensitive

Glc Glucose

GFP Green fluorescent protein

GMT Greenwich mean time

HXK Hexokinase

HPLC High performance liquid chromatography

II Irradiance indifferent

JVP Juvenile vegetative phase

Ler Landsberg erecta

LA Leaf area

LI Light integral

LOV Light, oxygen or voltage

LCMS Liquid chromatography mass spectrometry

LDP Long day plant

LD Long day

Mlt Maltitol

Mal Maltose

Mtl Mannitol

m/z Mass to charge ratio

miRNA microRNA

MST Monosaccharide transporter

mRNA messenger RNA

MS Murashige and Skoog

MI Myo-inositol

nRIU nano Refractive index units

NO Nitric oxide

PWD Phosphoglucan water dikinase

PPFD Photosynthetic photon flux density

PAR Photosynthetically-active radiation

RIL Recombinant inbred line

RID Refracted index detector

RH Relative humidity

RP Reproductive phase

Rha Rhamnose

Rbt Ribitol

RNA Ribonucleic acid

Rib Ribose

Rubisco Ribulose bisphosphate carboxylase

RNAi RNA interference

SAM Shoot apical meristem

SDP Short day plant

SD Short day

SNP Single nucleotide polymorphisms

SnRK Snf-related protein kinase

Sbl Sorbitol

SLA Specific leaf area

SEM Standard error of mean

Snf1 Sucrose non-fermenting1

SUS Sucrose synthases

SUC Sucrose transporter

SUT Sucrose transporter

Suc Sucrose

SURE Sugar responsive element

TILLING Targetting induced local lesions in genomes

T-DNA Transferred DNA

UVA Ultraviolet A

UVB Ultraviolet B

vpm Volume per million

Ws-4 Wassilewskija-4

WT Wild type

Xyl Xylitol

Xl Xylose

ZT Zeitgeber

Chapter 1

Introduction

1.1 The Juvenile Phase in Plants

Plant development between seedling emergence and flowering is characterized by a series of successive qualitative phases: (I) a post embryonic photoperiod-insensitive phase (JVP), during which plants are insensitive to photoperiod; (II) a photoperiod-sensitive inductive phase (AVP), in which plants require a number of short day (SD) or long day (LD) inductive cycles, depending on their age for rapid flowering, and (III) a photoperiod-insensitive post-inductive phase, in which plant development is no longer influenced by photoperiod.

The early phase of development during which the plants can not be induced to flower and are effectively insensitive to environmental influences of photoperiod and/or vernalisation has been called the juvenile phase (Thomas and Vince-Prue, 1984; Massiah, 2007; Jackson, 2009). This period differs from plant to plant from a period of a few days, for small herbaceous annual plants, through to periods that may last longer than 20 years (Hackett, 1985; Thomas and Vince-Prue, 1984, 1997; Meilan, 1997). From a physio-ecological perspective, by having a juvenile phase, plant species avoid the low seed yields that would occur if they were to flower precociously while still small and with limited photosynthetic capacity (Thomas and Vince-Prue, 1997).

1.2 The Importance of Juvenile Phase Studies

Juvenility has significant scientific and economic implications in plant biology. It has long attracted interest as an aspect of the fundamental topic of aging and also has practical implications, especially in the growth and development of those species in which it is striking and prolonged.

From a commercial perspective, understanding the timing and duration of JVP length is critical for scheduling in commercial horticulture and arable crops. Recently, emphasis on scheduling flowering has been greatly increased. This is driven

primarily by mass marketer demands for product consistency. In addition, increased price pressures have led plant scientists to explore molecular and physiological methods to hasten flowering, in order to reduce production costs. Knowledge of how photoperiod and/or irradiance influence flowering and JVP length could help with crop scheduling in commercial horticulture, decrease time to flowering and reduce waste with resulting benefits for the environment through lower inputs and energy required per unit of marketable product.

Furthermore, in many countries fast-growing tree species are being increasingly used for pulp and bioenergy production. In such cases, it may be equally important to explore molecular and physiological methods to prevent flowering and prolong JVP length. Moreover, the long JVP length of some species is one of several features limiting efficient breeding programs. Therefore, improving our knowledge of the ways by which species with long JVP length regulate their flowering time, it is of great interest, providing approaches to create early flowering phenotypes.

1.3 The Juvenile to Adult Phase Transition

1.3.1 Physiological Markers of Juvenility

The transition from juvenile to the adult phase of plant development has been associated with several morphological and physiological markers (Table 1.1; Telfer *et al.*, 1997; Jones, 1999; Bollman *et al.*, 2003; Brunner and Nilsson, 2004). However, these changes are often less distinct in herbaceous than in woody species. Furthermore, the association of these characteristics with juvenility varies from species to species and in many cases no clear association exists (Jones, 1999; Brunner and Nilsson, 2004). However, the end of the JVP can be identified by the beginning of floral induction. In this thesis floral competence is the major criterion to distinguish between the juvenile and adult vegetative phases of plant development.

Table 1.1: Comparison of Juvenile and Adult Characteristics in Plants

Character	Juvenile	Adult	Species	Reference
Shoot Apical Meristem				
Size	Small	Large	*Chrysanthemum morifolium*	Cockshull, 1985
Cell division	Active	Inactive	*Oryza spp.*	Itoh *et al.*, 2005
Leaf				
Size	Small	Large	*Typha spp.*	Grace, 1985
Shape	Round	Slender	*Zea mays*	Sylvester *et al.*, 2001
Phylotaxis	Alternate	Spiral	*Hedera helix*	Poethig, 1990
Trichomes	Adaxial	Adaxial-abaxial	*Arabidopsis*	Telfer *et al.*, 1997
Midrib	Absent	Present	*Oryza spp.*	Itoh *et al.*, 2005
Plastochron	Short	Long	*Oryza spp.*	Itoh *et al.*, 1998
Photosynthetic rate	Low	High	*Oryza spp.*	Itoh *et al.*, 2005
Response to photoperiod	Insensitive	Sensitive	*Antirrhinum spp.*	Matsoukas *et al.*, 2009
Stem				
Node	Absent	Present	*Carica papaya*	Hackett, 1985
Axillary bud	Growing	Dormant	*Zea mays*	Poethig, 1990
Orientation of vascular bundle	Irregular	Regular	*Oryza spp.*	Itoh *et al.*, 2005
Adventitious root	Many	Few or none	*Hedera helix*	Poethig, 1990
Whole Plant				
Trophism	Heterotrophic	Autotrophic	*Oryza spp.*	Itoh *et al.*, 2005
Disease resistance	Weak	Strong	*Oryza spp.*	Itoh *et al.*, 2005
Growth habit	Plagiotropic	Orthotropic	*Hedera helix*	Poethig, 1990
Pigmentation	Anthocyanin present	Anthocyanin absent	*Hedera helix*	Poethig, 1990
Flowering	Absent	Present	*Antirrhinum spp.*	Adams *et al.*, 2003

Physiological studies have shown that the juvenile to adult phase transition is a gradual and continuous process (Zimmerman *et al.*, 1985; Meilan, 1997). However, the length of the JVP is genetically controlled (Bradley *et al.*, 1997; Telfer *et al.*, 1997; Telfer and Poethig, 1998) and as with most genetic characters, there are interactions with the environment (Bohlenius *et al.*, 2006; Eriksson *et al.*, 2006; Hsu *et al.*, 2006). In general, conditions that promote vegetative growth have been found to hasten the end of the JVP; increased light integral (LI) [the product of photosynthetic photon flux density (PPFD) and photoperiod] and temperature have been found to shorten the length of the juvenile phase in a number of herbaceous species (Adams *et al.*, 1999; Adams and Jackson, 2004). Similarly, in tree species such as *Malus* x *domestica* Borkh., seedlings were induced to flower 16-20 months after germination, under optimum continuous growth conditions; otherwise 3-8 years was needed in the field (Zimmerman, 1971).

1.3.2 Biochemical Influence

Several biochemical markers can be employed to distinguish between juvenile and adult developmental phase in certain plants. For example, differences in peroxidase and esterase isozymes (Brand and Lineberger, 1992) and in protein phosphorylation (Huang *et al.*, 2000). Furthermore, the phytohormones auxin (De Zeeuw and Leopold, 1955), abscisic acid (Rogler and Hackett, 1975), cytokinin (Mullins *et al.*, 1979; Bouriquet *et al.*, 1985) and ethylene (Huang *et al.*, 2000) have been demonstrated to be intimately involved in phase change. In addition, gibberellic acid (GA) has promotional and repressive effects depending on plant species (Wilson *et al.*, 1992; Chien and Sussex, 1996; Telfer *et al.*, 1997; Telfer and Poethig, 1998). In *Arabidopsis,* GA mutations that affect GA biosynthesis (*ga1-3*, *ga4-1* and *ga5-1*) and GA sensitivity (*spindly-4*; *spy-4*) lengthen and shorten the vegetative phases, respectively (Telfer *et al.*, 1997).

1.3.3 Genetic Influence

Just as little is known about the physiological factors affecting the length of the juvenile phase, very little is known about the molecular mechanisms involved in this process. In *Zea mays*, several dominant mutations have been identified that affect the juvenile-adult phase change, such as *teopod1-3* (Dudley and Poethig, 1993) and *glossy15* (Moose and Sisco, 1994). Many of these show ectopic expression of specific juvenile traits in the adult phase. Furthermore, a recessive mutation has been identified in *Oryza sativa* that prevents the plant from entering the adult phase and maintains it in the JVP. The *mori-1* mutation is the only mutation to-date where the juvenile to adult phase transition is completely blocked (Asai *et al.*, 2002) and this indicates that *MORI-1* plays a central role in this phase change, by either initiating the adult phase or terminating the JVP. However, in *Arabidopsis* the appearance of trichomes marks the transition between juvenile and adult vegetative phase. Leaves of plants in JVP produce trichomes only on the adaxial surface, whereas leaves of plants in AVP produce trichomes on both the adaxial and abaxial surfaces. According to the appearance of trichomes, the autonomous pathway (Chapter 1.5.2.3) may be involved in controlling the length of a juvenile phase as certain autonomous pathway mutants such as *fpa-1* and *fve* display an extended JVP (Telfer *et al.*, 1997).

1.3.3.1 Post-Transcriptional Gene Silencing and Juvenility

Recent studies indicate that microRNA (miRNAs) and other endogenous small RNAs regulate transcription factors involved in organ morphogenesis and plant development. Several miRNAs have been shown to affect the JVP length in respect to leaf traits (Schwab *et al.*, 2005; Wu *et al.*, 2009). Studies on the juvenile to adult phase change have revealed a role for miR156 in prolonging the JVP length of *Arabidopsis* (Wu and Poethig, 2006; Chuck *et al.*, 2007). In addition, expression of another microRNA, miR172 hasten the JVP length by repressing AP2-like re-

pressors of FT (Aukerman and Sakai, 2003; Mathieu *et al.*, 2007; Jung *et al.*, 2007). It has been shown that the levels of both miRNAs exhibit contrasting expression patterns (Chuck *et al.*, 2007; Wu *et al.*, 2009). However, miR172 exhibits a similar temporal expression pattern in *Zea mays*, where it targets *GLOSSY15*, a gene required for the expression of juvenile epidermal traits (Lauter *et al.*, 2005).

Interestingly, a new category of mutations in *Arabidopsis* with precocious onset of adult traits has been characterized. These genes include *ZIPPY* (*ZIP/ ARGONAUTE-7*), which encodes an AGO-family protein (Hunter *et al.*, 2003) and SERRATE, a zinc-finger protein that has recently been indicated to be required for the production of miRNA (Clarke *et al.*, 1999; Grigg *et al.*, 2005). Mutations in the *Arabidopsis SQUINT* (*SQN*) gene reduce the number of juvenile leaves produced and thus cause a shortening of the JVP length (Berardini *et al.*, 2001) that is associated with a decrease in the activity of miR156 and an increase in the expression of its *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) gene targets. *SQN* gene encodes a cyclophilin 40, and a double mutant analysis suggests that it acts in parallel to *HASTY* (*HST*; Bollman *et al.*, 2003). The *HST* gene is proposed to promote a juvenile pattern of vegetative development by acting as the miR156 export receptor (Telfer *et al.*, 1997; Telfer and Poethig, 1998; Wu and Poethig, 2006; Chuck *et al.*, 2007). The mutation accelerates the appearance of adult characteristics such as abaxial trichomes on the leaves. However, *hst* mutants display a number of other developmental defects such as a delay of the transition to flowering, but only in nonpromotive photoperiods (Bollman *et al.*, 2003). Plants defective in *RNA-DEPENDENT RNA POLYMERASE-6* (*RDR-6*) and *SUPPRESSOR OF GENE SILENCING-3* (*SGS-3*), which were originally identified on the basis of their role in posttranscriptional gene silencing, also have an early onset of the adult phase and operate in the same pathway(s) as *ZIP* and *HST* (Peragine *et al.*, 2004).

1.4 Systems for Physiological and Molecular Studies of Juvenility

1.4.1 *Antirrhinum majus*

Antirrhinum majus (hereafter referred as *Antirrhinum*) is a native to the Mediterranean region, particularly to southwestern Europe and northern Africa. It was recently placed in the Plantaginaceae family (synonymous with Veronicaceae) following a revision of the classical Scrophulariaceae family (Olmstead *et al.*, 2001). It is a member of the asterid clade of flowering plants. Within the asterids, *Antirrhinum* belongs to the order Lamiales, a close relative of the order Solanales, which includes other model species such as *Petunia* and *Lycopersicon esculentum* (Olmstead *et al.*, 2001).

Antirrhinum is a herbaceous perennial model species, which is grown as bedding and as a cut flower in commercial horticulture. The large, brightly coloured and bilaterally symmetrical flowers are thought to have led to its cultivation from the Hellenistic period. Still a common garden feature, today hundreds of cultivated varieties and hybrids are available (Hudson *et al.*, 2008). It is a facultative LD species with a well define JVP length that is affected by light and with an approximate generation time of four months (Adams and Jackson, 2004). The high level of phenotypic variation combined with hardiness and ease of selfing and cross-pollination have made *Antirrhinum* attractive to many eminent scientists over the last 150 years, including Mendel, Darwin and de Vries (Schwarz-Sommer *et al.*, 2003; Hudson *et al.*, 2008).

In '*Variation of Animals and Plants under Domestication*', Darwin described the inheritance of different shaped *Antirrhinum* flowers and several colour variants. It was, however, the pioneering work of Baur, coinciding with the rediscovery of the research of Mendel, which established *Antirrhinum* as a model species in the early twentieth century (Schwarz-Sommer *et al.*, 2003). Baur began his work with *Antir-*

rhinum in 1907 and characterized *Aurea*, the first documented case of Mendelian segregation of a dominant lethal mutation. Along with his colleagues he maintained *Antirrhinum* at the forefront of plant genetics for several decades. Their discoveries included the first confirmation of genetic linkage, the identification of cytoplasmic inheritance, the quantification of the effects of environment on recombination and mutation rates and the correlation of recombination with *chiasma* formation (Schwarz-Sommer *et al.*, 2003).

Antirrhinum diverged from the more commonly used model species *Arabidopsis thaliana* an estimated 120 million years ago (Olmstead *et al.*, 2001). Today, due to this early diversion it is a useful species in comparative developmental studies (Hudson *et al.*, 2008). Recently, genomic resources have been developed to increase the usefulness of *Antirrhinum* as a model plant. Standard tools of molecular biology including complementary Deoxyribonucleic acid (cDNA) libraries, genomic libraries and various yeast two-hybrid libraries have been established. In order to facilitate gene identification and expression analysis, an expressed sequence tag (EST) database has been created containing ~12 000 unique sequences. The first ~2 500 EST sequences have been submitted to the European Molecular Biology Laboratory (EMBL) database and can be used searching at Dragon database (Dragon DB; `http://www.antirrhinum.net/blast/blast.html`; Schwarz-Sommer *et al.*, 2003).

1.4.2 *Arabidopsis thaliana*

Until the 1980s, most plant research focused on plant species such as *Pharbitis nil* (synonym *Ipomea nil*), *Xanthium strumarium* L., *Hordeum vulgare* and *Glycine max* L. *Arabidopsis thaliana* (hereafter referred as *Arabidopsis*), a small rosette species in the Brassicaceae family was first proposed as a model organism in 1943 by Frederick Laibach. He was drawn to *Arabidopsis* because of the large amount of phenotypic variation; it ranges from Northern Scandinavia (68 ° N latitude) to the mountains of Tanzania (0 ° N latitude; Laibach, 1951).

It is a model species that has been used to study many processes in plant development. It is a facultative LDP with some cultivars requiring vernalization (Chapter 1.5.2.2), thus making it a good model in order to study flowering time regulation. It has a small size, can be grown inexpensively in the laboratory; mature plants reach 15-20 cm height and it has a short life cycle of approximately six weeks. It also has high seed yield with production approximating 5 000 seeds per plant (Meinke *et al.*, 1998).

Arabidopsis has a small genome (120 megabases) organized in five chromosomes with approximately 30 000 genes, giving it the smallest known genome in a flowering species, making it suitable for genetic analysis (Meyerowitz, 2001). In addition, it is diploid and easy to manipulate, transform and mutate (Azpiroz-Leehan and Feldmann, 1997). The development of the floral dip method of transformation (Clough and Bent, 1998) has facilitated the establishment of large transformed populations of insertional mutants and has made both reverse and forward genetics easy (Krysan *et al.*, 1999). The entire genome sequence is available and many molecular tools exist which allow the altered genes in mutagenized populations to be rapidly identified (Lukowitz *et al.*, 2000). Moreover, several genome-wide sets of genetic markers, such as single-nucleotide polymorphism (SNPs) based on ESTs are available (Schmid *et al.*, 2003). Several databases are also available worldwide with easy and open data access.

The comparison of the *Arabidopsis* genome with other flowering plants has revealed extensive similarity between different species in the sequence and arrangement of homologous genes (Barnes, 2002). Molecular studies on *Arabidopsis* and *Oryza sativa* reveal that aspects of the genetic mechanisms controlling flowering time are conserved across different plant species (Hayama *et al.*, 2003; Andersen *et al.*, 2004). So, it is possible to take what is learned about the gene function in *Arabidopsis* and apply it to many other species of flowering plants. Thus the 'weed mustard' has become one of the most popular and well-studied model plant species.

1.5 Using Competence to Flower as an Assay to Study Juvenility

1.5.1 Historical Studies of Floral Induction

The first historical record of attempts to comprehend flowering behaviour are the writings of the Greek philosopher Theophrastus (~370-285 BC). Many centuries later Mendel included flowering among the traits he examined genetically but his data were never fully presented (Weller *et al.*, 1997). Detailed examination and quantification of flowering time variation actually begun between 1910 and 1920. It was during this time that the effects of daylength, low temperatures, and nutritional conditions were first quantified (Garner and Allard, 1920; Naylor, 1961; Evans, 1964).

The response of plants to the relative length of day and night, an important indicator of seasons is known as photoperiodism. The word is derived from the Greek roots for 'light' ($\varphi\acute{\omega}\varsigma$) and 'duration of time' ($\pi\epsilon\rho\acute{\iota}o\delta o\varsigma$). The photoperiodic response allows plant species to adapt to seasonal changes in their environment (Thomas and Vince-Prue, 1997). For example, shortening days can prepare for the low temperatures ahead. Julien Tournois was the first to realize in 1912 that daylength played an important role in the timing of flowering. He noted that early spring seedlings of *Cannabis sativa* and *Humulus japonicus* flowered very rapidly, whereas later seedlings did not flower and he was able to demonstrate that these two species required SDs to flower (Naylor, 1961). Tournois also noted that it was the scotoperiod [Greek roots for 'darkness' ($\sigma\kappa\acute{o}\tau o\varsigma$) and 'duration of time' ($\pi\epsilon\rho\acute{\iota}o\delta o\varsigma$)], not the day, which was most influential. In 1913, Klebs reported that the light probably acted as a catalytic factor rather than as nutritive one (Naylor, 1961). Klebs did not pursue this idea; however, he paved the way for others to seriously consider daylength as an important factor controlling physiological processes.

In 1920, Garner and Allard (1920) published that daylength was a major contrib-

utor to the timing of flowering in a wide variety of plants species. Their discovery was prompted by work on a mutant strain of *Nicotiana tabacum* called *Maryland mammoth*. This mutant line did not flower under normal summer conditions, but instead required winter-like SDs. Previous studies in species that required LDs had led to the conclusion that it was simply the increased amount of light, photosynthesis and growth that promoted flowering, not the LDs itself. The SD response of *Maryland mammoth* however could not be so easily explained and thus the effect of daylength itself was further examined (Garner and Allard, 1920). Garner and Allard (1920) examined the flowering response to daylength in many different plant species. Their work laid the foundation for the classification of plant species by their response to daylength. Short day plants (SDPs) flower after the daylength becomes shorter than a critical length (inductive photoperiod), LDPs flower when the daylength becomes longer than a critical length and day neutral plants (DNP) flower irrespective of daylength. Both SDPs and LDPs can be either obligate (also called qualitative) or facultative (quantitative). Obligate plants absolutely require inductive photoperiods to flower, whereas the flowering of facultative plants is only accelerated in the inductive photoperiod; they will still flower in unfavourable (non-inductive) daylengths (Thomas and Vince-Prue, 1997).

In order to distinguish between the SDs of spring and autumn some species have developed a dual daylength requirement. These species require either a series of SDs followed by LDs or LDs followed by SDs (Evans, 1975). The daylength requirement of some plants can be altered by changing ambient temperatures. For example *Pharbitis nil* is a strict SDP in warm conditions but flowers in any daylength at low temperatures (Vince-Prue and Gressel, 1985). Garner and Allard (1931) further defined the role of daylength by examining the effect of altering light and dark periods of equal duration. They found that LDPs flowered rapidly in short light-dark cycles whereas SDPs only flowered when they received long dark periods. These experiments supported Tournois' notion that it was the scotoperiod that was most important in sensing daylength.

Hamner and Bonner (1938), with work in *Xanthium strumarium*, further confirmed the importance of the scotoperiod. Definitively, they showed that it was the night duration that had to exceed a certain length to promote flowering in this SD species by exposing the plants to one minute of light in the middle of the scotoperiod which eliminated the promotion of flowering that normally occurred in a non-interrupted night (Hamner and Bonner, 1938). Additionally, they demonstrated that the temperature during the light period had little effect on flowering whereas in the dark period temperature had a large effect on flowering. SD species show a more complex response to daylength. A short night break will not induce flowering in most LDPs and only a few species can be induced to flower by a night break of under 30 min (Thomas and Vince-Prue, 1997).

The regulation of flowering in many plants involves more than just daylength signals; in addition exposure to extended of low temperatures between 1°C and 7°C is often required to promote flowering (Michaels and Amasino, 2000). Over the years physiological studies have led to three models for the control of flowering time (Bernier, 1988; Thomas and Vince-Prue, 1997). The first is the concept of a universal flowering hormone, which was first postulated by Chailakhyan (1936). The florigen concept was based on the transmissibility of substances or signals across grafts between reproductive "donor" shoots and vegetative "recipients" in *Nicotiana tabacum* plants. It was proposed that florigen was produced in leaves under favourable photoperiods and transported to the SAM via the phloem. The identification of a graft-transmissible floral inhibitor also led to the concept of a competing "antiflorigen." Many research years were consumed hunting for florigen in the phloem sap, but its chemical nature has remained elusive. Several lines of evidence have demonstrated that the FT/TSF protein complex is a large part of the florigenic signal across diverse species (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Lin *et al.*, 2007; Mathieu *et al.*, 2007; Tamaki *et al.*, 2007).

The inability to separate the hypothetical flowering hormones from assimilates led to a second model; the nutrient diversion hypothesis. This model proposed that

inductive treatments result in an increase in the amount of assimilates moving to the apical meristem, which in turn induces flowering (reviewed in Sachs and Hackett, 1983; Bernier, 1988).

The view that photosynthate assimilates are the only important component in directing the transition to flowering was superseded by the multifactorial control model, which proposed that a number of promoters and inhibitors, including phytohormones and assimilates, are involved in controlling the developmental transition (Bernier, 1988). According to this model, flowering can only occur when the limiting factors are present at the apex in the appropriate concentrations and at the right times. This model attempted to account for the diversity of flowering responses by proposing that different factors could be limiting for flowering in different genetic backgrounds and/or under particular environmental conditions.

1.5.2 Genetics and Physiology of Flowering Time

Flowering time has been genetically explored in *Arabidopsis* and many loci have been cloned through the study of natural variation and induced mutations (Table A.1). This has led to the conclusion that in *Arabidopsis* there are at least four major pathways that control flowering initiation (Figure 1.1).

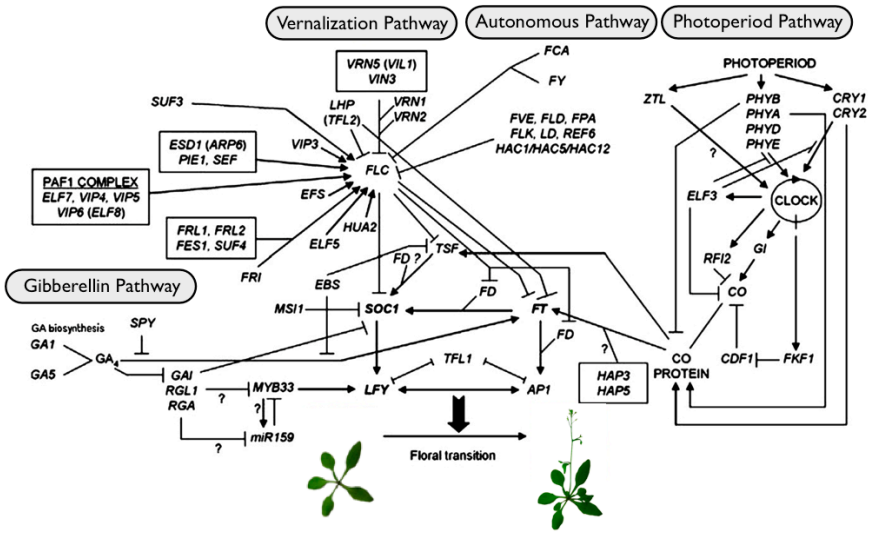

Figure 1.1: Genetic Control of Time to Flowering in *Arabidopsis*

Schematic representation of the major pathways regulating flowering and their interactions (Adapted from Massiah, 2007).

The autonomous and gibberellin pathways promote flowering in response to endogenous factors and the vernalization and photoperiod pathways promote flowering in response to environmental factors (Koornneef *et al.*, 1998; Simpson *et al.*, 1999; Mouradov *et al.*, 2002; Simpson and Dean, 2002; Massiah, 2007; Jackson, 2009). In addition, a pathway involving light quality and a thermosensory pathway have also been recently proposed (Koornneef *et al.*, 1998; Mouradov *et al.*, 2002; Blazquez *et al.*, 2003; Massiah, 2007; Jackson, 2009).

1.5.2.1 The Photoperiod Pathway

Photoperiod is an important environmental cue which regulates the floral transition, especially in temperate zones where the change in seasons throughout the year has a major effect on daylength (Jackson, 2009). The pathway starts with photoreceptors (Chapter 1.5.4), which initiate signals that interact with a circadian clock (Chapter 1.5.5) and entrain the circadian rhythm (Thomas and Vince-Prue, 1997; Thomas, 2006). Classic photoperiod pathway mutants were defined as 'blind'

(photoperiod insensitive) to photoperiod because they can not respond to inductive photoperiod conditions. Such mutants flower late in LDs or early in SDs, exhibiting similar leaf numbers and flowering time in both LDs and SDs. Furthermore, they show little or no response to vernalization, which means after vernalization they flower with the same or similar leaf number as in LDs and SDs (Koornneef *et al.*, 1998; Mouradov *et al.*, 2002; Simpson and Dean, 2002).

In order to explain how photoperiod regulates flowering, two models have been proposed: the internal and the external coincidence models (Thomas and Vince-Prue, 1997). The internal coincidence model proposes that two internal rhythms are in phase under inductive photoperiods and as a result it promotes flowering; under non-inductive photoperiods these two rhythms are out of phase and as a consequence flowering is inhibited. On the other hand, the external coincidence model and the most prominent, proposes that an external signal (daylength) interacts with an internal light-sensitive rhythm during a certain time of the day, which means under inductive photoperiods the interaction of light and the light sensitive rhythm occurs and plants are induced to flower; under non inductive photoperiods there is no such interaction and flowering is repressed. Furthermore, the appropriate flowering time requires not only temporal, but also spatial integration of promotive signal(s). Classical studies have demonstrated that the leaves of a photoperiodically induced plant species can initiate flowering when grafted to a non-treated plant. This led to the hypothesis that there is a mobile floral signal that moves from the leaf to the SAM, in response to the floral promotive cues (Chapter 1.5.1; (Chailakhyan, 1936; Bernier *et al.*, 1993; Thomas and Vince-Prue, 1997)).

The most striking advances in our understanding of the genetic control of the timing of developmental transitions have come from the study of *Arabidopsis* (Levy and Dean, 1998; Simpson *et al.*, 2003; Turck *et al.*, 2008) and several genes such as *LEAFY* (*LFY*), *APETALA-1* (*AP-1*), *TERMINAL FLOWER-1* (*TFL-1*), and *FLOWERING LOCUS T* (*FT*) (Bradley *et al.*, 1997; Kardailsky *et al.*, 1999;

Kobayashi *et al.*, 1999; Corbesier *et al.*, 2007). Furthermore, genetic and molecular approaches have identified genes, which are responsible for the photoperiod response. Some of these genes encode proteins that specifically regulate flowering time, are involved in the regulation of light signal inputs or are components of the circadian clock. Genes such as *GIGANTEA* (*GI*), *CONSTANS* (*CO*), *FT*, *CRYPTOCHROME-2* (*CRY-2*; *FHA-1*) and FLOWERING WAGENINGEN (*FWA*) act in the photoperiod pathway (Mouradov *et al.*, 2002; Hayama and Coupland, 2003; Simpson and Dean, 2002; Massiah, 2007; Turck *et al.*, 2008; Jackson, 2009). *GI* is regulated by the circadian clock, and binds to the promoter of the *CO* gene as part of a larger protein complex to promote expression of *CO* (Mizoguchi *et al.*, 2005). Mutations in genes *GI, EARLY FLOWERING-3* (*ELF-3*), *EARLY FLOWERING-4* (*ELF-4*) and *LATE ELONGATED HYPOCOTYL* (*LHY*), which are responsible for clock function, affect *CO* expression and flowering time (Suarez-Lopez *et al.*, 2001; Doyle *et al.*, 2002; Yanovsky and Kay, 2002). The *CO* expression pattern is consistent with the external coincidence model, which under LDs activates *FT* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS-1* (*SOC-1*) to promote flowering (Samach *et al.*, 2000; Suarez-Lopez *et al.*, 2001; Yanovsky and Kay, 2002). *CONSTANS* is also controlled by the clock and modulated by daylength, which provides strong evidence that *CO* acts as a link between the clock and flowering initiation. It encodes a protein with two zinc fingers, which is related to GATA transcription factors, and has a carboxyl-terminal CCT motif (Putterill *et al.*, 1995; Robson *et al.*, 2001).

FT, a 23 kDa protein which shows similarities to the RAF-1 kinase inhibitors of mammals, belongs to the CETS family. *CENTRORADIALIS* (*CEN*), *SELF PRUNING* (*SP*) and *TFL-1* are also members of the CETS family, with approximately six members in *Lycopersicon esculentum* and six in *Arabidopsis* (Bradley *et al.*, 1997; Kardailsky *et al.*, 1999). They were named CETS after the first three plant genes with identified biological functions: *CEN, TFL1*, and *SP* (Pnueli *et al.*, 1998). The *Arabidopsis* genome encodes four proteins similar to FT and TFL-1, namely TWIN SISTER OF FT (TSF), MOTHER OF FT AND TFL-1 (MFT),

BROTHER OF FT AND TFL-1 (BFT) and *Arabidopsis thaliana* RELATIVE OF CENTRORADIALIS (ATC; Turck *et al.*, 2008; Jackson, 2009). *FT* shares 59% a homology with *TFL-1* (Ohshima *et al.*, 1997; Ahn *et al.*, 2006). Interestingly, although FT antagonizes TFL-1 function, a change of a single amino acid can convert *TFL-1* as a repressor of flowering to an activator of flowering (Hanzawa *et al.*, 2005).

In *Arabidopsis*, expression of FT is regulated in LDs by the photoperiodic pathway (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Yanovsky and Kay, 2002; Turck *et al.*, 2008; Jackson, 2009), by the light-quality pathway (Halliday *et al.*, 2003; Cerdan and Chory, 2003) and by the autonomous pathway (Samach *et al.*, 2000). β-glucuronidase staining indicated that both *FT* and *CO* promoters are active in the companion cells of the phloem minor veins (An *et al.*, 2004). High CO expression levels promote FT expression in the leaf, and its paralog, TSF, in the stem. TSF shares redundant temporal and spatial pattern of expression, but in a lower expression level. Both proteins are unloaded to the phloem and transported to the SAM (Yamaguchi *et al.*, 2005; Turck *et al.*, 2008; Jackson, 2009). At the SAM, FT and TSF interact with the locally expressed FLOWERING LOCUS D (FD) protein (Abe *et al.*, 2005; Yamaguchi *et al.*, 2005; Wigge *et al.*, 2005; Jang *et al.*, 2009) and promote transcription of the MADS-box genes *AP-1* and *FRUITFUL* (*FUL*; Wigge *et al.*, 2005; Conti and Bradley, 2007). Once *AP-1* expression is stabilised FT is no longer required and the SAM becomes totally committed to flower development. Under SD conditions, *FT* expression levels are reduced. However, as plant growth and development proceeds, *FT* expression levels show a clear increase (Yanovsky and Kay, 2002).

Ectopic expression of *Arabidopsis FT* shortens the vegetative phase, causing early flowering (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Kojima *et al.*, 2002; Bohlenius *et al.*, 2006; Lifschitz and Eshed, 2006; Yan *et al.*, 2006; Lin *et al.*, 2007). In addition, genes orthologous to *FT* have been identified in other Brassicaceae species, in Curcubitaceae and species such as *Lycopersicon, Pharbitis*, *Gentiana*,

Oryza, *Hordeum*, *Populus*, *Picea* and *Citrus* (Kardailsky *et al.*, 1999; Kojima *et al.*, 2002; Bohlenius *et al.*, 2006; Lifschitz and Eshed, 2006; Yan *et al.*, 2006; Lin *et al.*, 2007; Gyllenstrand *et al.*, 2007; Turck *et al.*, 2008). Ectopic over-expression of these also shortens the vegetative phase in transgenic homologous and heterologous plants. This conservation together with the small protein size makes FT partially capable of fulfilling the requirements of one of the florigen candidates, or one of its important components. However, the fact that *Arabidopsis* eventually flowers also under SDs, suggests that other molecules could also contribute to the long-distance induction of flowering, with carbohydrates and hormones, specifically gibberellins being attractive candidates.

1.5.2.2 The Vernalization Pathway

The requirement of winter for flowering was first observed in arable crops. Winter varieties of *Triticum spp.* and *Hordeum vulgare* are able to flower only after they go through a period of low temperature, although some winter cereals often fail to survive the harsh winter conditions present in some areas. In 1915, the plant physiologist G. Gassner speculated that he could mimic the effect of winter on flowering of such cereals by keeping imbibed seeds at near freezing temperature (1°C~7°C) for 4-12 weeks. The Russian agronomist T. Lysenko adopted this controlled winter treatment of cereals to extend arable land in Northern Russia. This controlled winter treatment procedure came to be known as vernalization (from Latin *vernus*, meaning "of the spring"). The practice of vernalization specifically refers to the promotive effect of low temperatures on flowering, rather than the breakage of dormancy by cold (Lang, 1965). Since its discovery as agriculture practice, physiological and genetic studies have been used to elucidate its mechanisms in various plant species. However, the molecular mechanisms involved in vernalization have been one of the mysteries in plant biology until recent advances were made in *Arabidopsis*.

Biennial plants require vernalization to flower. Crosses between annual and biennial

varieties have established the genetic basis for the vernalization requirement in many plant species. Hybrids of naturally occurring winter annual and summer annual accessions of *Arabidopsis* have revealed that variation at one or both of two loci, *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*), is responsible for the winter-annual habit in *Arabidopsis* (Clarke and Dean, 1994; Koornneef *et al.*, 1994). Both genes synergistically act to delay flowering in winter annual accessions of *Arabidopsis* and a loss-of-function mutation in either gene results in the loss of the late flowering phenotype (Michaels and Amasino, 2001). The cloning of *FLC* (Michaels and Amasino, 1999; Sheldon *et al.*, 1999) provided the first insight into the molecular nature of vernalization in *Arabidopsis*. *FLC* is a repressor of flowering and the presence of a dominant allele of *FRI* increases *FLC* expression to levels that repress flowering. Vernalization overcomes the effect of *FRI* by repressing *FLC* expression and this repression is stably maintained after plants are returned to warm growth conditions (Michaels and Amasino, 1999; Sheldon *et al.*, 1999).

Generally, vernalization does not directly cause flowering but provides competence to flower. This is through stable epigenetic repression of *FLC*. The establishment and maintenance of silenced chromatin at the *FLC* locus has been associated with a series of covalent modifications introduced to both DNA and histones (Sung and Amasino, 2004; Bastow *et al.*, 2004). Thus, the epigenetic repression of *FLC* is a key feature of vernalization. *FLC* is expressed predominantly in mitotically active regions such as shoot and root apical meristems, which are the sites of cold perception and the tissues that achieve the vernalized state. It should be noted that although most of the flowering promotion by vernalization in *Arabidopsis* is due to *FLC* repression, there is clearly a component of flowering promotion that is *FLC* independent.

Besides FLC, the family of the closely related MADS-domain proteins includes the five MADS AFFECTING FLOWERING (MAF; Ratcliffe *et al.*, 2003; De Bodt *et al.*, 2003) and FLOWERING LOCUS M (FLM, also known as MAF-1; Scortecci *et al.*, 2001; Ratcliffe *et al.*, 2001) proteins. It has been hypothesized that all MAF

proteins function also as repressors of flowering (Ratcliffe *et al.*, 2003).

Summer annual accessions of *Arabidopsis* often contain loss-of-function mutations in *FRI* (Johanson *et al.*, 2000). However, it has also been indicated that certain summer annual types contain an active FRI allele but also contain an allele of FLC that is not up-regulated by *FRI* (Gazzani *et al.*, 2003; Michaels *et al.*, 2003). Thus there are at least two routes by which winter annual types of *Arabidopsis* have become summer annuals.

Although the involvement of *FLC* and *FRI* in the vernalization requirement seems to be conserved in the Brassicaceae family (Osborn *et al.*, 1997; Schranz and Osborn, 2004); cereals appear to have different loci that are responsible for winter-annual behavior. Genetic analyses of the vernalization requirement in spring and winter varieties of *Triticum spp.* and *Hordeum vulgare* have revealed three different gene classes; the *REDUCED VERNALIZATION RESPONSE* (*VRN*) genes, the *PHOTOPERIOD RESPONSE* (*PPD*) genes and the *EARLINESS PER SE* (*EPS*) genes. Differences in the *VRN* genes divide them to winter and spring classes, whereas differences in *PPD* genes divide the temperate cereals into photoperiod-sensitive and photoperiod-insensitive classes. The *EPS* genes regulate flowering independently of vernalization and photoperiod. Their expression is critical for *Triticum spp.* flowering time under different environments (Snape *et al.*, 2001).

Molecular cloning of *VRN-1*, *VRN-2* and *VRN-3* genes from *Triticum spp.* and *Hordeum vulgare* has shown that quite distinctive sets of genes control winter annual behavior in cereals (Yan *et al.*, 2003, 2004). *VRN-1* encodes a MADS-box transcription factor with high similarity to *Arabidopsis* meristem identity genes *AP1, CAULIFLOWER* (*CAL*), and *FUL* that promotes flowering and thus confers the spring habit of *Triticum spp.* (Yan *et al.*, 2003; Danyluk *et al.*, 2003; Trevaskis *et al.*, 2003). *VRN-2* region includes two similar ZCCT genes encoding proteins with a putative zinc finger and a CCT domain that have no clear homologs in *Arabidopsis*. Both ZCCT genes act as floral repressors and are downregulated by vernalization (Yan *et al.*, 2004). *VRN-3* encodes a RAF kinase inhibitor like pro-

tein with high homology to *Arabidopsis FT* (Yan *et al.*, 2006). Thus the simplest model in *Triticum spp.* is that *VRN-2* is a repressor, which prevents flowering by repressing *VRN-1* expression. Vernalization promotes flowering by "shutting off" *VRN-2* and relieving the repression of *VRN-1*. Thus *VRN-2* may play a role analogous to that of *FLC*; both are repressors that target genes required for flowering (*VRN-3*) and both repressors are "turned off" by vernalization.

In addition, it has long been hypothesized that the GA may be involved in vernalization. Exogenous application of GA to the biennial *Hyoscyamus niger* is capable of inducing flowering under LD conditions in the absence of cold (Lang, 1957; Zeevaart, 1983). Furthermore, in *Raphanus sativus* the endogenous GA level was found to increase during the course of a vernalizing cold treatment (Suge, 1970). These observations suggest that GA may play a role in vernalization. Nevertheless, studies indicate that, at least in *Arabidopsis*, GA is probably not required for this process. In one such study, exogenous GA application did not affect the expression of the *FLC* gene (Sheldon *et al.*, 1999). In another experiment, the endogenous GA level of vernalization responsive *Arabidopsis* strains was genetically reduced by a mutation in the *GIBBERELLIN INSENSITIVE* (*GAI*) gene, which encodes an enzyme that catalyses the first step of GA biosynthesis (Sun *et al.*, 1992). Although this *gai* mutation can cause a very severe GA deficiency (Sun *et al.*, 1992), it did not cause a loss of vernalization response (Michaels and Amasino, 1999; Chandler *et al.*, 2000).

1.5.2.3 The Autonomous Pathway

The autonomous pathway promotes flowering independently of environmental factors (Boss *et al.*, 2004). Autonomous pathway mutants flower late in LDs and even later in SDs but flower rapidly after vernalization (Koornneef *et al.*, 1998; Levy and Dean, 1998; Mouradov *et al.*, 2002; Simpson and Dean, 2002). Genetic analysis of double mutants between several autonomous pathway genes suggests the existence of two different subgroups: the *FLOWERING TIME CONTROL*

PROTEIN ALPHA (*FCA*)/*FLOWERING TIME CONTROL PROTEIN ALPHA* (*FY*) subgroup and the *FLOWERING TIME CONTROL PROTEIN PI ALPHA* (*FPA*)/*FVE* subgroup. Moreover it also suggests that these genes may be involved in other developmental processes and that they interact in a complex way since some of the double mutants are not viable and others show complex synergistic interactions (Koornneef *et al.*, 1998).

Autonomous pathway genes *FPA, FVE, LUMINIDEPENDENS* (*LD*), *FCA* and *FY* promote flowering by repressing *FLC*, which acts in a dosage-dependent manner to delay flowering. Autonomous pathway mutants are late flowering because *FLC* mRNA levels are high (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). The late flowering phenotype of autonomous pathway mutants depends on the presence of FLC since in an *flc* null background this late flowering phenotype is suppressed (Michaels and Amasino, 2001). The only mutant that still shows a slightly late flowering phenotype in the absence of *FLC* is *fpa* (Michaels and Amasino, 2001).

1.5.2.4 The Gibberellin Pathway

Gibberellins are plant specific hormones that regulate many aspects of plant development such as leaf and stem elongation, fruit ripening, fertility, apical dominance and flowering (Hedden and Phillips, 2000; Olszewski *et al.*, 2002). Exogenous GA promotes flowering in a range of plants, including *Arabidopsis,* although in other plants it can have inhibitory or negligible effects on flowering (Lang, 1965; Zeevaart, 1983; Pharis and King, 1985). Many cold requiring LDPs flower early in response to GAs under SD conditions; however, SDPs rarely respond to GAs in non-inductive LDs (Zeevaart, 1969). Interestingly, plants that require both LDs and SDs respond to GAs, but GA treatment only by passes the LD photoperiod requirement and not the SD requirement (Zeevaart, 1969). These results correlate with LDs causing increases in GA levels in such plants (Zeevaart, 1983; Gocal *et al.*, 2001a).

In *Arabidopsis* numerous mutants in the GA biosynthesis pathway and in GA signal transduction have been isolated (Peng *et al.*, 1997; Hedden and Phillips, 2000). Phenotypically these mutants are dwarfed, dark green, with reduced apical dominance and late flowering phenotypes (Koornneef and Veen, 1980). Null mutations in the early steps of GA biosynthesis (e.g. *ga1-3*) do not flower in SD conditions, whereas weak mutants (e.g. *ga1-6*), or GA signal transduction mutants (e.g. *gai*), flower later than WT (Wilson *et al.*, 1992). This late-flowering phenotype is fully reversible by the addition of small amounts of GAs; in fact, *ga1-6* mutants flower only slightly later than wild type (WT), but still show the GA-deficient dwarf phenotype. This result shows that the GA levels required for flowering are much lower than the GA levels required for normal growth (Wilson *et al.*, 1992).

Gibberellin promotion of flowering is independent of other flowering pathways. *FLOWERING PROMOTING FACTOR-1* (*FPF-1*) was isolated based on the up-regulation of *FPF-1* in the apical meristem immediately after the transition to flowering. Overexpression of *FPF-1* causes in GA mediated early flowering in late flowering mutants, implying that *FPF-1* may lead to increased sensitivity of meristems to GAs (Kania *et al.*, 1997). Similar to other GA mutants, *ga1-3* has a slightly late flowering phenotype in LDs, suggesting that alternate flowering pathways can bypass the GA requirement for flowering in LDs. Indeed, it appears that the photoperiod promotion pathway can override the requirement for GAs because mutations in *CO* greatly enhance the late flowering phenotype of *ga1-3* mutants in LDs (Reeves and Coupland, 2001). Thus, GAs must promote flowering through a pathway independent of the photoperiod pathway. Likewise, the GA promotion pathway is independent of the vernalization pathway because *ga1-3* mutants respond to vernalization (Michaels and Amasino, 1999; Chandler *et al.*, 2000). Further evidence for the independence of the GA promotion pathway comes from autonomous pathway *ga1-3* double mutants that are synergistically later flowering than either single mutant, indicating that these pathways promote flowering independently of each other (Chandler *et al.*, 2000; Reeves and Coupland, 2001).

Little is known about the mechanisms by which GAs promote flowering. However, recent evidence has demonstrated a link between GA regulation and downstream flowering genes. Identified based on similarity to GAMYB, a transcription factor that activates transcription of GA-responsive promoters in *Hordeum vulgare*. The *AtMYB33* possesses GAMYB activity (as in *Hordeum vulgare*), is up-regulated by GAs and binds to and up-regulates the *LFY* promoter (Gocal *et al.*, 2001a). This photoperiod transition is accompanied by increased expression of *AtMYB33*, and induction of flowering (Gocal *et al.*, 2001a). In *Arabidopsis*, GA levels and sensitivity to GAs increase after the transitions from SD to LD conditions, which act to promote flowering through activation of the floral integrators *SOC1* and *LFY* (Blazquez *et al.*, 1998; Moon *et al.*, 2003).

1.5.2.5 Other Phytohormone Pathways

Physiological changes associated with the transition to reproductive phase can be caused by treatments that do not themselves cause floral initiation. For example, one of the earliest events observed at the SAM following photoperiodic induction is a transitory increase in the number of cells undergoing mitosis (Bernier *et al.*, 1977). When cytokinins are applied to the SAM of the LDP *Sinapis alba,* they cause an increase in mitotic activity similar to that caused by exposure to a single LD. Cytokinin may therefore be a component of the floral signal moved at the SAM, although it is insufficient by itself to intiate flowering (Bernier *et al.*, 1977). Other potential phytohormone pathway includes the polyamines. Photoperiodic induction of the *Sinapis alba* was correlated with a large increase in the concentration of putrescine, the major polyamine in the leaf phloem exudates (Havelange *et al.*, 1996). *Sinapis alba* leaves treated with an inhibitor of putrescine biosynthesis decrease the presence of putrescine in the phloem exudate and also repressed flowering initiation. This result suggests that putrescine may be a component of the floral stimulus in *Sinapis alba* (Havelange *et al.*, 1996). Moreover, several experiments support a role for salicylic acid (SA) in control of flowering in *Pharbitis*

nil and *Arabidopsis*. This phytohormone is involved in stress-induced early flowering in *Arabidopsis*, and might interact with the autonomous pathway through an *FCA*-independent branch and the photoperiod-dependent pathway, through a *CO*-independent process (Hatayama and Takeno, 2003; Martinez *et al.*, 2003).

1.5.2.6 The Nitric Oxide Pathway

It has been revealed that nitric oxide (NO), a signaling molecule, whose production is induced by various abiotic and biotic stresses, represses the transition to reproductive phase in *Arabidopsis*. The NO-deficient mutant *nos-1* has an early flowering phenotype while plants overproducing NO, due to disruption of the chloroplast phosphoenolpyruvate/phosphate translocator gene *NOX-1*, are late flowering. Further molecular studies indicated that NO inhibits flowering by repressing the *CO* and *GI* genes in photoperiod pathway, and upregulating the expression of floral repressor *FLC* (He *et al.*, 2004). However, the biological relevance of NO-mediated repression of the transition to reproductive phase remains unclear.

1.5.2.7 The Light Quality Pathway

Light quality affects flowering time in a way different from the light input in the photoperiod pathway. This is due to information provided about the local environment in which the plant grows, rather than the seasonal changes (Thomas, 2006; Thomas *et al.*, 2006). A well-described example is the shade-avoidance response (Devlin *et al.*, 1999). In *Arabidopsis,* the importance of light quality in the control of flowering time has been confirmed by molecular-genetic studies. Light quality is perceived by the phytochromes (PHYs) and cryptochromes (CRYs) . Among the PHYs , *PHYB* has a predominant role. The *phyB* mutant flowers early, suggesting that *PHYB* is a repressor of flowering (Halliday *et al.*, 1994; Aukerman *et al.*, 1997; Devlin *et al.*, 1998, 1999). The *phyA* mutant flowers slightly late under LD conditions, and flowering is strongly delayed when the light is far-red enriched at the end

of the LD period or when the night is interrupted by a short period of light (Reed *et al.*, 1994). This indicates that *PHYA* promotes flowering under these conditions.

Far-red and blue light promote flowering through *PHYA*, *CRY-1* and *CRY-2*, respectively. This is supported by the flowering phenotype of photoreceptor mutants. For example, *cry-2* mutant flowers late under LDs demonstrating that *CRY-2* functions to promote flowering (Lin, 2000). Interestingly, it has recently been shown that light quality affects the expression of miR172, a regulator which is involved in juvenile phase change (Lauter *et al.*, 2005; Wu *et al.*, 2009), with its levels being higher under LDs and blue light enriched conditions (Jung *et al.*, 2007). However, the elements acting downstream from *PHYA*, *CRY-1* and *CRY-2* in this pathway remain unclear.

1.5.2.8 The Light Quantity Pathway

LI is also known to have an impact on flower initiation in some plant species (Thomas, 2006). Exposure to low or high LI levels can delay or hasten the time to flowering, respectively. For instance, *Achillea millefolium* grown under a 16 h d^{-1} photoperiod in controlled environment (CE) conditions flowered after 57, 45 and 37 d when grown under 100, 200 or 300 μmol m^{-2} s^{-1}, respectively (Zhang *et al.*, 1996). Similarly, Adams *et al.* (1999) demonstrated that *Petunia* flowering was hastened by LDs, but that decreased LI prolonged the time to flowering.

However, the effect of LI on time to flowering can be unpredictable in several species. Hence, the term 'facultative irradiance response' (FI) has been coined to describe a developmental hastening of flowering by addition of supplemental light (Erwin and Warner, 2000). Species such as *Antirrhinum* (LDP), *Nicotiana* (LDP or SDP) and *Petunia* (LDP) that exhibit a FI response, show a decrease in leaf numbers and days to flower as irradiance increases. In contrast, the term 'irradiance indifferent' (II) refers to plants such as *Salvia* [SDP or facultative LDP (FLDP)] and *Zinnia* [DNP or facultative SDP (FSDP)] that do not show any response

to increased irradiance (Seeley, 2000; Thomas and Vince-Prue, 1997; Erwin and Warner, 2000; Mattson and Erwin, 2005; Thomas, 2006).

Despite the high sensitivity of FI species to elevated levels of LI, the majority does not show a hastened flowering phenotype with increasing irradiance. It has been shown for *Pelargonium* x *hortorum* that a linear relationship between LI and days to flower, for an increased irradiance developmental response exists until a threshold level between 6.89 and 9.01 μmol m^{-2} d^{-1} (Erickson *et al.*, 1980). However, some species require greater threshold levels. For instance, absolute flowering of *Digitalis* was reached with LI greater than 11 μmol m^{-2} d^{-1} (Fausey *et al.*, 2001). Furthermore, giving supplemental irradiance (at 30, 60 and 90 μmol m^{-2} s^{-1}) to *Gerbera* hastened flowering by up to 23 d in the winter, but only up to 11 d during the spring (Gagnon and Dansereau, 1989), suggesting that the impact of supplemental irradiance on flowering can be dependent on season's ambient light conditions and species's threshold requirement.

1.5.2.9 The Carbohydrate Pathway

Carbohydrates have many important functions in plants. It is not surprising that the addition of sugars to plant growth media affects many developmental processes and gene expression (Gibson, 2005). Studies have implicated carbohydrates as playing a role in the control of vegetative to reproductive phase change of plant development. However, there have been contradictory reports on the role of sugars in the regulation of flowering. Several *Arabidopsis* mutants such as *carbohydrate accumulation mutant-1* (*cam-1*), *phosphoglucomutase-1* (*pgm*-1), *ADP-glucose pyrophosphorylase-1* (*adg-1*), *starch excess 1* (*sex-1*) and *chloroplastic beta-amylase-3* (*bam-3*), which exhibit altered starch metabolism and catabolism lead to increased tissue sugar concentrations; all flower later than WT under inductive LD conditions (Caspar *et al.*, 1985; Lin *et al.*, 1988; Caspar *et al.*, 1991; Eimert *et al.*, 1995; Lao *et al.*, 1999; Yu *et al.*, 2001).

Various plant organs are supplied with carbohydrates via the sieve tubes. A major transport form is the disaccharide sucrose (Suc). It is the most extensively studied compound that might participate in long-range signalling for flowering. The late flowering phenotype of a starch-deficient mutant in *Arabidopsis* can be rescued by addition of Suc to the growth medium (Yu *et al.*, 2000). Furthermore, in *Sinapis alba* and *Arabidopsis* plants exposed to inductive photoperiods Suc levels increase rapidly and transiently in phloem leaf exudates (Bernier *et al.*, 1993; Corbesier *et al.*, 1998). The increased Suc export in these studies, however, appears not to be due to increased photoassimilate production; instead it derives from starch mobilisation, as shown by analysis of mutants impaired in starch metabolism and catabolism. Furthermore, according to defoliation experiments (Corbesier *et al.*, 1996), the Suc export increase coincides with the start of the mobile signal transport and occurs before the activation of cell division in the SAM (Bernier *et al.*, 1993; Corbesier *et al.*, 1998). Consistent with this, in *Nicotiana tabacum* decreasing phloem loading of Suc by antisense repression of *Nicotiana tabacum* SUCROSE TRANSPORTER-1 (NtSUT-1) causes delayed flowering (Burkle *et al.*, 1998).

Suc can be supplied to the aerial part of the plants by growing them on vertical plates. Under these conditions, flowering of otherwise late-flowering *Arabidopsis* genotypes is accelerated, both in LDs and darkness, indicating that Suc affects flowering not only under SD conditions (Roldan *et al.*, 1999). Furthermore, using the vertical-plates assay, evidence has been provided for a Suc florigenic effect via FT, the major photoperiodic pathway output, by the failure of exogenous Suc to complement the late flowering phenotype of the *Arabidopsis ft-1* mutant, despite its effectiveness with other late flowering mutants (*co-2*, *gi-4*; Roldan *et al.*, 1999). Moreover, the *constitutive photomorphogenesis1* (*cop-1*) mutant can flower in darkness if Suc is supplied in media (McNellis *et al.*, 1994). *COP-1* is an ubiquitin ligase, a major regulator of seedling photomorphogenesis which regulates CO protein abundance in the vascular tissue of adult plants, as part of the mechanism by which *Arabidopsis* discriminates between LDs and SDs during flowering time regulation (Deng *et al.*, 1992).

Arabidopsis plants grown on 6% Glucose (Glc) -Murashige and Skoog (MS) media exhibit a late flowering phenotype compared with plants grown on 2% Glc (Zhou *et al.*, 1998). Similarly, a delayed flowering phenotype of *Arabidopsis* plants grown in the dark on medium containing 6% Suc compared to medium containing 2% Suc has been also observed. In contrast, the *glucose-insensitive mutant-1* (*gin-1*) grown under similar conditions, did not exhibit such sensitivity (Zhou *et al.*, 1998). In addition, Ohto *et al.* (2001) observed an 8 d delay in flowering of *Arabidopsis* plants grown on medium containing 5% compared with 1% Suc. This response coincided with a significant increase in the number of adult rosette leaves and overall plant size at flowering. Furthermore, the time-period that plants received more Suc increased the severity of the delay, suggesting a Suc threshold level for the response of flowering to additional sugar (Ohto *et al.*, 2001). Interestingly, a recent study on *Arabidopsis SUCROSE-PROTON SYMPORTER-9 (AtSUC-9)* indicates that it is involved in the control of flowering time, as *atsuc9* mutant plants flowered earlier than WT under SD conditions (Sivitz *et al.*, 2007). This might be by preventing premature flowering through maintaining a low threshold level of Suc (Sivitz *et al.*, 2007). Moreover, it has been reported that in WT *Arabidopsis* plants elevated Suc level can slightly delay flowering and reduce *FT* and *SOC-1* mRNA abundance, whereas in *co* mutants it accelerates flowering without an increase the expression of *FT* and *SOC-1* (Ohto *et al.*, 2001).

Further evidence supporting the role of carbohydrates in controlling flowering time through altered gene expression comes from a study by Wilson *et al.* (2005). Several *Arabidopsis* mutant genotypes were screened for flowering time genes by generating global gene expression profiles with genome microarray technology. This study concluded that genes with functions associated with carbohydrate metabolism exhibited the largest changes in expression across all the flowering time mutants, indicating a link between flowering time and carbohydrate metabolism (Wilson *et al.*, 2005). Other recent evidence also strengthens the link between carbohydrates and the control of flowering time. *Arabidopsis* plants with a mutation in the *TREHALOSE-6-PHOSPHATE SYNTHASE* (*TPS*), a gene closely associated

with both sugar signaling and starch metabolism, failed to undergo the transition to the reproductive phase (Dijken *et al.*, 2004).

Several lines of evidence have demonstrated that there is a synergistic interaction between GAs and Suc in the activation of *LFY* transcription (Blazquez *et al.*, 1998; Eriksson *et al.*, 2006). Levels of GA_4 and Suc increase dramatically in the SAM shortly before floral initiation and the regulation of genes involved in GA metabolism suggests that this increase is possibly due to transport of GAs and Suc from outside sources to the SAM (Eriksson *et al.*, 2006).

Given the links between carbohydrate metabolism and flowering time, it is possible that excess sugars produced at elevated carbon dioxide [CO_2] may also contribute to signal the transition to flowering via a phototrophic induction pathway. Plants in the C_4 dicarboxylic acid pathway of photosynthesis grown at elevated [CO_2] commonly accumulate excess leaf carbohydrates (Curtis and Wang, 1998; Long *et al.*, 2004); this may be one possible mechanism through which elevated [CO_2] may be influencing flowering time. However, all the above mentioned data indicate that the role of carbohydrates on the vegetative to reproductive phase change of plant development depends on their concentration, source of origin, and spatial and temporal supply.

1.5.2.10 The Thermosensory Pathway

One of the environmental factors that affects flowering is temperature, including both ambient and near-freezing temperatures (Chapter 1.5.2.2). For some species high ambient temperatures promote flowering. For example, WT *Arabidopsis* plants flower earlier at 23°C than at 16°C (Blazquez *et al.*, 2003) and perhaps night temperature is more important than day temperature (Thingnaes *et al.*, 2003).

The molecular basis of ambient temperature in flowering time control was studied and it was found that this mechanism is probably mediated through the blue light

receptor *CRY-2* and the red light receptor *PHYA*. As Blazquez *et al.* (2003) reported, the late flowering phenotype of the *cry-2* mutant is exacerbated at 16°C whereas *phyA* mutants flower slightly late at 23°C. However, the *cry-2/phyA* double mutant growing at 23°C flowers as late as the *cry-2* single mutant growing at 16°C (Blazquez *et al.*, 2003). It has also been suggested that the *fca* and *fve* mutants were found to flower as late at 23°C as 16°C (Blazquez *et al.*, 2003). These observations suggest that photoreceptors *CRY-2* and *PHYA* and the autonomous pathway genes *FCA* and *FVE* mediate the promotive effect of elevated ambient temperatures redundantly.

1.5.3 Floral Integration

Pathways that promote the floral transition coordinate to induce transcription of three important genes called floral pathway integrators (FPIs): *FT, SOC-1* and *LFY*. Signals that actively inhibit flowering repress their expression (Koornneef *et al.*, 1998; Levy and Dean, 1998; Simpson *et al.*, 1999; Mouradov *et al.*, 2002; Boss *et al.*, 2004; Massiah, 2007; Jackson, 2009). *FT* coordinates inputs from the photoperiod and light quality pathway, and from the vernalization and autonomous pathways through the activity of FLC (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Samach *et al.*, 2000). *LFY* coordinates inputs by both the GA and photoperiod pathways through activation of a *cis*-element in the *LFY* promoter (Blazquez and Weigel, 2000), whereas *SOC1* expression is influenced by all the pathways (Samach *et al.*, 2000; Hepworth *et al.*, 2002; Moon *et al.*, 2003; Lee *et al.*, 2004). Nevertheless, *FT*, *SOC1* and *LFY* can not be the only FPIs, as the triple *lfy/ft/soc-1* mutant can still flower under LD conditions (Moon *et al.*, 2005). The FPI genes control a second set of genes that direct the formation of the various flower organs, the floral meristem identity (FMI) genes which in *Arabidopsis* include *AP1, APETALA2* (*AP2*), *FUL, CAL,* and *LFY* (Boss *et al.*, 2004).

1.5.4 Light Perception and Signalling

In order to adapt to changes in the environment, circadian clocks need to be adjusted through the perception of environmental light-dark signals (Yanovsky and Kay, 2002; Thomas, 2006). The photoreceptor genes are responsible for perceiving different light durations and qualities and initiating signals that interact with the circadian clock to entrain the circadian rhythm (Thomas, 2006). Mutations in photoreceptor genes have varied effects on flowering time. Some mutants flower early, whereas other flower late indicating that these genes are involved in both the promotion and inhibition of flowering (Chapter 1.5.2.7; Simpson and Dean, 2002). Physiological, biochemical and molecular genetic studies have led to the identification of four families of photoreceptors in higher plants: PHYs (Quail, 2002), CRYs (Lin, 2002), phototropins (PHOTs; Briggs *et al.*, 2001) and the unidentified ultraviolet B photoreceptors (UVB; Frohnmeyer and Staiger, 2003; Brosche and Strid, 2003; Jiao *et al.*, 2007).

PHYs are photochromic proteins absorbing light in the red/far red end of the spectrum (600-700 nm). It has been suggested that PHYs evolved from bacterial bilinsensory proteins, a hypothesis that is supported by the discovery of PHY-like proteins in photosynthetic bacteria, nonphotosynthetic eubacteria and fungi (Montgomery and Lagarias, 2002). They are involved in a variety of plant functions including hypocotyl elongation, stem and leaf expansion, vegetative growth and development, and flowering (Ahmad, 1999). The PHYs exist in two reversible isomeric forms; Pr absorbs red-light and is converted into Pfr whereas Pfr absorbs far-red light and is converted back to Pr. Exposure to red light leads to a late flowering phenotype and thus, the activated Pfr form is inhibitory to floral induction (Goto *et al.*, 1991; Bagnall, 1992, 1993). In *Arabidopsis*, five PHY genes (*PHYA to E*) have been isolated and sequenced (Sharrock and Quail, 1989; Clack *et al.*, 1994). Sequence analysis showed that *PHYB, PHYD*, and *PHYE* have 80% amino acid similarity and they all function as inhibitors of floral induction (Devlin *et al.*, 1998; Neff and Chory, 1998; Devlin *et al.*, 1999). In contrast, *PHYA* acts as a

promoter of floral initiation (Neff and Chory, 1998), whereas the role of *PHYC* has not been established.

CRYs comprise flavoproteins and are able to detect blue light (400-500 nm). *CRY-1*, together with *CRY-2* (*FHA1*; *FHA-1* is a *CRY-2* allele in Landsberg erecta (Ler) background) are very important during de-etiolation, the transition of a dark grown seedling living from its seed reserves to a photoautotrophically competent seedling. This developmental transition includes a massive reorganization of the transcriptional program, inhibition of hypocotyl growth, promotion of cotyledon expansion, and synthesis of a number of pigments including chlorophyll and anthocyanins (Lin, 2002; Liscum *et al.*, 2003). In addition, these photoreceptors are important for photoperiod-dependent flowering induction and for resetting the circadian oscillator(Cashmore *et al.*, 1999; Yanovsky and Kay, 2002). Analysis of multiple PHY and CRY mutants has revealed that interactions exist between these photoreceptors (Lin, 2000). *PHYB, PHYD* and *PHYE* have redundant functions in directly mediating red-light inhibition of floral induction. *PHYA* and *CRY-2*, promote floral induction by suppressing the effect of these inhibitory PHYs (Thomas, 2006).

The PHOTs (*PHOT-1* and *PHOT-2* in *Arabidopsis*) perceive blue and ultraviolet A (UVA; 340-480 nm) wavelengths and mediate a number of photomorphogenic responses. They are protein kinases harboring two light, oxygen or voltage (LOV) domains that can perceive light and in response, to control phototropism, stomatal aperture and chloroplast movements (Briggs and Christie, 2002). However, their contribution to transcriptional regulation is relatively small, as only a limited number of genes are under their control (Jiao *et al.*, 2007).

Ultraviolet B (UVB; 280–320 nm) light is sensed by plants as both an informational signal and an environmental stress factor. *Arabidopsis UVB RESISTANCE-8* (*UVR-8*) is a UVB-specific signalling component that orchestrates the expression of a range of genes with vital UVB protective responses. Under low fluence UVB rates, *UVR-8* regulates the expression of *ELONGATED HYPOCOTYL-5 (HY-5)*,

a bZIP transcription factor which is involved in many light rsponsive pathways (Brown *et al.*, 2005). In addition, *COP-1*, a negative regulator of the visible light response, is also regulated under low fluence UVB rates (Oravecz *et al.*, 2006). However, it is not clear if both loci function as UVB receptors or are downstream parts of the mechanism which control plant development in response to UVB wavelengths.

How photoreceptors initiate the signal transduction pathway for floral induction is still unclear. There have been reports that PHYs can act as protein kinases (Fankhauser *et al.*, 1999) or they can interact with other nuclear proteins that modulate expression of light-regulated genes (Ni *et al.*, 1998). Ahmad and Cashmore (1996a) isolated early flowering *Arabidopsis* mutant *phytochromes-signalling early-flowering*-1 (*pef-1*), *pef-2* and *pef-3* and these loci might code for proteins involved in the early steps of the PHY signalling pathway. Several studies have shown that multiple related bHLH (basic helix–loop–helix) class transcription factors play key roles in PHY signal transduction (Quail, 2002). Members of the bHLH family appear to be particularly important because several of them specifically interact with the light-activated Pfr (Quail, 2002). Surprisingly, these transcription factors primarily act as negative regulators of PHY signalling. Moreover, in some cases, the PHYs inhibit those negative regulators (Duek and Fankhauser, 2005). Photoreceptors may also affect flowering time indirectly through their effects on the biological clock. Clock entrainment is known to involve different photoreceptors in different light conditions (quality and irradiance) and novel blue light photoreceptors of the *ZEITLUPE/FLAVIN-BINDING, KELCH REPEAT, F-BOX-1/LOV KELCH PROTEIN-2* (*ZTL/FKF-1/LKP-2*) family have been demonstrated to regulate clock components Boss *et al.* (2004).

1.5.5 The Circadian Oscillator in Plants

Identification of a central oscillator in plants has proven more difficult in *Arabidopsis* than in other model systems. There is evidence that plants may contain

multiple oscillators (Hall *et al.*, 2002; Michael *et al.*, 2003) with partially redundant functions. Recent microarray analyses suggest that large numbers of physiological events are under the control of the circadian clock, including metabolite biosynthesis and hormone responses (Covington *et al.*, 2008; Mizuno and Yamashino, 2008).

A basic circadian clock system has three primary components (Somers, 1999). First is the central oscillator/pacemaker that generates the 24 hr oscillators. In animals and cyanobacteria, this is made up of proteins whose interactions create an autoregulatory negative feedback loop, which in turn generates the 24 hr oscillator. In plants, it is unknown how the oscillator operates but a number of genes have been identified as potential oscillator elements. *CIRCADIAN CLOCK ASSOCIATED-1* (*CCA-1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), *LUX ARRHYTHMO* [*LUX* , also called *PHYTOCLOCK-1* (*PCL-1*)], *PSEUDO RESPONSE REGULATOR-9* (*PRR-9*), *PRR-7*, *PRR-5* and *GI* are thought to form a feedback loop (Millar *et al.*, 1995; Schaffer *et al.*, 1998; Wang and Tobin, 1998; Makino *et al.*, 2000; Strayer *et al.*, 2000; Alabadi *et al.*, 2001; Mizoguchi *et al.*, 2002; McClung, 2006). In addition, the *TIMING OF CAB EXPRESSION-1* (*TOC-1*, also called *PRR-1*) has been cloned (Strayer *et al.*, 2000). *TOC-1* is involved in a transcriptional feedback loop to control its own expression and that of *CCA-1* and *LHY* (Wang and Tobin, 1998; Schaffer *et al.*, 1998). *TOC-1* has also been identified as a link between environmental information and clock outputs, with an essential function in constant darkness and in the integration of red light signals to the clock (Mas *et al.*, 2003).

The second component is the input pathway that synchronizes or entrains the oscillator with environmental cues. In a light-coordinated circadian clock this function is carried out by the photoreceptors (Millar *et al.*, 1995; Somers *et al.*, 1998). *PHYB* is the primary photoreceptor for high intensity red light whereas *PHYA* acts under low intensity red light. *CRY-1* and *PHYA* both act to relay low-fluence blue light to the circadian clock. Analysis of the *Arabidopsis* early flowering mutants *cop-*

1, *de-etiolated-1* (*det-1*) and *early flowering-3* (*elf-3*) showed that the disrupted genes might also be a part of the input pathway (Deng *et al.*, 1991; Hicks *et al.*, 1996; Kwok *et al.*, 1996). The third component is the output pathway that links the oscillator to plant processes under circadian rhythm (McClung, 2006).

1.5.6 Flowering Time Genes in Other Species

Plants show diverse flowering responses to environmental cues. To what extent are the results from *Arabidopsis* applicable to other species? Several lines of evidence from *Oryza sativa* and other species indicate that many of the genes identified in *Arabidopsis* are present in other angiosperms and perform a similar function (Hayama and Coupland, 2003; Andersen *et al.*, 2004). Flowering time in *Oryza sativa* is promoted by SDs. Even though *Arabidopsis* and *Oryza sativa* show opposite photoperiodic responses, several flowering time genes identified in *Arabidopsis* seem to have similar functions in *Oryza sativa. HEADING DATE-1* (*HD1*) encodes a gene homologous to *CO* (Yano *et al.*, 2000) and *HD3A* encodes a gene highly related to *FT* (Kojima *et al.*, 2002). *Oryza sativa GI* (*OsGI*), promotes expression of *HD1*, consistent with *Arabidopsis.* However, data suggest that *HD1* negatively regulates *HD3A*, the opposite of what happens in *Arabidopsis* (Simpson *et al.*, 2003). Whereas the promotion under LDs in *Arabidopsis* results from *CO* activating *FT*, the delay in flowering in LDs in *Oryza sativa* results from *HD1* repressing *HD3A* (Simpson *et al.*, 2003). *HEADING DATE-3A* promoter activity was studied using *Hd3a::GUS* fusion and found to be active in the leaf vascular tissue. The *Hd3a::GUS* fusion gene, driven by the phloem specific *rolC* promoter, caused early flowering and the fluorescence of green fluorescent protein (GFP) was specifically detected in vascular tissues (Hayama and Coupland, 2004). These results are consistent with the hypothesis that the HD3A protein is produced in the vascular tissues of leaves transported to the SAM via the vasculatre and there induces the transition to reproductive phase.

1.6 Carbohydrate Metabolism and Transport

In leaves of higher plants, photosynthesis generates adenosine-5'-triphosphate (ATP) and reducing power produces triose phosphates. Triose phosphates are simple carbohydrates that can be used as substrates in multiple anabolic and catabolic pathways. They can be converted to hexose phosphates which are used for starch, cell wall, hexose and Suc synthesis. Sucrose and its metabolic derivatives serve as raw materials for the synthesis of nucleic acids, proteins, lipids, starch and polysaccharides in the cell wall, as well as providing energy for plant growth and development. To be utilized by plant cells in the form of hexoses, Suc is first cleaved by either cell wall invertases (CIN) or sucrose synthases (SUS).

Translocation through the phloem provides the most important long distance transport conduit of the plant. Whilst the xylem tubes transport mainly mineral-containing water from the roots to the shoots, the phloem is responsible for the translocation of organic compounds from the sites of synthesis (source organs) to the developing and non-photosynthetic tissues (sink organs). Plant species from different families translocate Suc or raffinose together with one of three different polyols in their phloem (Oparka and Cruz, 2000). However, Suc is the dominant transport metabolite for long distance carbon transport between source and sink organs. Generally, two mechanisms of phloem loading exist: plasmodesmata provide a symplastic pathway to channel Suc directly from cytoplasm to cytoplasm within adjacent cells. In an alternative mode of phloem loading, Suc can be apoplasticaly (via cell wall) imported into cells by Suc transporters (called SUTs or SUCs), or cleaved by CIN and imported by monosaccharide transporters (MST; Koch, 2004; Rolland *et al.*, 2006). In many plant species the nature of the predominantly used route is controversially debated. Nevertheless, the MSTs have been isolated from various plant species and have a broad range of substrate specificities, including Glc, 3-O-methylglucose, 2-deoxyglucose, mannose, fructose (Fru) and polyols. Hexose transporters are believed to function in hexose uptake in sink cells and subcellular sugar compartmentation. Suc transporters are responsible for the uploading of Suc

into the phloem, maintaining the mass flow of phloem sap and unloading of Suc into the sink apoplast, which is reflected by the diverse locations of these transporters in source and sink tissues. The *Arabidopsis* genome encodes nine putative SUTs.

The phloem contains, in addition to photosynthate and other small molecules (Richardson *et al.*, 1982; Lohaus *et al.*, 2000; Fiehn, 2003), a variety of macromolecules, including mRNA (Jorgensen *et al.*, 1998; Sasaki *et al.*, 1998), small RNA (Yoo *et al.*, 2004a), peptides and proteins (Fisher *et al.*, 1992). As the florigen hypothesis is now accepted as more complex than the movement of a single type of signal molecule, these endogenous compounds along with their involvement in sink-strength regulation, may have florigenic or antiflorigenic functions.

1.7 Carbon Partitioning in Plants

Starch is the predominant storage carbohydrate in most higher plants. In developmental biology its importance is demonstrated by the late flowering phenotype of starch-deficient mutants and their reduced growth and photosynthetic rates compared with WT plants under inducted floral conditions (Chapter 1.5.2.9; Caspar *et al.*, 1985; Schulze *et al.*, 1991; Geiger *et al.*, 1995).

There are two types of starch: the transitory and the reserve starch. Transitory starch is synthesised at the same time as Suc during photosynthesis. It is stored inside chloroplasts and broken down to sustain metabolism during the dark period, when CO_2 cannot be photoassimilated. Under dark conditions, starch is converted to maltodextrin by several enzymes, such as debranching enzyme (*DBE*), and appears to be influenced by a glucan water dikinase (GWD) and phosphoglucan water dikinase (PWD) (Smith *et al.*, 2003; Zeeman *et al.*, 2004; Kotting *et al.*, 2005). α-amylase (*AtAMY*), a key enzyme in hydrolytic degradation of starch in the endosperm, was thought to be involved in the conversion of starch to maltodextrin

in leaf tissue (Trethewey and Smith, 2000). However, recent data indicate that *AtAMY* is not required for transitory starch breakdown in *Arabidopsis* leaves (Yu *et al.*, 2005). Maltodextrin is converted to maltose (Mal) and Glc by β-amylase (*BAM*) and *DISPROPORTIONATING ENZYME-1* (*DPE-1*) in the chloroplast (Critchley *et al.*, 2001; Scheidig *et al.*, 2002b; Schneider *et al.*, 2002). Several lines of evidence indicate that Mal and Glc are the two major forms of carbon exported from chloroplasts under dark conditions (Weber *et al.*, 2000; Servaites and Geiger, 2002; Weise *et al.*, 2004). Mal is exported by the Mal transporter *MALTOSE EXCESS-1* (*MEX-1*; Niittyla *et al.*, 2004) and is metabolized in the cytosol by several enzymes, including *DISPROPORTIONATING ENZYME*-2 (*DPE-2*) and possibly glucan phosphorylase (Chia *et al.*, 2004; Lu and Sharkey, 2004).

Reserve starch accumulates over several days, weeks, or months in amyloplasts. This type serves as mid to long-term reserve. In cereals it represents 65% to 75% and in *Solanum tuberosum* even 80% of the total dry weight (DW).

In some species such as *Beta vulgaris* and *Arabidopsis* starch is the main storage form (Fondy *et al.*, 1989; Zeeman and Ap Rees, 1999), whereas in others such as *Pisum sativum* and *Spinacia olearacea* Suc is the end product of photosynthesis, and only its surplus is directed to the starch biosynthetic path (Stitt *et al.*, 1983). However, the regulation of carbon partitioning between starch and Suc is complex and not completely understood. Current models suggest that assimilates is channelled into starch when the demand for Suc synthesis (export, storage, and utilisation) is exceeded by the rate of photosynthesis (Stitt and Quick, 1989). In that case changes in metabolite levels in the cell inhibit Suc synthesis and stimulate starch biosynthesis. However, this 'surplus' model is unlikely to apply to all species. In plants such as *Arabidopsis, Glycine max, Gossypium hirsutum,* and *Nicotiana tabacum* starch synthesis is more likely to be 'constitutive' in that some photosynthate is partitioned into starch even if there is still a demand for Suc.

1.8 The Aims of this Study

The purpose of this study was to study the physiological and genetic influences on juvenility with the specific aims being:

1. To study the effect of irradiance and [CO_2] in assimilation of carbohydrates and how these influence the transition within the vegetative phase of plant development.

2. To gain a better understanding of the genetic basis underlying juvenility using *Arabidopsis* mutants.

Chapter 2

General Materials and Methods

2.1 Materials

The Materials and Methods described in this chapter are common to two or more of the results chapters. Materials and methods specific to individual chapters will be described within those chapters.

2.1.1 Enzymes and Biochemicals

Enzymes were grade II and purchased from Roche™ (Basel, CH) and Invitrogen Life Technologies™ (Paisley, UK).

All chemicals were of analytical purity and used as received. They were purchased from either BDH Merck Ltd™ (Poole, UK), Merck House™ (Poole, UK), Sigma-Aldridge™ (St. Louis, USA), Fluka™ (Milwaukee, USA) or SUPELCO™ (Bellefonte, USA) unless otherwise stated. Carbohydrate standards were purchased from Sigma-Aldridge™ (Monosaccharides Kit Catalog No. 47267; Sugar Alcohols Kit Catalog No. 47266; St. Louis, USA). Water and solvents used for LC and LC-MS were of HPLC grade and purchased from Fisher Scientific, (Loughborough, UK). General solutions and buffers used were prepared as described by Sambrook *et al.* (2001) unless otherwise stated. All solutions were made up in reverse osmosis water purified by a MilliQ® (Milford, USA) system. Water was sterilized by autoclaving at 15 psi at 120°C for 20 min. Any other materials were supplied as stated in the specific Materials and Methods sections.

2.1.2 Plant Resources

2.1.2.1 *Antirrhinum majus*

The *Antirrhinum* Bells Red F1 hybrid, which has a dwarf habit and early flowering phenotype, was obtained from Colegrave Seeds (LOT: BELR03RWGL32769-07; Banbury, UK) and Goldsmith Seeds Inc., (LOT: BELR01RWGL12583-02,

LOT:BELR01RWGL22173-02; CA, USA). This line will be referred to as Bells F1.

The Recombinant Inbred Line (RIL) No57 was derived from an interspecific cross between *Antirrhinum majus* and *Antirrhinum charidemi* (Langlade *et al.*, 2005). It was provided by Prof. E. Coen at John Innes Centre, UK. In this study, the F9 generation was used. This line will be referred to as RIL57.

2.1.2.2 *Arabidopsis thaliana*

Mutant and WT *Arabidopsis* plants were Columbia-0 (Col-0), Ler and Wassilewskija-4 (Ws-4). All the genotypes are rapid cycling summer annual ecotypes. The background and stock number of each genotype used in this study is listed in Table 2.1.

2.1.2.3 Statistical Software Packages

Data were analysed by using the regression and analysis of variance techniques of Sigma Plot 11® (Systat Software, Chicago, USA) and GenStat® (GenStat V12, Rothamsted, UK) statistical software.

2.2 Methods

2.2.1 *Antirrhinum* Growth and Development

Seeds of Bells F1 and RIL57 were sown into Plantpak P40 (HSP, Essex, UK) module trays containing Levington F2 compost (6 parts compost, 1 part sand and 1 part vermiculite; Levington Horticulture, Suffolk, UK). Germination was carried out in CE growth cabinets (PEL Rooms) set at 22°C with light providing ~80 µmol m^{-2}

s^{-1} PPFD at tray height for 8h d^{-1}. When 50% of seedlings emerged the trays were distributed into CE cabinets (Chapter 3.2.1.1) or glasshouse (GH) compartments (Chapter 3.2.1.2; Chapter 3.2.1.3) and the daylength or [CO_2] treatments initiated.

Table 2.1: **Information on the *Arabidopsis* Genotypes Used in this Study**

Genotype	**Function**	**EASC ID**[a]	**Reference**
Col-0	WT	N1092	Meinke and Scholl 2003
Ler	WT	NW20	Koornneef *et al.* 1991
Ws-4	WT	N5390	Bechtold *et al.* 1993
fha-1 (Ler)	Photoreceptor	N108[1]	Guo *et al.* 1998
*phyb (*Ler)	Photoreceptor	N6211[1]	Reed *et al.* 1993
gi-4 (Ler)	Photoperiod	N181[1]	Koornneef *et al.* 1991
co-2 (Ler)	Photoperiod	N175[1]	Koornneef *et al.* 1991
ft-1 (Ler)	Photoperiod	N56[1]	Koornneef *et al.* 1991
tfl-1 (Ler)	Repressor	N3091[1]	Ratcliffe *et al.* 1999
lhp-1 (Ws-4)	Repressor	V. Gaudin[2]	Gaudin *et al.* 2001
hst-1 (Ler)	Repressor	N3811[3]	Telfer and Poethig 1998
gin1-1 (Ler)	Transporter	J. Sheen[1]	Zhou *et al.* 1998
gin2-1 (Ler)	Transporter	J. Sheen[1]	Moore *et al.* 2003
pgm-1 (Col)	Starch deficient	N210[1]	Caspar *et al.* 1985
adg1-1 (Col)	Starch deficient	N3094[1]	Wang *et al.* 1998
sex1-1 (Col)	Starch excess	N3093[1]	Yu *et al.* 2001
sex-4 (Col)	Starch excess	S. Zeeman[4]	Zeeman *et al.* 1998
bam-3 (Col)	Starch excess	S. Zeeman[5]	Lao *et al.* 1999
abi-4 (Col)	Aba insensitive	N122591[6]	Tissier *et al.* 1999
eto-1 (Col)	Ethylene overproducer	N3072[1]	Guzman and Ecker 1990

Parenthesis and superscript numbers in Genotype and EASC ID columns indicate WT background and mutagen, respectively. Names in EASC ID column indicate the donor other than EASC. [1]EMS: Ethylmethanesulfonate, [2]T-DNA: Transferred DNA, [3]DEB: diepoxybutane, [4]X-rays, [5]TILLING: Targeting Induced Local Lesions in Genomes, [6]Transposon; EASC: European *Arabidopsis* Stock Centre; Aba: Abscisic acid.

Plants were initially watered and subsequently fed with Peter Excel™ (18:10:18; Scotts International B.V., NL) liquid feed at a rate of 500 mg l^{-1}. Plants were potted up into 9 cm pots (Optipot FP 9M; volume of 0.49 l) containing Levingtons M2 peat based potting compost (Levington Horticulture, Suffolk, UK) when they

reached the six leaf stage.

2.2.2 *Arabidopsis* Growth and Development

Mutant and WT seeds were sown into Plantpak P24 module trays (HSP, Essex, UK) containing Levingtons F2 compost (Levington Horticulture, Suffolk, UK). To improve synchronous germination, seeds were stratified for 4 d at 4°C in dark conditions before they were distributed in growth cabinets. Trays were covered with plastic domes to keep the moisture level high until the first vegetative leaves were visible. The seals of the domes were then broken to allow the plants to acclimate to a less humid environment. The domes were completely removed two days after the seal was broken. Plants were grown in CE cabinets (Saxcil®, Chester, UK) under 100 μmol m^2 s^{-1} PAR with 22 ± 0.5°C temperature and 70 ± 2% relative humidity (RH). The soil was kept moist by application of tap water three times a week.

2.2.3 Estimation of the Length of Juvenile Vegetative Phase

An analytical approach developed by Adams *et al.* (2003) which is based on temporal response to flowering was used to estimate the length of JVP and AVP in *Antirrhinum* and *Arabidopsis* plants grown under different experimental conditions.

The approach determines the phases of photoperiod sensitivity by conducting reciprocal transfer experiments in which plants are transferred from LDs to SDs and *vice versa* at regular intervals, from seedling emergence to flowering. It enables the analysis of reciprocal transfer experiments data in terms of the following parameters (Figure 2.2.3): The photoperiod-insensitive juvenile phase (a_1); the photoperiod-sensitive flower inductive phases (AVP) P_{IS} and P_{IL} in SDs and LDs, respectively; and the photoperiod-insensitive post inductive phase (a_3). For LDPs such as *Antirrhinum* and *Arabidopsis*, the photoperiod-sensitive flowering inductive

phase in LDs P_{L} can be subdivided into a photoperiod-sensitive flower induction phase (P_{IL}), and a photoperiod-sensitive flower development phase (P_{dL}).

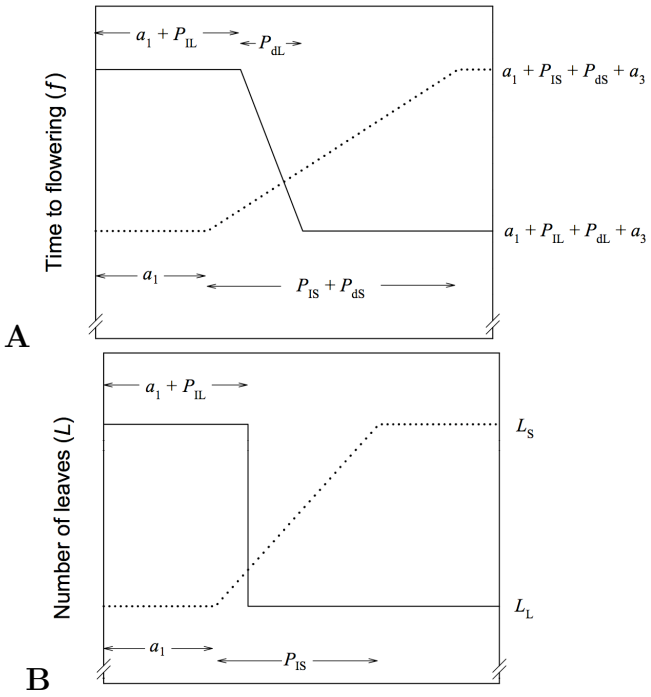

Figure 2.1: Schematic Representation of Phases of Photoperiod Sensitivity as Revealed by the Reciprocal Transfer Approach

Schematic representation of (A) the response of time from seedling emergence to flowering (f) and (B) the number of leaves below the inflorescence (L) for LDPs transferred from LDs to SDs (continuous line) and from SDs to LDs (broken line) at various times from seedling emergence. The duration from seedling emergence to flowering consists of a photoperiod-insensitive juvenile phase (a_1), followed by photoperiod-sensitive flower induction and development phases in LDs (P_{IL} and P_{dL}, respectively) or SDs (P_{IS} and P_{dS}, respectively). The final phase of flower development corresponds to the photoperiod-insensitive flower development phase (a_3). L_{S} and L_{L} denote the number of nodes beneath the corolla of the first opened flower under continuous SDs and LDs, respectively. Figures adapted by Adams *et al.* (2003).

Similarly in SDs, P_{S} can be subdivided into P_{IS} and P_{dS}. The model also enables flowering time data sets from SDs (L_{S}) and LDs (L_{L}) to be combined and analysed simultaneously to determine the phases of photoperiod sensitivity.

2.2.3.1 Flowering Time Analysis

Flowering time in *Antirhinum* was measured as either the time in days from 50% of seedling emergence or the number of nodes beneath the corolla of the first opened flower. Flowering in *Arabidopsis* was measured as the number of days from 50% of seedling emergence or the number of rosette leaves to the first elongation of the floral bolt at 1 cm height. The experimental design was a randomized complete block.

2.2.4 Extraction of Soluble Carbohydrates from *Antirrhinum* and *Arabidopsis* Plant Material

Plant material was collected into liquid N_2 and subsequently freeze dried (FreeZone 12 Liter Cascade Freeze Dry System, LABCONCO, Kansas, USA) and homogenised using a ball mill (Glen Creston Ltd., London, UK) for 2-3 min. One ml 80% ($^{V}/_{V}$) ethanol (EtOH) at 75°C was added per 100 mg tissue, mixed and incubated at 75°C for 5 min. Samples were centrifuged at 3 000 x g (Biofuge® Heraeus, Osterode, DE) for 10 min, the supernatant removed and the pellet re-extracted with 0.5 volume 80% ($^{V}/_{V}$) EtOH as described previously. The two supernatants were combined, placed in a 2 ml microfuge tube and concentrated at 40°C using a Techne® 36-well sample concentrator (Techne, Staffordshire, UK). Pellets were kept in 0.5 ml 40% ($^{V}/_{V}$) EtOH at 2°C for starch analysis (Chapter 2.2.6.2). Samples were re-dissolved in 1 ml 80% ($^{V}/_{V}$) acetonitrile (ACN) and vortexed for 5 s. Ion exchange resin (10 mg; Amberlite MB 150, Sigma-Aldridge™, St. Louis, USA) was added and the samples mixed overnight in an orbital incubator at 23°C. Samples were centrifuged at 13 000 x g (Biofuge® Heraeus, Osterode, DE) for 30 s at ambient temperature and the carbohydrate-containing supernatants removed.

2.2.5 Resolution of Total Soluble Carbohydrates in *Antirrhinum* by HPLC

Chromatographic analyses were carried out with an Agilent 1100 high performance liquid chromatography (HPLC) 3D system (Instrument model G1322A, Agilent Technologies, Santa Clara, USA) equipped with an Agilent refractive index detector [(RID) Model 1100, Agilent Technologies, Santa Clara, USA]. An Apex carbohydrate column (Jones Chromatography, Hengoed, UK) was used with an ACN:water (80:20 $^{V}/_{V}$) mobile phase for isocratic elution. Refractometric detection at 590 nm was performed at ambient temperature. Separations were carried out at a flow rate of 0.75 ml min^{-1} for 22 min, pressure of 40 mmHg and 30 μl sample injection volume. Prior to injection, samples and carbohydrate standards were centrifuged using a Micro-Spin® centrifuge filter (Costar Nylon 66, 0.2 μm, Cornig Life Science, Lowell, USA) for 2 min at 7 000 x g (Biofuge® Heraeus, Osterode, DE). One ml aliquots (in screw cap vials, Agilent Technologies, Waldbronn, DE) were then analyzed by HPLC.

Chromatographic measurements were standardized using the absolute calibration method. Calibration curves were constructed in triplicate using a series of carbohydrate standard solutions (Sigma-Aldridge™, St. Louis, USA) of 18 different monosaccharides and oligosaccharides [Arabinose (Ara), Arabitol (Arb), Erythritol (Ery), Fru, Galactose (Gal), Galactitol (Glt), Glc, Maltitol (Mlt), Mal, Mannitol (Mtl), Myo-inositol (MI), Rhamnose (Rha), Ribitol (Rbt), Ribose (Rib), Sorbitol (Sbl), Suc, Xylitol (Xyl) and Xylose (Xl)] containing 250, 500, 1 000 and 2 000 μg ml^{-1}. Various eluent combinations and flow rate modes were tested to enable their separation.

2.2.6 Determination of Total Soluble Carbohydrates in *Antirrhinum* by HPLC

Retention times of standards were used as the criteria for chromatographic peak identification. Quantitative analysis was performed by absolute calibration using peak areas as the reference parameters. Data were collected directly from the Agilent 1100 HPLC 3D system (Agilent Technologies, Santa Clara, USA) with the Agilent ChemStation for LC 3D 1100 software (Rev. A.10.02, Agilent Technologies, Santa Clara, USA). Statistical analysis of the data generated in Agilent ChemStation 1100 was performed using SigmaPlot 11® (Systat Software, Chicago, USA). After plotting the data for each treatment, regression analysis was performed to produce the equation of the regression line and correlation coefficient to estimate the total sugar concentration in tissue from individual plants.

2.2.6.1 Liquid Chromatography–Mass Spectrometry

Liquid Chromatography–Mass Spectrometry (LC–MS) method development was carried out on a Thermo Accela (Thermo Electron, San Jose, USA), HPLC system coupled to an ion trap mass spectrometer (LTQ DECA XL, Thermo Electron, San Jose, USA) equipped with a Thermo orthogonal electrospray interface.

Carbohydrates were extracted from *Antirrhinum* Bells F1 adult vegetative plants, as described in Chapter 2.2.4. Carbohydrate standards were purchased from Sigma-Aldridge™ (Monosaccharides Kit Catalog No. 47267; Sugar Alcohols Kit Catalog No. 47266; St. Louis, USA). Chromatographic separation was performed on an Apex carbohydrate column (Jones Chromatography, Hengoed, UK). The flow rate was 400 μl min^{-1}, the sample injection volume was 20 μl and the porous graphitic carbon column was used at ambient temperature. The triple stage mobile phase was composed of (A) water, (B) ACN, and (C) 15% aqueous formic acid (Sigma-Aldridge™, St. Louis, USA). The LC run time was 20 min using the gradient elution profile: 0–5 min, 96% A 4% B to 92% A 8% B; 5–7 min, 92% A 8% B

to 75% A 25% B and maintained for 3 min; 10–20 min, 75% A 25% B to 25% B 75% C followed by column re-equilibration: 20–22 min, 25% B 75% C to 50% A 50% B and maintained for 5 min; 27–30 min, 50% A 50% B to 96% A 4% B and maintained for 10 min.

The ion trap mass spectrometer was operated in the negative ion mode with the ion source voltage set to −3.0 kV, capillary voltage −20 V, tube lens offset −60 V, capillary temperature 300 °C, sheath gas 40 (arbitrary units) and auxiliary gas 30 (arbitrary units). Mass spectra were acquired over the scan range mass-to-charge ratio (m/z) 50–800. Data were processed using Xcalibur 1.2 software (Thermo, San Jose, CA, USA). Optimum negative ion ESI-MS parameters were determined for each standard compound using a solution of 50 µg ml^{-1} in 80% ($^{V}/_{V}$) ACN by direct infusion with a syringe pump at a flow rate of 3 μl min^{-1} into solvent flow from the LC at a flow rate of 400 μl min^{-1}, over the scan range m/z 50–800.

2.2.6.2 Enzymatic Assay of Sucrose, Reducing Sugars and Starch

For analyses and quantification of Glu, Fru and Suc, plant material was sampled and immediately frozen in liquid nitrogen. The freeze-dried materials were ground, and amount of 50-100 mg were used for the analysis. Sugars were determined enzymatically in EtOH extracts at 340 nm by a UV/VIS V530 JASCO (Easton, USA) spectrophotometer, after digestions with β-fructosidase, hexokinase (HXK), glucose-6-phosphatdehydrogenase and phosphoglucose isomerase, using the EZS 864+ kit (Diffchamb; Lyon, FR), following the manufacturer's guidelines.

Starch was determined from the pellets of the soluble sugar extractions (Chapter 2.2.4) after extensive washing with water. Two ml water was added per pellet, re-suspended and centrifuged at 3 000 x g (Biofuge® Heraeus, Osterode, DE) for 5 min. The supernatant was removed and the pellet re-extracted twice using the same procedure. Starch from the air-dried pellets was quantitatively dissolved in hot dimethyl sulfoxide (DMSO). Briefly, pellets were suspended in 85% ($^{V}/_{V}$)

DMSO and heated for 30 min at 90°C. After cooling, 8 M HCl was added and the solution was incubated for a further 30 min at 60°C. The sample was then centrifuged at 4 000 x g for 15 min. After adjusting pH to 4.5 with NAOH (5 M), the starch was precipitated with EtOH (96%; $^{V}/_{V}$) part of the suspension was digested with amyloglucosidase and HXK/glucose-6-phosphatdehydrogenase. Starch was determined by a UV/VIS V530 JASCO (Easton, USA) spectrophotometer at 340 nm, using the EnzyPlus™ determination kit (Diffchamb, Västra Frölunda, SE), following the manufacturer's guidelines.

Chapter 3

Identification of Phases of Photoperiod Sensitivity Using Reciprocal Transfer Experiments in *Antirrhinum*

3.1 Introduction

The ability to schedule or predict time to flowering requires an understanding of how the environment affects the pathways involved in this process. Quantitative modelling has become a major research tool in plant science, as in other areas of biology because it facilitates the consistent integration of observed physiological responses that explain and predict complex processes of plant development such as the time to flowering. However, most quantitative flowering models are very simplistic as they assume that plants are equally sensitive to photoperiod throughout development (Adams *et al.*, 1998). It has been indicated that several species, including *Hordeum* (Roberts *et al.*, 1988), *Oryza* (Collinson *et al.*, 1992) *Triticum* (Slafer and Rawson, 1995), *Papaver* (Wang *et al.*, 1997), *Sorghum* (Ellis *et al.*, 1997), *Chrysanthemum* (Adams *et al.*, 1998), *Petunia* (Adams *et al.*, 1999) and *Antirrhinum* (Adams *et al.*, 2003) exhibit distinct phases of development, which respond differently to environmental triggers such as photoperiod.

In annual plants such as *Arabidopsis,* the SAM progresses through a vegetative phase where leaf primordia are initiated on its flanks to form a rosette (Clarke *et al.*, 1999). This critical phase of plant development is characterized by two subphases: The JVP and the AVP. The JVP is insensitive to photoperiod and plants are often incapable of flowering, irrespective of environmental conditions. The suggestion has been that they must first attain a given size or age before they can be induced to flower (Thomas and Vince-Prue, 1984, 1997). The JVP provides a mechanism by which plants avoid the low seed yields that would occur if they were to flower precociously while still small and with limited photosynthetic capacity (Thomas and Vince-Prue, 1984). However, it is during the AVP phase that the SAM acquires the competence to respond to floral inducers required for the transition to flowering.

Days to flower and leaf numbers are a direct measure of plant's chronological and developmental age respectively, and in most species are highly dependent on envi-

ronmental factors such as photoperiod, temperature and [CO_2]. Throughout the last decades, much work was done in the field of environmental control of plant growth and development by conducting research on the effect of elevated [CO_2] on photosynthesis (Gunderson and Wullschleger, 1994; Norby *et al.*, 2002; Long *et al.*, 2004; Rogers *et al.*, 2004), plant growth (Ferris *et al.*, 2001; Taylor *et al.*, 2003; Hattenschwiler *et al.*, 2002), and carbohydrate accumulation (Masle *et al.*, 1993; Kehr *et al.*, 2001). It has been concluded that the effects of elevated [CO_2] on plant growth and development can be divided into primary and secondary effects. Primary effects comprise increasing net photosynthesis (Stitt, 1991; Long and Drake, 1992), decreasing stomatal conductance (Mousseau and Saugier, 1992; Gonzalez-Meler *et al.*, 1996) and influence on dark respiration (Curtis and Wang, 1998; Davey *et al.*, 2004). Secondary effects include alterations in more complicated characteristics such as anatomy, morphology, physiology and development (Ceulemans and Mousseau, 1994). Despite the ample evidence regarding the effect of elevated [CO_2] on flowering, data on its effect on JVP are limited. Some studies have shown that the effect of CO_2 on flowering can be entirely explained by its effect upon growth (He and Bazzaz, 2003), whereas others have found that the effect of CO_2 on growth is poorly correlated with its effect on time to flowering (Marc and Gifford, 1984; Reekie and Bazzaz, 1991; Reekie *et al.*, 1994). However, it can be hypothesized that elevated [CO_2] might affect time to flowering by having a direct impact on the pre-flowering developmental phases such as the JVP. It has been shown that the presence of CO_2 might be required by photoperiod sensitive species for their temporal response to photoperiod (Bassi *et al.*, 1975; Langston and Leopold, 1954) via a yet unknown mechanism.

In addition to these variables, LI is also known to have an impact on flower initiation in some plant species. Exposure to low or high LI levels can delay or hasten the time to flowering, respectively (Chapter 1.5.2.8).

In this Chapter, the effects of different environmental variables on the phases of photoperiod sensitivity in *Antirrhinum* were studied. Plant reciprocal transfer

experiments between inductive and non-inductive conditions were conducted. The temporal response to photoperiod, as revealed by the number of days to flowering and the number of nodes beneath the corolla of the first opened flower, was used as an assay to estimate the length of JVP and AVP in plants. Furthermore, plant material was generated in clearly defined growth phases, to enable juvenile and adult phase material to be sampled for molecular and physiological analyses.

3.2 Specific Materials and Methods

3.2.1 Photoperiod Experiments

Photoperiod experiments were carried out in photoperiod chambers in GH compartments and CE growth chambers (Figure A.1).

3.2.1.1 Effect of LI on JVP Length in *Antirrhinum* Bells F1

Experiments using *Antirrhinum* Bells F1 were setup as described in Chapter 2.2.1. Summer, winter and spring experiments were initiated on May 12, 2005, Nov 17, 2006 and March 4, 2008, respectively. The summer and winter reciprocal transfer experiments were setup and carried out by A. Massiah, A. Jackson and V. Valdes as a part of the HH3728SX DEFRA project. In all experiments four photoperiod chambers (1.26 m x 2.05 m x 2.4 m tall; Figure A.1A) in the same GH compartment (9.9 m x 9.6 m) were used. Plants were grown on automated trolleys (1.7 m^2, Figure A.1A), which received natural daylight for 8 h d^{-1}. At 16:00 h Greenwich Mean Time (GMT) each day the trolleys were programmed to move into light-tight chambers, where they remained until 08:00 h the following day. For all experiments two daylengths were exploited: SDs (8 h d^{-1}) and LDs (16 h d^{-1}). SD grown plants did not receive any further light. LDs were provided using 8 h d^{-1} extension lighting within the photoperiod chambers from a combination of incandescent (Philips 32W, NL) and fluorescent (General Electric 60W, HU) light tubes (ratio = 6:1) providing approximately 4 μmol m^2 s^{-1} photosynthetically-active radiation (PAR) at plant height.

Photoperiod compartments were ventilated at night to minimise any temperature increase caused by the lamps operation. A fan built into the top of each chamber was used to draw air into the compartments from light-tight vents built into the base of the trolleys, providing approximately 17 air changes per hour. The aerial

environment (temperature, RH, [CO_2] and PPFD) was monitored independently and logged on a computer via the Orchestrator SCADA™ (Measurement Systems Ltd., Berkshire, UK) software.

To investigate the effect of different levels of LI on the length of JVP and AVP within the same experiment, SDs and LDs were applied in combination with two levels of shading (Figure A.1A). Two trolleys (one in SDs and one in LDs) were left unshaded, while the other two (one in SDs and one in LDs) were shaded with XLS 16F shade material (37% transmission; AB Ludvig-Svennson, Kinna, SE). Plant reciprocal transfers between SD and LD treatments were started 7 days after 50 % seedling emergence. Ten plants were transfered weekly for up to 11 weeks, within both shaded and unshaded treatments. A further 20 plants were grown until flowering in constant SDs and LDs under each level of shade as controls. The growth phases and JVP length were clearly defined as described in Chapter 2.2.3. Plant material was generated in known growth phases, to enable juvenile and adult phase material to be sampled for further studies. All the experiments had an unreplicated 2 x 2 factorial design. Flowering time was recorded as described in Chapter 2.2.3.1.

3.2.1.2 Defining the Juvenile Phase Length in *Antirrhinum* RIL57

The reciprocal transfer experiment was carried out in Sanyo Fitotron (Loughborough, UK) growth chambers under 300 μmol m^2 s^{-1} PAR. The experiment was setup as described in Chapter 2.2.1. SD conditions (8 h d^{-1}) were provided by using a combination of fluorescent (General Electric 60W, HU) and incadescent (Philips 32W, NL) light tubes. LDs were provided using 8 h d^{-1} extension lighting within the photoperiod chambers from a combination of incandescent (Philips 32W, NL) and fluorescent (General Electric 60W, HU) light tubes providing approximately 4 μmol m^2 s^{-1} PAR at plant height (Figure A.2). Day and night temperatures were set to 20°C. Five replicate plants were randomly selected from each photoperiod and transferred from SDs to LDs and *vice versa* weekly, for up

to 9 weeks. A further 20 plants were grown in constant SDs and LDs conditions as controls. The JVP was clearly defined as described in Chapter 2.2.3. Flowering time was recorded as described in Chapter 2.2.3.1.

3.2.1.3 Effect of [CO_2] on JVP Length in *Antirrhinum* Bells F1

The reciprocal transfer experiment was carried out in Saxcil (Chester, UK) growth cabinets using the *Antirrhinum* Bells F1. The experiment was setup as described in Chapter 2.2.1. Two chambers were setup to SD conditions and two for LDs. SD conditions (106 µmol m^2 s^{-1} PAR) were achieved by using a combination of fluorescent (General Electric 60W, HU) and incandescent (Philips 32W, NL) light tubes. LD conditions consisted of a combination of fluorescent (General Electric 60W, HU) and incandescent (Philips 32W, NL) light for the first 8 h d^{-1} (100 μmol m^2 s^{-1} PAR) and low intensity (6 μmol m^2 s^{-1} PAR) incandescent (Philips 32W, NL) light for the 8 h d^{-1} extension. The spectra of experimental light are shown in Figure A.3. Day and night temperatures were set to 20°C.

One of the two SD and LD cabinets were provided with CO_2 at 1000 vpm, and the other at ~385 vpm CO_2. Plant reciprocal transfers were carried out weekly, for up to 12 weeks. Five plants were transferred from SD to LD and *vice versa*, between cabinets set at the same [CO_2]. A further 10 plants were grown until flowering in constant SDs and LDs at each [CO_2] as controls.

3.2.2 Estimation of Phases of Photoperiod Sensitivity

Phases of photoperiod sensitivity were estimated using flowering time data as described in section 2.2.3. Data were fitted in the model by S. R. Adams and V. Valdes by using the FITNONLINEAR directive of GenStat (GenStat V12, Rothamsted, UK).

3.3 Results and Discussion

3.3.1 Quantifying the Effect of LI on Flowering in *Antirrhinum* Bells F1

The effect of LI on the length of the JVP was investigated under natural differences in LI in experiments carried out during summer, winter and spring light conditions (Chapter 3.2.1.1). Additionally, LI was manipulated within each experiment by imposing a shaded treatment. To ensure plant growth and development under LD conditions received similar LI to those grown under SDs, daylength was artificially increased without modifying the total quantity of light available for photosynthesis, by extending the SD treatment with low intensity incandescent light. *Antirrhinum* plants grown during the winter experiment received the lowest PPFD (Table 3.1) compared to plants grown during the spring and summer experiments. This is due to the time of year and the use of shade screens.

Table 3.1: **The Effect of LI on Flowering Times Under SD and LD Conditions in *Antirrhinum* Bells F1**

	Days to flowering		Nodes to flowering	
PPFD	LD	SD	LD	SD
Summer				
Unshaded [13.9]	52.6 (±0.2)	62.1 (±0.2)	10.0 (±0.1)	15.8 (±0.1)
Shaded [4.4]	66.7 (±0.5)	87.8 (±0.9)	16.0 (±0.3)	23.6 (±0.1)
Winter				
Unshaded [7.3]	94.6 (±0.5)	119.3 (±0.4)	14.2 (±0.2)	22.6 (±0.5)
Shaded [1.6]	121.6 (±0.6)	151.4 (±0.7)	16.1 (±0.2)	29.0 (±0.5)
Spring				
Unshaded [11.5]	60.9 (±0.7)	74.5 (±1.3)	12.3 (±0.3)	20.1 (±0.4)
Shaded [4.0]	65.2 (±1.5)	91.2 (±1.5)	16.3 (±0.4)	26.2 (±0.2)

Number of days and number of nodes beneath the corolla of the first opened flower of *Antirrhinum* Bells F1. Plants were grown in SDs and LDs, under shaded and unshaded conditions. PPFD is expressed as mol m^{-2} d^{-1} in square brackets. Standard error of mean (SEM) indicated in parenthesis.

In all experiments, node numbers at flowering and days to flowering exhibited a quantitative response to photoperiod, defining SD and LD responses. For all three seasonal experiments, plants grown under constant LDs flowered earlier than SD grown plants within each shade treatment. Reducing LI by artificial shading increased time to flowering as did natural reductions in LI.

Increasing LI level has been reported to reduce time to flowering for several species, including *Arabidopsis* (Bailey *et al.*, 2001; Moharekar *et al.*, 2007) *Achillea* (Zhang *et al.*, 1996), *Pelargonium* (Armitage *et al.*, 1981) and *Rosa* (Mortensen and Moe, 1993). However in some species, increased LI levels alone cannot adequately affect flowering time, because temperature also influences floral initiation (Adams *et al.*, 1999; Blazquez *et al.*, 2003; Samach and Wigge, 2005). Studies on these four species have shown that time to flowering was reduced by increasing LI. Nevetheless, no mention is made of the effect of temperature. Therefore, it is unclear whether the reduction in days to flowering was a result of increased plant temperature, resulting from increased irradiance, or a reduction in days to flowering by the absolute irradiance effect. However, in *Antirrhinum* the latter is the most likely case; as shown for *Petunia* LI had a greater effect on *Antirrhinum* flowering time than temperature (Adams *et al.*, 1999; Adams, S. R., in preparation).

3.3.2 Quantifying the Effect of LI on JVP Length in *Antirrhinum* Bells F1

Previous studies to determine the length of JVP in several species using reciprocal transfer experiments between inductive and non inductive conditions have combined and analysed all of the flowering data simultaneously (Ellis *et al.*, 1992, 1997; Adams *et al.*, 1999).

The analytical approach developed by Adams *et al.* (2003), utilising both days to flowering and number of nodes at flowering was applied to estimate durations of photoperiod-insensitive and photoperiod-sensitive phases of plant development

under different LI. A combined analysis of this type has the advantage of helping to separate the effects of photoperiod on flower induction and flower development in terminal flowering species such as *Antirrhinum*, since no more leaves are produced on the main stem once flower initiation has occurred. The reciprocal transfer experiments were carried out under three different seasons (Figure 3.1; Figure 3.2; Figure 3.3).

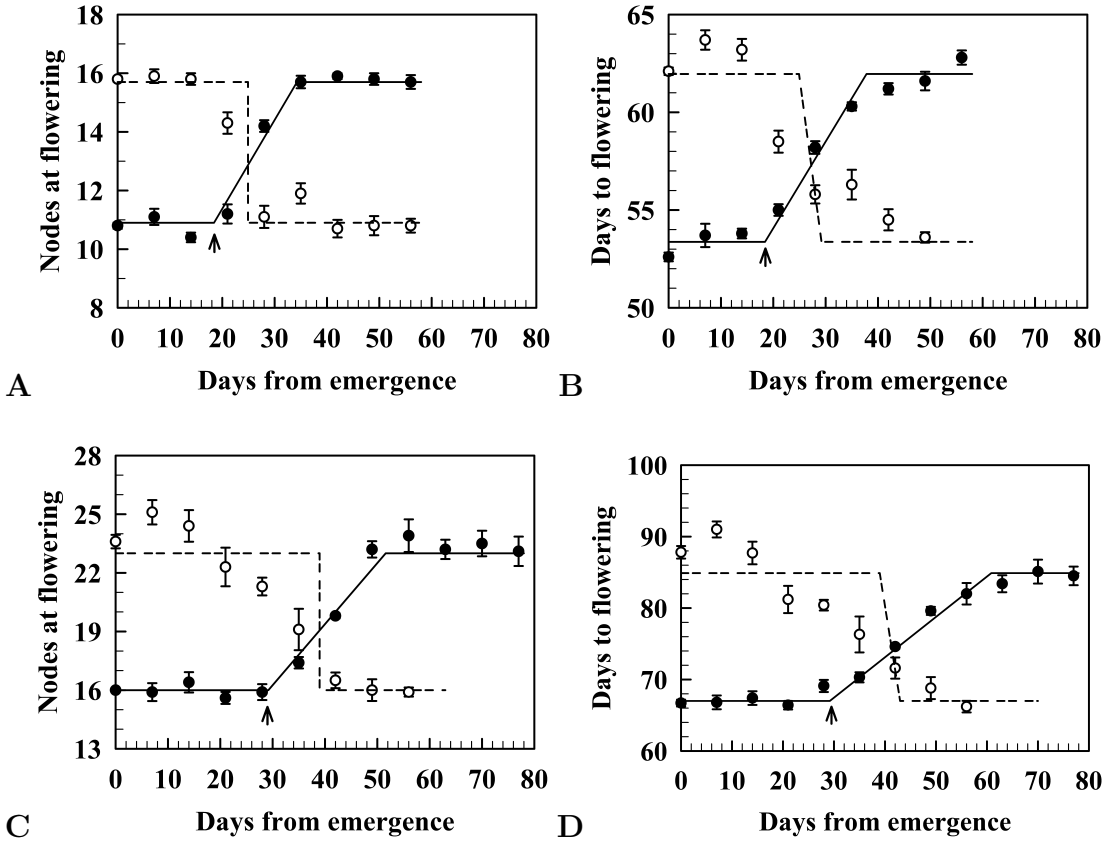

Figure 3.1: **The Effect of LI on Phases of Photoperiod Sensitivity During the Summer Experiment in *Antirrhinum* Bells F1**

Antirrhinum Bells F1 plants grown under unshaded (A, B) and shaded (C, D) conditions during the summer experiment. (A, C) number of nodes at flowering; (B, D) number of days to flowering. Plant reciprocal transfers from SDs to LDs and *vice versa* were conducted weekly. Solid (–) and broken (- -) lines show the fitted relationships for SDs to LDs (●) and LDs to SDs (○) transfers, respectively. Arrows indicate the end of JVP. Error bars indicate the SEM.

For all experiments, exposure to SD conditions during JVP had no effect on the time or the node number at flowering, as *Antirrhinum* plants were effectively insensitive to photoperiod during this period. However, after the end of the JVP, SDs delayed flowering and increased the number of nodes at flowering. Similarly, when plants were transferred from LDs to SDs, exposure to LDs during the JVP had no effect on the node number or time to flowering (Figure A.4).

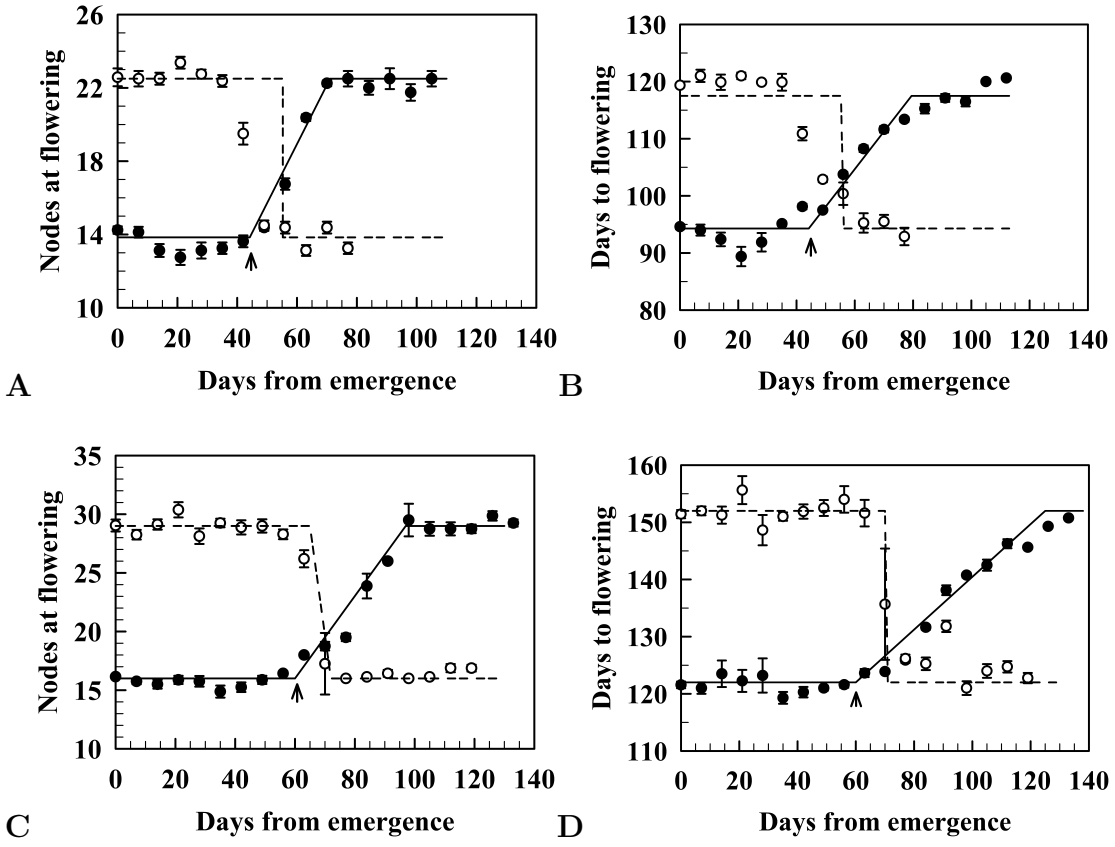

Figure 3.2: **The Effect of LI on Phases of Photoperiod Sensitivity During the Winter Experiment in *Antirrhinum* Bells F1**

Antirrhinum Bells F1 plants grown under unshaded (A, B) and shaded (C, D) conditions during the winter experiment. (A, C) number of nodes at flowering; (B, D) number of days to flowering. Plant reciprocal transfers from SDs to LDs and *vice versa* were conducted weekly. Solid (–) and broken (- -) lines show the fitted relationships for SDs to LDs (●) and LDs to SDs (○) transfers, respectively. Arrows indicate the end of JVP. Error bars indicate the SEM.

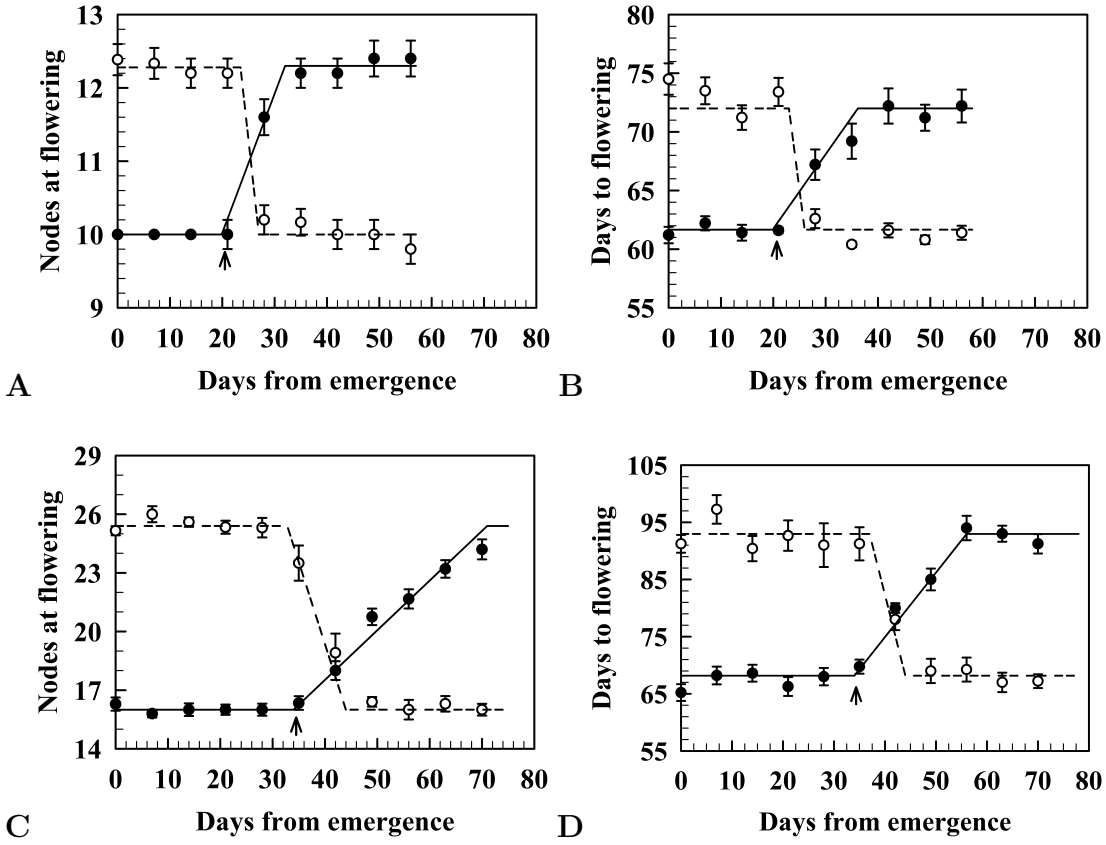

Figure 3.3: **The Effect of LI on Phases of Photoperiod Sensitivity During the Spring Experiment in *Antirrhinum* Bells F1**

Antirrhinum Bells F1 plants grown under unshaded (A, B) and shaded (C, D) conditions during the spring experiment 2008. (A, C) number of nodes at flowering; (B, D) number of days to flowering. Plant reciprocal transfers from SDs to LDs and *vice versa* were conducted weekly. Solid (–) and broken (- -) lines show the fitted relationships for SDs to LDs (●) and LDs to SDs (○) transfers, respectively. Arrows indicate the end of JVP. Error bars indicate the SEM.

A hastening of time to flowering and decrease in the node number at flowering was seen once sufficient LDs had been received for flower commitment, following the end of the juvenile phase (start of AVP).

Reducing LI in all experiments by shading, caused a significant increase in the length of JVP (Figure 3.1; Figure 3.2; Figure 3.3; Table 3.2). This increase was more dramatic in winter experiment with 15.8 d, due to this season's reduced ambient LI levels, followed by the spring and summer experiments with 14.1 and 10.7 d.,

respectively. In addition to prolonged JVP under shaded conditions, modification of AVP was also observed (Table 3.2). Plants grown under shaded conditions in summer, winter and spring experiments remained photoperiod sensitive 6.6, 10.3 and 5.7 d longer than plants grown under unshaded conditions, indicating that both developmental phases are subject to modification.

Table 3.2: **The Effect of LI on Juvenile and Adult Phases Length in *Antirrhinum* Bells F1**

	Phases of Plant Development	
PPFD	**JVP**	**AVP**
Summer		
Unshaded [13.9]	18.4 (±0.9)	15.9 (±1.5)
Shaded [4.4]	29.2 (±1.4)	22.4 (±2.0)
Winter		
Unshaded [7.3]	44.2 (±1.5)	26.9 (±2.4)
Shaded [1.6]	60.0 (±0.8)	37.2 (±1.4)
Spring		
Unshaded [11.5]	19.9 (±0.3)	16.3 (±1.2)
Shaded [4.0]	34.0 (±0.5)	22.0 (±2.1)

Differences in the duration (d) of the phases of sensitivity to photoperiod fitted using the FITNONLINEAR directive of GenStat (GenStat V12, Rothamsted, UK). Plants were grown under shaded and unshaded conditions. PPFD is expressed as mol m^{-2} d^{-1} in square brackets. SEM indicated in parenthesis.

These results are in agreement with studies on the effect of shading on the length of JVP in *Petunia* spp. (Adams *et al.*, 1999). However, it was unclear whether other species will be as responsive to LI or whether it is species specific. The length of the JVP in *Antirrhinum* has been shown to be sensitive to LI since reducing levels by shading cause a dramatic increase in the length of JVP.

For many years the horticulture industry has manipulated light environment to enhance useful commercial properties (Mortensen and Strømme, 1987; Moe *et al.*, 1992; Mortensen and Moe, 1993; Mattson and Erwin, 2005). Photoperiodic regulation of flowering is a regular practice in many species such as *Chrysanthemum*

spp. (Cathey and Borthwick, 1970) and *Euphorbia pulcherrima* (Moe *et al.*, 1992). However, this practice is currently not used to its full potential. Under protected cultivation, photoperiod manipulation can be achieved with either light extension by using supplementary electric lighting systems with specific wave bands (Mortensen and Strømme, 1987; Mortensen and Moe, 1993) or spectral filtering GH screens (black-out screens; Rajapakse and Kelly, 1995; Haeringen *et al.*, 2008). However, the data in this Chapter have shown that the timing of such treatments is critical. Using day extension or night-break lighting for photoperiodic regulation of flowering too early, while plants are in JVP will be ineffective in hastening flowering in LDPs as they are incompetent to respond to the photoperiodic stimulus. Therefore, manipulating the length of JVP by increasing the LI levels would have an important impact in crop profitability.

Light constitutes a critical environmental plant growth indicator, which is estimated by the duration, quality, direction and intensity (Thomas, 2006), as well as the essential energy source for photosynthesis (Bjorkman, 1981). It has been demonstrated that responses of the photosynthetic apparatus to shade conditions can be at two different levels: either at the structural level such as leaf anatomy, or at the level of the biochemistry of the photosynthetic apparatus (Bjorkman, 1981). More specifically, plants that grow in shaded environments invest relatively more of their photosynthetic products and other resources in leaf area (LA) and they have a high LA ratio. Their leaves are relatively thin: They have a high specific LA and low leaf mass density (Bjorkman, 1968; Goodchild *et al.*, 1972). This is associated with relatively few, small palisade mesophyll cells per unit area (Bjorkman, 1968; Goodchild *et al.*, 1972; Bjorkman, 1981). However, the leaves of plants grown under shaded conditions have a high chlorophyll concentration per unit fresh mass, which results in a rather similar chlorophyll concentration per unit LA as that in leaves of unshaded grown plants, but relatively less protein per unit chlorophyll (Bjorkman, 1968, 1981; Lichtenthaler and Babani, 2004).

On the other hand, one of the mechanisms by which unshaded grown plants achieve

a high light-saturated rate of net CO_2 assimilation at ambient [CO_2] is by producing thicker leaves, which provides space for more chloroplasts per unit LA. The increased thickness is largely due to the formation of longer palisade cells in the mesophyll (Bjorkman, 1981; Boardman, 1977). Furthermore, leaves of plants grown under unshaded conditions have larger amounts of Calvin-cycle enzymes per unit LA as compared with leaves of plants grown under shaded conditions, due to more cell layers, a larger number of chloroplasts per cell, and a larger volume of stroma where these enzymes are located, compared with the leaves of plants grown in shaded conditions (Bjorkman, 1968; Goodchild *et al.*, 1972; Boardman, 1977; Bjorkman, 1981). Moreover, leaves of plants grown under unshaded conditions also have more stroma-exposed thylakoid membranes, which contain the b_6f cytochromes and ATPases (Anderson *et al.*, 1973; Evans and Seemann, 1989). It is possible that all these components might be involved in the enhanced photosynthetic capacity of leaves of the unshaded grown plants.

Therefore, it can be hypothesized that the prolonged JVP length of plants grown under shaded conditions is due to insufficient supply of photosynthates produced in leaves under these conditions. Hence, plant material from clearly defined developmental growth stages was collected from plants grown under shaded and unshaded conditions, during the summer and winter experiments for qualitative and quantitative carbohydrate determinations (Chapter 4.3.2.2).

3.3.3 Controlled Environment Juvenility Experiments

3.3.3.1 Defining the Juvenile Phase Length in *Antirrhinum* RIL57

A reciprocal transfer experiment was conducted under CE conditions (Chapter 2.2.1) to determine the phases of photoperiod sensitivity in *Antirrhinum* RIL57 (Chapter 2.2.3; Figure 3.4).

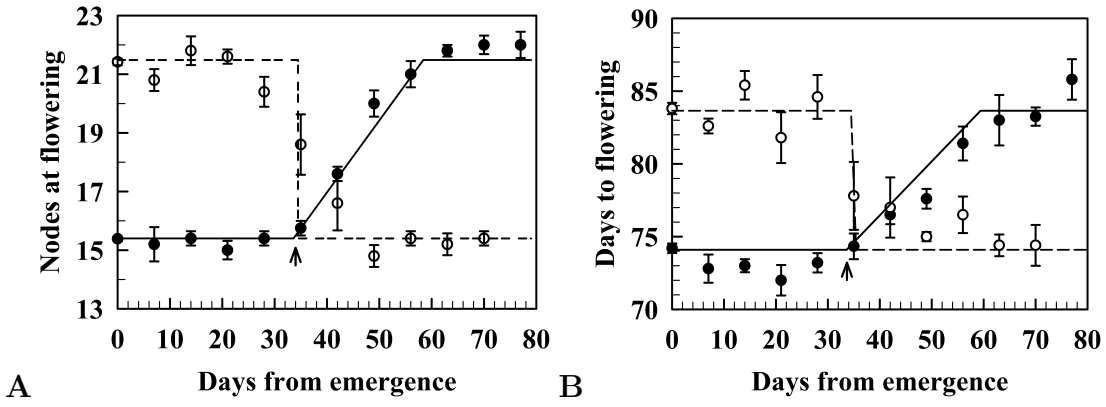

Figure 3.4: **Phases of Photoperiod Sensitivity in *Antirrhinum* RIL57**

Phases of photoperiod sensitivity in *Antirrhinum* RIL57 as revealed by the reciprocal transfer approach. Plants grown under 300 μmol m^2 s^{-1} PAR in Sanyo Fitotron (Loughborough, UK) growth cabinets. (A) number of nodes at flowering; (B) number of days to flowering. Plant reciprocal transfers from SDs to LDs and *vice versa* were conducted weekly. Solid (–) and broken (- -) lines show the fitted relationships for SDs to LDs (●) and LDs to SDs (○) transfers, respectively. Arrows indicate the end of JVP. Error bars indicate the SEM.

The study of time to flowering in RIL57 indicates that *Antirrhinum* plants grown in continuous LDs flowered with 15 nodes within 74 d, whereas those grown under continuous SDs flowered with 21 nodes within 84 d (Figure 3.4). Under the experimental conditions used, the length of the JVP was estimated as 34 d whereas the AVP 25 d. (Table 3.3).

Table 3.3: **The Duration of Developmental Stages in *Antirrhinum* RIL57**

Experimental Conditions		**Phases of Photoperiod Sensitivity**	
		JVP	**AVP**
[CO_2]	**PAR**		
Ambient	300	33.6 (±1.8)	24.8 (±3.2)

Differences in the duration (days) of the phases of sensitivity to photoperiod fitted using the FITNONLINEAR directive of GenStat (GenStat V12, Rothamsted, UK). PAR is expressed as μmol m^2 s^{-1}. SEM are shown in parenthesis.

This experiment was carried out to provide plant material at clearly defined de-

velopmental stages from which to extract phloem exudates. These were used to qualitatively and quantitatively determine carbohydrates (Chapter 4.3.2.4).

3.3.3.2 Quantifying the Effect of [CO_2] on Flowering in *Antirrhinum* Bells F1

The effect of daylength under ambient and elevated [CO_2] on flowering is summarized in Table 3.4.

Table 3.4: **The Effect of [CO_2] on *Antirrhinum* Flowering Time in Plants Grown Under Continuous SD and LD Conditions**

Experimental Conditions		Days to flowering		Nodes at flowering	
		LD	SD	LD	SD
LI	**[CO_2]**				
2.5	Ambient	70.4 (±0.7)	117.9 (±0.8)	14.8 (±0.2)	42.8 (±0.4)
2.5	Elevated	56.0 (±0.5)	106.5 (±0.8)	12.6 (±0.1)	41.1 (±0.3)

Flowering time data obtained from experiments using *Antirrhinum* Bells F1. Plants were grown in constant SDs and LDs under ambient and elevated [CO_2]. LI is expressed as mol m^{-2} d^{-1}. SEM indicated in parenthesis. Elevated and ambient [CO_2] were 1000 and ~385 vpm, respectively.

Antirrhinum plants flowered at a higher node number in SDs than LD conditions, whereas under ambient [CO_2], flowering was significantly delayed as evidenced from both flowering indicators. Under elevated [CO_2] time to flowering was accelerated in plants grown in LDs compared to plants grown in SDs. [CO_2] has been shown to have variable effects on flowering time. Elevated [CO_2] sometimes hastens flowering (Zhang and Lechowicz, 1995) but some other times have shown no effect (Zhang and Lechowicz, 1995; Ward and Strain, 1999). Some studies have shown that the effect of elevated [CO_2] on time to flowering can be entirely explained by its effect upon growth (He and Bazzaz, 2003), whereas others have found that the effect of elevated [CO_2] on growth is poorly correlated with its effect on time to flowering (Marc and Gifford, 1984; Reekie and Bazzaz, 1991; Reekie *et al.*, 1994).

However, it has been demonstrated that the presence of CO_2 is required by the photoperiod sensitive species for their temporal response to photoperiod (Langston and Leopold, 1954; Evans, 1969; Bassi *et al.*, 1975), whereas an extremely high [CO_2] (approx. 10000-50000 μl l^{-1}) can reverse the photoperiodic stimulus, allowing plants to flower in a non-inductive photoperiod (Purohit and Tregunna, 1974; Hicklenton and Jolliffe, 1980). It can be hypothesized that elevated [CO_2] might affect time to flowering by influencing the pre-flowering developmental phases.

3.3.3.3 Quantifying the Effect of [CO_2] on JVP Length in *Antirrhinum* Bells F1

The effect of CO_2 on JVP length is not well understood. However, as increased LI levels hastened juvenility and time to flowering in *Antirrhinum*, possibly due to photosynthate availability, it can be hypothesized that elevated [CO_2] affects time to flowering by having a direct impact on the JVP. This hypothesis was tested by carrying out CE reciprocal transfer experiments to investigate the impact of CO_2 enrichment on the growth phases of photoperiod sensitivity in plants grown under low LI levels (2.5 mol m^{-2} d^{-1} PPFD).

It has been demonstrated that LI below a particular compensation point limits photosynthesis and thus net carbon accumulation and plant growth. Therefore, it was further hypothesized that the effect of [CO_2] enrichment is or will be more pronounced at low PPFD, as it is likely at this particular compensation point photosynthates would be a limiting factor.

The phases of photoperiod sensitivity were estimated by fitting flowering time data in the model (Adams *et al.*, 2003) using the FITNONLINEAR directive of GenStat (Figure 3.5). In some cases, particularly under ambient and low PPFD conditions, even though plants had initiated a terminal inflorescence the flowers did not develop, a phenomenon related to phototrophic effects known as blindness (Nell and Rasmussen, 1979); plants subsequently flowered on a secondary branch. In these

cases the leaf number on the main stem was used, but the flowering time was ignored in the data analysis and presentation.

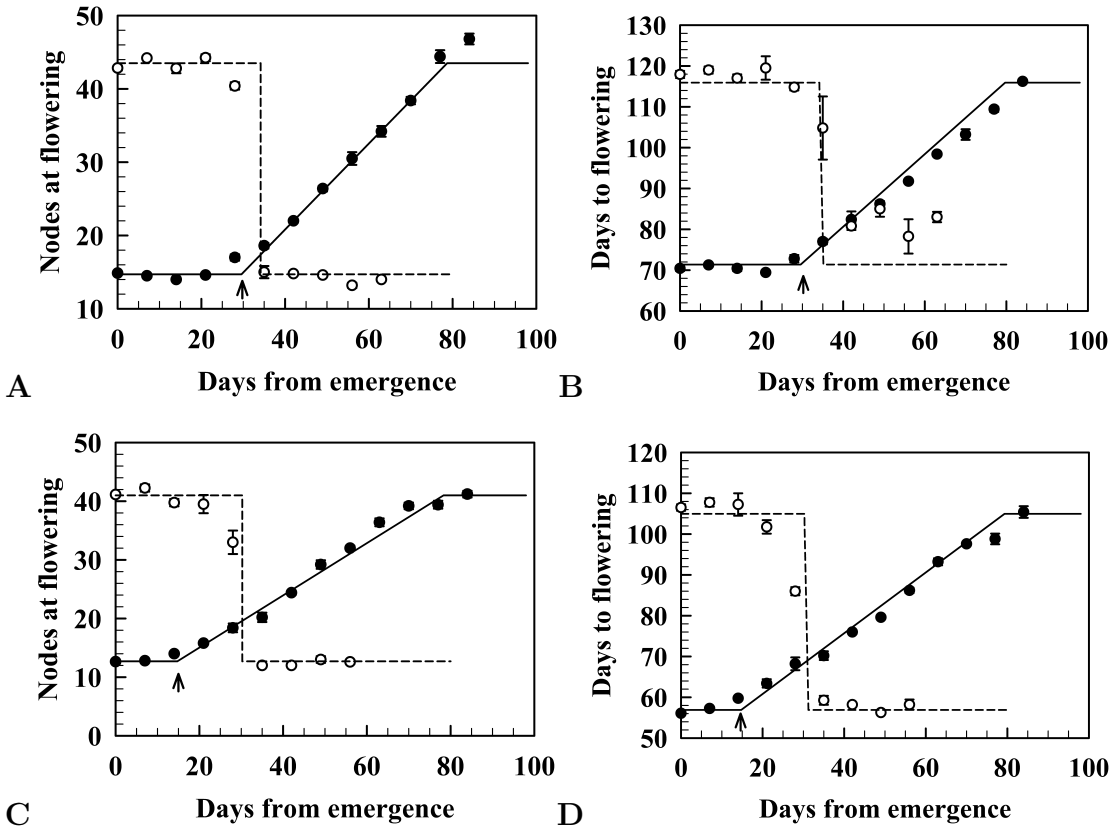

Figure 3.5: **Phases of Photoperiod Sensitivity of *Antirrhinum* Bells F1 Grown Under Ambient and Elevated [CO_2]**

Plants grown under ambient (A, B) and elevated (C, D) [CO_2] under 2.5 mol m^{-2} d^{-1} PPFD. Plant reciprocal transfers from SDs to LDs and *vice versa* were conducted weekly. Solid (–) and broken (- -) lines show the fitted relationships for SDs to LDs (●) and LDs to SDs (○) transfers, respectively. Arrows indicate the end of JVP. Error bars indicate the SEM. Elevated and ambient [CO_2] were 1000 and ˜385 vpm, respectively.

Estimates of the duration of the JVP (Adams *et al.*, 2003) are summarized in Table 3.5. CO_2 enrichment hastened the end of juvenility under 2.5 mol m^{-2} d^{-1} PPFD by approximately 14.1 d., compared to JVP of plants grown under ambient CO_2 conditions. Previous studies (Adams and Jackson, 2004) quantifying the effect of CO_2 enrichment on the length of JVP under 8 mol m^{-2} d^{-1} PPFD, also revealed an effect of elevated [CO_2] on shortening the JVP length. However, the extent

of the response was rather small (5-6 d), possibly due to the saturated LI level of 8 mol m^{-2} d^{-1} PPFD used in this experiment. However, elevated [CO_2] under reduced PPFD (2.5 mol m^{-2} d^{-1}) had a dramatic impact on time to flowering and JVP length. Such a response indicates that the effect of elevated [CO_2] on time to flowering and JVP length is accentuated under limiting LI.

Table 3.5: **The Effect of [CO_2] on the Duration of JVP in *Antirrhinum* Bells F1**

Experimental Conditions		**Plant Developmental Phase JVP**
LI	**[CO_2]**	
2.5	Ambient	28.8 (±0.7)
2.5	Elevated	14.7 (±1.1)

Plants were grown under ambient (~385 vpm) and elevated (1000 vpm) [CO_2]. LI is expressed as mol m^{-2} d^{-1}. SEM indicated in parenthesis.

It has been demonstrated that photosynthesis of plants utilizing the C_3 photosynthetic pathway is promoted by elevated [CO_2] due to the insufficient saturation of ribulose bisphosphate carboxylase (Rubisco), by ambient [CO_2] and its inhibitory affect on the competing process of photorespiration (Long and Drake, 1992; Drake *et al.*, 1997).

Therefore, it can be hypothesized that the hastened JVP length of plants grown under elevated [CO_2] is likely to be due to a enhanced supply of photosynthates produced in leaves under elevated [CO_2]. To test this hypothesis, plant material from clearly defined developmental growth stages were collected from plants grown under ambient and elevated [CO_2] for qualitative and quantitative carbohydrate determinations (Chapter 4.3.3.2; Chapter 4.3.3.3).

3.4 Conclusions

Antirrhinum has been described as a photoperiod sensitive facultative LD species (Cremer *et al.*, 1998; Adams *et al.*, 2003). The estimation of the phases of photoperiod sensitivity in the GH reciprocal transfer experiments revealed a relationship between LI and JVP length, validating previous data (Adams and Jackson, 2004). Reduced LI levels extend the length of JVP and delay floral initiation. This is probably due to the existence of a LI threshold level, which is naturally dependent on the season's ambient light conditions. The concept of critical LI threshold is not new; it has been already reported for some species (Armitage *et al.*, 1981; Foggo and Warrington, 1989; Adams, S. R., in preparation).

The effect of LI on JVP length and time to flowering may be due to assimilate availability. This is supported by results quantifying the effect of elevated [CO_2] under limited PPFD. Elevated [CO_2] significantly shortened the JVP length under 2.5 mol m^{-2} d^{-1} PPFD, showing a significant interactive effect with LI. Such a response indicates that elevated [CO_2] may accentuate the effects of increasing LI levels on JVP length and time to flowering.

Collectively, it is possible that the prolonged JVP length and time to flowering of plants grown under decreased LI and ambient [CO_2] is linked with an insufficient supply of photosynthates produced under these conditions. This is a hypothesis that is tested in Chapter 4, which reveals the relationships between assimilate levels and the transition within the vegetative phase in *Antirrhinum.*

Chapter 4

Carbohydrate Assimilate Levels and Juvenile to Adult Phase Transition Relationships in *Antirrhinum*

4.1 Introduction

Irradiance and elevated [CO_2] have profound effects on plant growth and development. Irradiance is known to have spatial and temporal impacts, such as ontogenetic modifications in unshaded and shaded leaves (Sack *et al.*, 2003; Terashima *et al.*, 2006), acclimation responses to plant canopy architecture (Niinemets *et al.*, 2004b,a) and crucial modification to flowering time (Adams *et al.*, 1999, 2001, 2003; Mattson and Erwin, 2005). Irradiance is captured by the chloroplasts and the absorbed energy is converted into photosynthate assimilates via a photochemical reaction and the reductive pentose phosphate cycle (Nobel, 2009). An increase in LI thus leads to increasing levels of ATP and $NADPH^+$ created by the light dependent reactions, increasing the contents of starch, Suc and Glc in plant tissues (Bjorkman, 1981; Nobel, 2009)

In most plant species overall growth is promoted by elevated [CO_2]. Enrichment with CO_2 in the phyllosphere often causes a reduction in stomatal density and a higher rate of CO_2 assimilation (Woodward and Kelly, 1995; Drake *et al.*, 1997; Woodward *et al.*, 2002). It is commonly accepted that carbohydrate accumulation, plant growth and [CO_2] are positively correlated (Drake *et al.*, 1997; Pritchard *et al.*, 1999; Tocquin *et al.*, 2006). For a number of plant species data have shown that photosynthesis is increased by elevated [CO_2] (Poorter and Navas, 2003; Long *et al.*, 2004), as it is its major target. In plants grown at ambient [CO_2], especially those utilizing the C_3 photosynthetic pathway, photosynthesis is still limited by the competition between CO_2 and O_2 at the active site of Rubisco, which is involved in both photosynthesis and photorespiration. Any CO_2 increase thus leads to increasing plant tissue contents of soluble and insoluble carbohydrates (Poorter *et al.*, 1997). Because of the temporal and spatial requirement of plant growth and development for photosynthate assimilates, which might be where irradiance and CO_2 metabolic pathways come together, it is critical that plants produce carbohydrates in sufficient quantities.

The determination of the abundance of low molecular weight carbohydrates is important for characterizing physiological and biochemical processes in plant species. HPLC has been extensively used for sugar separation and quantification. However, the main drawback to HPLC is that sugars do not absorb UV light at a wavelength longer than 200 nm. Despite this limitation, improvements in RID have resulted in the routine use of HPLC for sugar analysis (Peris-Tortajada, 2000; Folkes and Jordan, 1984).

In Chapter 3 it was demonstrated that a strong inverse relationship between irradiance and JVP length exists and that at limiting PPFD, $[CO_2]$ can reduce the length of the JVP in *Antirrhinum*. In this Chapter the following hypothesis is examined: the prolonged JVP length and time to flowering of plants grown under decreased LI and ambient $[CO_2]$ is linked with an insufficient supply of photosynthate assimilates produced under these conditions.

4.2 Specific Materials and Methods

4.2.1 Leaf Phloem Exudate Studies in Juvenile and Adult Vegetative Plants

The EDTA-facilitated exudation method described by King and Zeevaart (1974), Costello *et al.* (1982) and Fellows and Zeevaart (1983) was adapted to juvenile and adult vegetative *Antirrhinum* plants grown under constant SD and LD conditions as follows. Twenty leaves (the first 2 leaves below the half-expanded young leaf of 10 plants) were placed together in a 0.2 ml microfuge tube containing 180 μl 20 mM EDTA (pH 7.5) after the petioles had been re-cut under distilled water. The leaf exudation took place in airtight containers containing water to ensure maximum RH and to prevent EDTA uptake by the leaves, within a CE chamber (Sanyo Fitotron, Loughborough, UK) with 22±1°C temperature. Light was provided by a series of cool white fluorescent bulbs (120V, 0.5 Amps, 60 Hz, Sylvania, DE) delivering 100 μmol $m^{-2}s^{-1}$ light intensity.

The phloem exudate samples collected during the first 30 min were discarded and the leaf tissues were transferred to new tubes. The collection started at zeitgeber (ZT) 3 and ZT 11 at 2 h intervals for plants grown in SD and LD conditions respectively and lasted for 6 h. At the end of the procedure, leaves and phloem exudate samples were collected and stored at -20°C for further analysis. EDTA sample elimination was completed by drying the exudates and redissolving them into 1 ml of sterilized distilled water followed by 20 ml absolute MeOH, as described in Lejeune *et al.* (1988). Contents of Suc, Fru and Glc were determined as described in Chapter 2.2.4.

4.3 Results and Discussion

4.3.1 Development of the HPLC Assay to Measure Carbohydrate Contents

Carbohydrate contents (Chapter 2.2.5) present in leaf extracts were assayed by HPLC using a RID. An extraction protocol was developed using EtOH as an extraction agent (Chapter 2.2.4). Method development was carried out in leaf tissue extracts and standard solutions of 16 different monosaccharides and oligosaccharides (Chapter 2.2.5). The effect of the mobile phase flow rate was investigated so that the optimal conditions for a monosaccharide–oligosaccharide mixture determined (data not presented). Various eluent combinations and elution modes were tested to enable their separation. Optimum separations were achieved at a flow rate of 0.75 ml min^{-1} for 22 min, pressure of 40 mmHg and 30 μl sample injection volume.

Identification of HPLC peaks was postulated by retention times in relation to carbohydrates standards and using (for Glc, Fru and Suc) an LCMS approach (data not presented).

4.3.2 Effect of Light Integral on Carbohydrate Accumulation in *Antirrhinum* Bells F1

Reciprocal transfer experiments were carried out in order to examine the effect of LI on carbohydrate accumulation and their putative role within the vegetative phase change in *Antirrhinum* Bells F1 (Chapter 3.2.1.1). The findings that a strong inverse relationship between LI and juvenile phase length exists (Chapter 3.3.2), have led to an investigation to determine whether the length of JVP could, partially, be controlled by photosynthetic assimilate availability.

Plant material from clearly defined developmental growth stages was collected from plants grown under shaded and unshaded conditions, during the summer and winter experiments, respectively (Chapter 3.2.1.1).

4.3.2.1 Effect of Light Integral on Dry Weight Partitioning

The increase of biomass in aerial parts was used as a parameter to evaluate environmental effects on plant growth. DW was analyzed to evaluate relative biomass accumulation in *Antirrhinum* Bells F1 plants grown under different irradiances. A dramatic and rapid increase in DW partitioning in plants grown under unshaded conditions was observed (Figure 4.1).

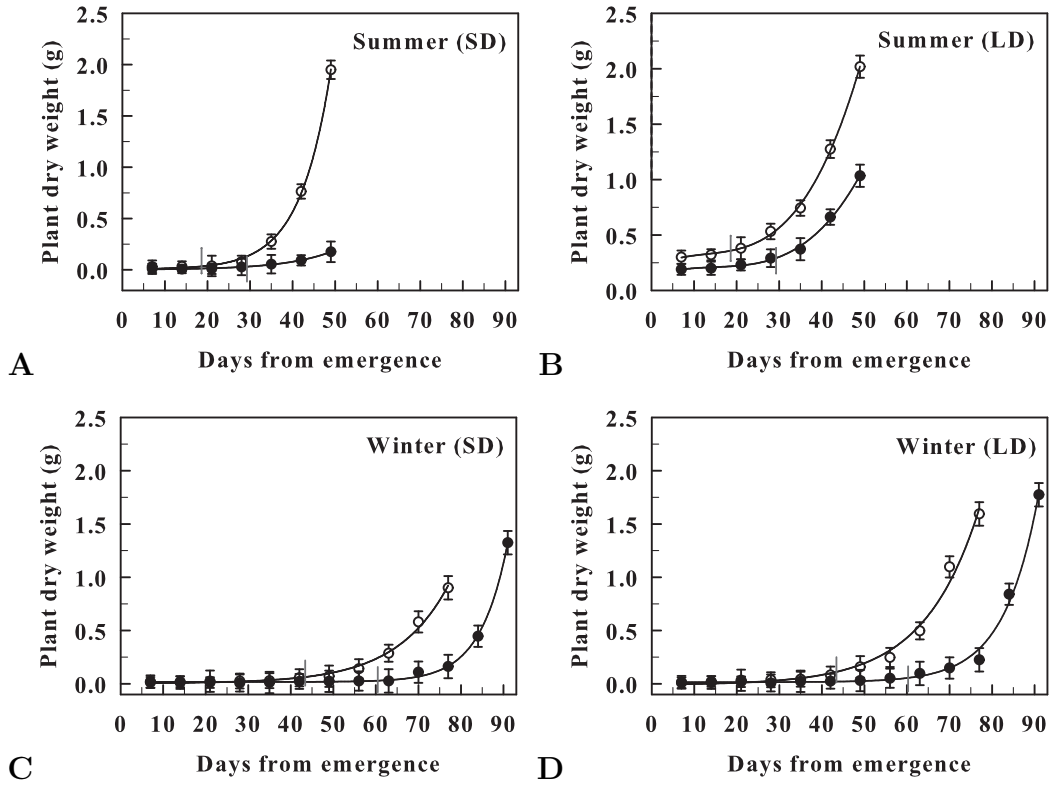

Figure 4.1: **Effect of Light Integral on Plant Dry Weights of *Antirrhinum* Bells F1**

DW of *Antirrhinum* Bells F1 plants grown under unshaded (○) and shaded (●) SDs (A, C) and LDs (B, D) during the summer (A, B) and winter (C, D) experiments. The end of JVP is indicated by a barrier (|).

The increase in total DW observed under unshaded conditions could be due to an increase in total LA and/or specific leaf area (SLA; data not presented; S.R. Adams, personal communication). The leaf number could not be included as an explanation, as unshaded plants flower with less leaf numbers than shaded plants (Table 3.1).

Antirrhinum plants are light limited under shaded conditions. Reduced LI has direct effects on DW accumulation. The reduction in DW yield of plants utilizing the C_3 photosynthetic pathway grown under shaded conditions, is primarily due to their greatly reduced photosynthetic rate under low LI (Clements and Long, 1934; Evans and Hughes, 1962; Blenkinsop and Dale, 1974). However, alterations in the photosynthetic mechanism including a decline in electron transport chain constituents and carboxylation enzymes are also critical (Boardman, 1977).

4.3.2.2 Effect of Light Integral on Soluble Carbohydrate Accumulation in *Antirrhinum* Bells F1

In order to test the hypothesis that low levels of light density decrease the accumulation of carbohydrates in plant tissues, and plants with decreased carbohydrate availability would have long length of JVP, two experiments were carried out (Chapter 3.2.1.1).

The length of JVP (Figure 3.1; Figure 3.2) as revealed by the reciprocal transfer approach, differed in plants grown under shaded and unshaded conditions in summer and winter experiments, with *Antirrhinum* plants grown under unshaded conditions having a significantly shorter JVP (Chapter 3.3.2). Compared to plants grown under shaded conditions, analysis of HPLC data for most of the carbohydrates indicated that the accumulation rate in plants grown under unshaded conditions was hastily and efficaciously increased (Summer experiment: Figure A.7 to Figure A.13; Winter experiment: Figure A.14 to Figure A.20). These observations are consistent with a linkage between LI, length of JVP and assimilate availability

in *Antirrhinum*.

The increase in the allocation of carbon in plants grown under unshaded conditions has been reported in many species (Bjorkman and Holmgren, 1963; Bjorkman *et al.*, 1972; Boardman, 1977; Pearcy *et al.*, 1987). This is especially true for the plant species grown under these conditions, in which photosynthesis occurs faster because a high quantity of ATP and $NADPH^{+}$ created by the light dependent reactions, resulting in increasing carbohydrate accumulation and DW (Blenkinsop and Dale, 1974; Boardman, 1977; Bjorkman, 1981; Chapter 4.3.2.1).

On the other hand, it has been attested that reduced LI decreases stromal volume and photochemical capacity (Bjorkman *et al.*, 1972; Crookston *et al.*, 1975) and maximal leaf photosynthetic rate at saturating irradiance (Bjorkman *et al.*, 1972; Bowes *et al.*, 1972; Mahendra *et al.*, 1974; Crookston *et al.*, 1975; Besford, 1986). Furthermore, the reduction in photosynthetic capacity of leaves grown under low irradiance is accompanied by a reduction in amount and activity of Rubisco, the key enzyme which catalyzes the Calvin cycle-fixation step of the pentose phosphate reduction pathway, and in total leaf protein (Bjorkman *et al.*, 1972; Blenkinsop and Dale, 1974; Gauhl, 1976).

In addition to reducing assimilation, reduced LI caused less carbon to be partitioned into Suc (Figure A.12 A, B; Figure A.19 B), the predominant form of photoassimilate that is imported to heterotrophic organs (Ho, 1976; Logendra and Janes, 1992; Lalonde *et al.*, 1999; Barker *et al.*, 2000), and Glc (Figure A.9; Figure eA.16), the most dominating hexose signaling molecule for gene regulation in plants (Rolland *et al.*, 2002b; Smeekens, 2000; Rolland *et al.*, 2006).

Qualitative analysis of HPLC data obtained from samples collected during the summer and winter experiments revealed the presence of the following compounds: Ery, Fru, Glu, MI Mtl, Suc and Xyl. Representative examples of the HPLC profiles generated by material collected from plants grown in constant SD and LD conditions, during the summer experiment (Figure 4.2), showed that carbohydrate levels are low during the JVP and increase once the AVP is attained.

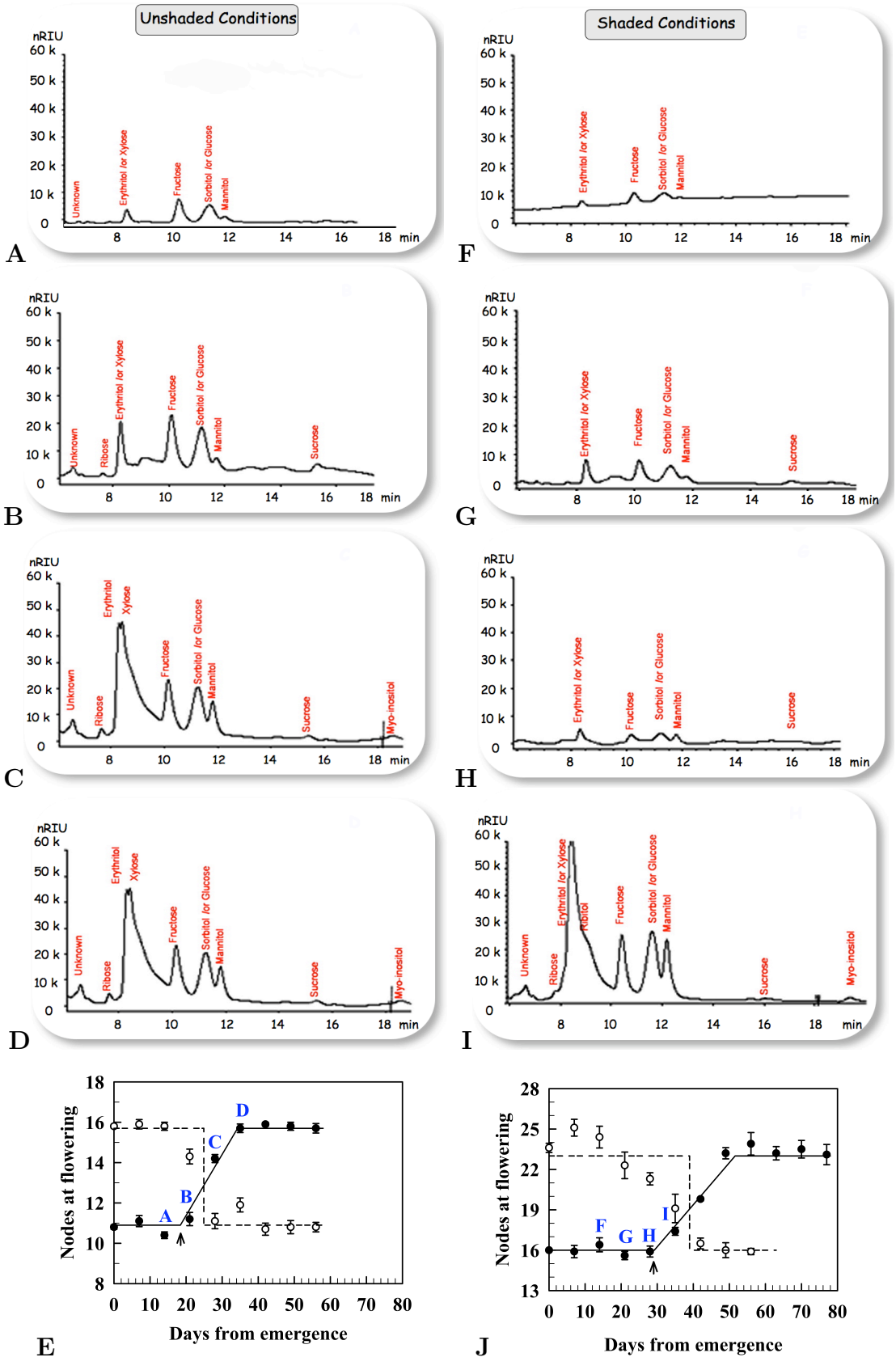
Unshaded Conditions
Shaded Conditions
nRIU
60 k
50 k
40 k
30 k
20 k
10 k
0
8
10
12
14
16
18 min
Unknown
Ribose
Erythritol /or Xylose
Erythritol
Xylose
Fructose
Sorbitol /or Glucose
Mannitol
Sucrose
Myo-inositol
A
B
C
D
E
F
G
H
I
J
Nodes at flowering
Days from emergence

Figure 4.2: Carbohydrate Profiles at Selected Stages of Plant Development from Leaf Tissue Harvested from the Unshaded and Shaded Treatments During the Summer Experiment

The corresponding stages of development represented by the carbohydrate profiles shown are indicated on the transfer experiment plots (E and J for the unshaded and shaded treatments, respectively) by letters. The end of JVP in plots E and J is indicated by a black arrow. nRIU: nano Refractive index units.

Comparing the HPLC profiles in unshaded (Figure 4.2 A, B, C and D) and shaded (Figure 4.2 F, G, H and I) conditions, it can be speculated that a total carbohydrate threshold level may be required before plants undergo the transition from a JVP to an AVP of plant development. This can be supported by comparison of profiles for juvenile stages A and F to H within the unshaded and shaded summer experiment, respectively. Comparison between experiments of profiles at stages C and H, where leaf tissue was harvested from plants at the same chronological age, but where plants from the unshaded treatment were in AVP, and plants from the shaded treatment were in JVP, further supports this hypothesis.

Further confirmation of the low carbohydrate levels during the JVP can be obtained by the HPLC profiles generated by material collected from plants grown in constant SD and LD conditions, during the winter experiment. Furthermore, a similar trend to summer experiment can be observed by studying the HPLC profiles for juvenile stages A and F to H within the unshaded and shaded winter experiment (Figure 4.3), respectively.

In plants grown under shaded conditions this carbohydrate threshold level is attained by increasing the shoot/root ratio (data not presented; S. R. Adams personal communication), by increasing the number of leaves produced (Table 3.1), and possibly by increasing the leaf longevity. However, the present results confirm an earlier suggestion that there might be a delay in the transition within the vegetative phase of plant development with respect to the attainment of maximal photosynthetic capacities and carbohydrate accumulation (Tsai *et al.*, 1997).

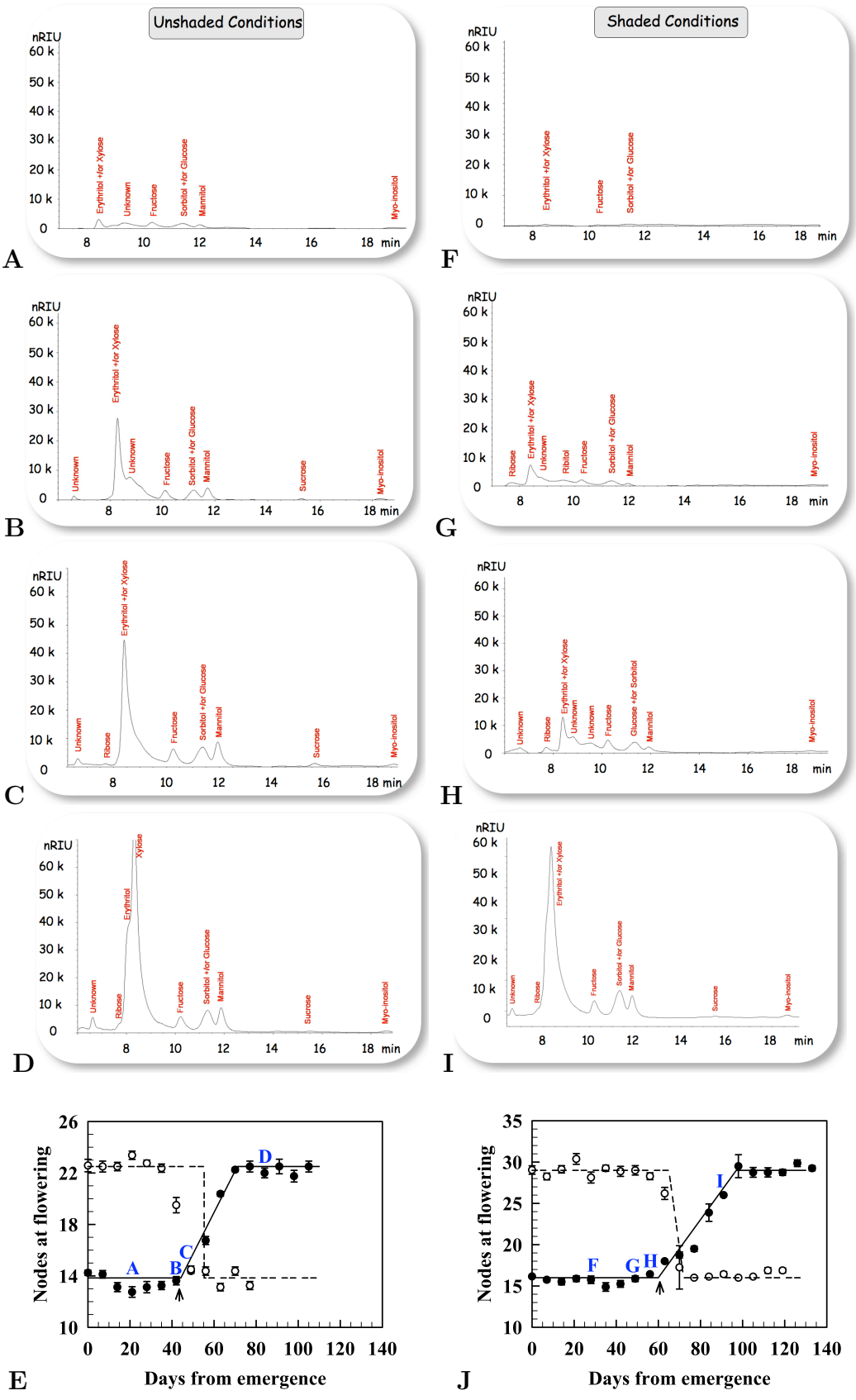
Unshaded Conditions
Shaded Conditions
nRIU
min
A
B
C
D
E
F
G
H
I
J
Nodes at flowering
Days from emergence
Erythritol +/or Xylose
Unknown
Fructose
Sorbitol +/or Glucose
Mannitol
Myo-inositol
Ribose
Sucrose
Ribitol
Glucose +/or Sorbitol
Xylose
Erythritol

Figure 4.3: Carbohydrate Profiles at Selected Stages of Plant Development from Leaf Tissue Harvested from the Unshaded and Shaded Treatments During the Winter Experiment

The corresponding stages of development represented by the carbohydrate profiles shown are indicated on the transfer experiment plots (E and J for the unshaded and shaded treatments, respectively) by letters. The end of JVP in plots E and J is indicated by a black arrow.

It has been corroborated that decreased photosynthetic rates by antisense suppression of the Rubisco small subunit in *Nicotiana tobacum*, delayed the transition from juvenile to adult phase, suggesting that plants might have a source strength threshold for full, adult shoot morphogenetic growth (Masle *et al.*, 1993; Jiang and Rodermel, 1995; Tsai *et al.*, 1997). Moreover, some other developmental responses have been found be controlled by a phototrophic pathway, as monitored by the effects of particular carbohydrate threshold levels. For instance, it has long been hypothesized that leaf senescence is initiated when the photosynthetic rate, and subsequently assimilate levels, drop below a certain threshold level (King *et al.*, 1995; Gan and Amasino, 1997; Wingler *et al.*, 1998). Furthermore, systemic acquired resistance in plants has been mediated by ectopic expression of invertase, through carbohydrate accumulation. It was shown that a particular threshold level of hexoses is necessary to activate pathogenesis-related protein genes in *Nicotiana tobacum* (Herbers *et al.*, 1996).

In comparison to the later developmental stages, a quantitative analysis of data obtained by HPLC was consistent with carbohydrate levels being a limiting factor during the JVP (Summer experiment: Figure A.7 to Figure A.13; Winter experiment: Figure A.14 to Figure A.20). *Antirrhinum* plants grown under different LI, which resulted in different juvenile phase lengths (Chapter 3.3.2), had roughly a similar carbohydrate accumulation level at the end of the JVP (Table 4.1; Figure 4.4).

Table 4.1: **Effect of Season and Light Integral on Soluble Carbohydrate Accumulation in *Antirrhinum* Bells F1**

Experimental Conditions	Soluble Carbohydrates (at the end of JVP)								JVP
	Ery	Fru	Glc	MI	Mtl	Suc	Xyl	Total	
Summer									
Shaded [4.4]	2.1	2.44	4.0	0.01	2.3	1.69	5.2	17.8	29.2
Unshaded [13.9]	2.9	2.65	2.1	0.05	0.6	1.79	4.3	14.4	18.4
Winter									
Shaded [1.6]	7.4	2.4	3.16	0.01	1.3	2.6	12.6	29.7	60
Unshaded [7.3]	8.2	2.4	3.8	0.06	2.85	1.7	16	35.1	43

Total sugars refer as the sum of Ery, Fru, Glc, MI, Mtl, Suc and Xyl. The experimental conditions for the summer and winter experiments are indicated in Chapter 3.2.1.1. Sample preparation and determination are described in Chapter 2.2.4 and Chapter 2.2.5, respectivelly. LI is expressed as mol m^{-2} d^{-1} PPFD in square brackets. JVP denotes the length of JVP in d.

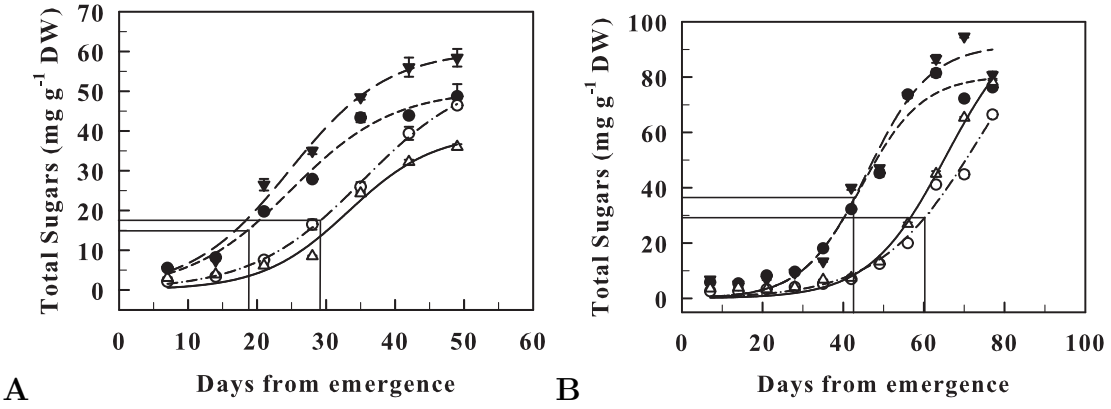

Figure 4.4: **Effect of Light Integral on Total Soluble Carbohydrates in *Antirrhinum* Bells F1**

Total soluble carbohydrate accumulation across plant development under shaded SDs (○), unshaded SDs (●), shaded LDs (▽) and unshaded LDs (▼) in summer (A) and winter (B) experiments. For the estimation of JVP length reciprocal transfer experiments were carried out (Chapter 3.3.2; Figure 3.1; Figure 3.2). Solid and broken lines show the fitted relationships. The horizontal and vertical lines indicate the total carbohydrate accumulation at the end of JVP. Total carbohydrates are refered as the sum of Ery, Fru, Glc, MI, Mtl, Suc and Xyl; they expressed as mg g^{-1} DW. The results are means ± SEM (3 separate samples, each of fifteen individual plants).

This carbohydrate threshold level was estimated for plants grown in both experimental seasons, under shaded and unshaded conditions by a non linear regression analysis (Figure 4.4; Table 4.1). However, differences in the total carbohydrate threshold values between the summer and winter experiments might be associated with the environmental conditions such as season's ambient light conditions and/or with differences in plant nutrition.

During photoperiod experiments, the photo-morphogenetic response to photoperiod is light quality dependent. Hence, during the reciprocal transfer, the photomorphogenetic response to photoperiod was correctly assayed by providing the additional hours of light in the LD treatment, with very low intensity in wavelengths that are less efficient for photosynthesis, and more efficient for a photoperiodic response (Chapter 3.2.1.1). This is reflected by the only slight differences in sugar accumulation in plants grown under SD and LD conditions in both experiments (Summer experiment: Figure A.7 to Figure A.13; Winter experiment: Figure A.14 to Figure A.20; Figure 4.4).

It is possible that the sufficient accumulated resources in plant tissues is a signal for the transition from JVP to AVP of plant development in *Antirrhinum*. This might be modulated by carbohydrate concentrations, either based on substrate supply or based on chemical signals, or both. However, the mechanisms that might be involved in this process are not known.

Carbohydrates are known to affect plant growth and development in two fundamentally different ways. Firstly, an increasing body of evidence suggests that carbohydrates have a function as hormone-like signaling molecules (Moore and Sheen, 1999; Rolland *et al.*, 2002b). Currently, there is much interest in the metabolic regulation of plant gene expression by carbohydrates and many plant genes appear to be regulated at the transcriptional level by sugar concentrations (Koch, 1996; Smeekens, 2000; Koch, 2004; Jansson, 2005; Rolland *et al.*, 2006). In the case of photosynthetic genes, it has been proposed that carbohydrates *per se* are not the direct signals controlling gene transcription but rather metabolic factor(s) related

to sugar concentrations (Krapp *et al.*, 1993) or to flux through HXK (Jang and Sheen, 1994; Jang *et al.*, 1997; Moore *et al.*, 2003). Secondly, they are important as substrates in carbon and energy metabolism in both source and sink tissues. Several lines of evidence have demonstrated that increases in assimilate transport and carbohydrate availability in the SAM may increase the rates of cell division and meristem growth and development (Bernier *et al.*, 1993; Kinsman *et al.*, 1996; Corbesier *et al.*, 1998). Therefore, naturally the phototrophic pathway might affect the transition within the vegetative phase in a more indirect manner, perhaps by altering the sink/source ratio.

4.3.2.3 Effect of Light Integral on Insoluble Carbohydrate Accumulation in *Antirrhinum* Bells F1

In most plant species, starch and Suc are the primary products of photosynthesis. They are synthesised concurrently in the leaves. Leaf starch is degraded throughout the night and the carbohydrates released used to provide substrates for respiration of the leaf, and for continued Suc synthesis and export (Zeeman and Ap Rees, 1999; Zeeman *et al.*, 2004, 2007). In order to reveal the relative changes in starch accumulation across plant development, leaf material was generated in defined growth phases by carring out a reciprocal transfer experiment (Chapter 3.2.1.1). The starch content was determined by measuring the Glc released from the insoluble fraction after treatment with α-amylase and amyloglucosidase (Chapter 2.2.6.2).

The length of JVP as revealed by the reciprocal transfer approach, was significantly shorter in plants grown under unshaded conditions (Chapter 3.3.2; Figure 3.3). Starch accumulation in plants grown under both irradiances increased following germination (Figure 4.5 A, B). However, compared to plants grown under shaded conditions, starch synthesis in *Antirrhinum* plants grown in unshaded conditions rapidly increased. It might be the increasing levels of starch accumulation in the leaves of adult *Antirrhinum* plants to be a symptom that carbohydrates are available in excess for plant growth demand and phloem transport capacities.

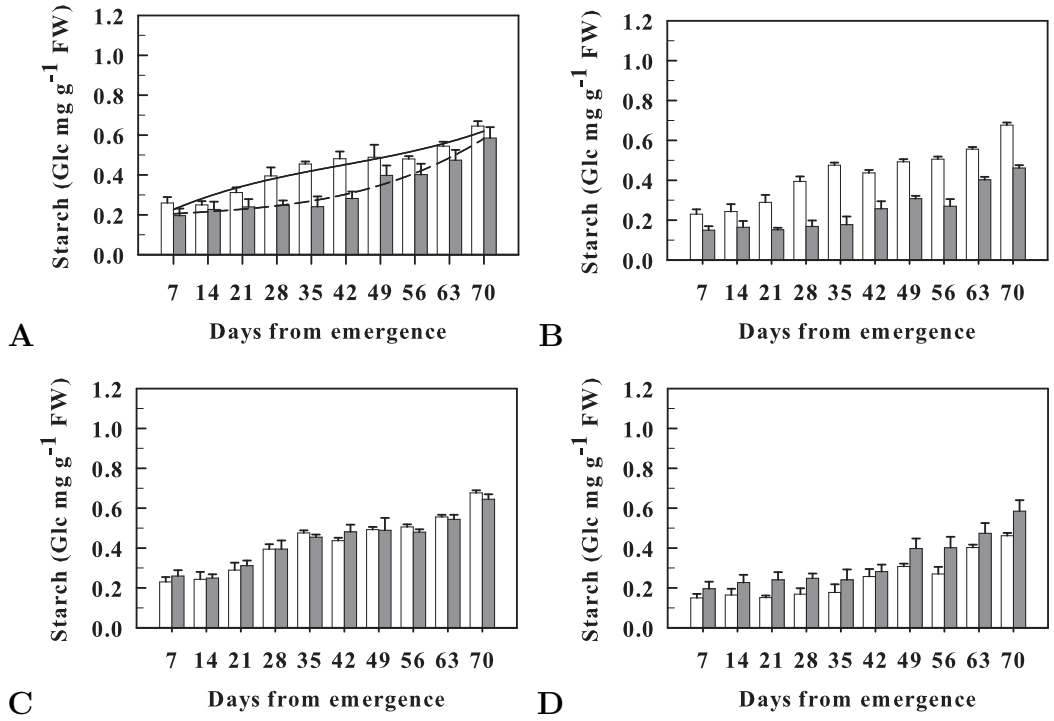

Figure 4.5: **Effect of Light Integral on Starch Accumulation in *Antirrhinum***

(A, B) Starch accumulation across plant development under unshaded (□) and shaded (■) conditions, in SD (A) and LD (B) photoperiods. (C, D) Starch accumulation across plant development in LD (□) and SD (■) photoperiods, under unshaded (C) and shaded (D) conditions. Solid lines in plot A show the fitted relationships. For the estimation of JVP length a reciprocal transfer experiment was carried out (Chapter 3.2.1.1; Figure 3.3) The length of juvenile phase was estimated to 19 d and 34 d from seedling emergence, in unshaded and shaded conditions, respectively. The results are means ± SEM (3 separate samples, each of fifteen individual plants).

Similar to the soluble carbohydrate accumulation (Chapter 4.4), slight differences in starch accumulation in plants grown under both photoperiods were observed (Figure 4.5 C, D). This is due to low intensity incandescent light provided in the 8 h^{-1} extension in the LD treatment (Vince-Prue and Guttridge, 1973; Martinez-Zapater and Somerville, 1990; Bagnall *et al.*, 1995; Chapter 3.2.1.1). Furthermore, small differences in the starch contents between leaf tissue collected from plants grown in both irradiances at the end of JVP, were observed (Table 4.2). Taken together, these results could suggest that a sufficient starch accumulation level

is critical in order to sustain a steady supply of several hexoses and Suc for the transition within the vegetative phase.

Table 4.2: **Effect of Light Integral on Starch Accumulation in *Antirrhinum* Bells F1**

Spring Experiment	**Starch Accumulation** (at the end of JVP)	**JVP**
LI		
Unshaded [11.5]	0.34	19.9
Shaded [4.0]	0.27	34.0

The experimental conditions for the spring experiment are indicated in Chapter 3.2.1.1. Sample preparation and determination are described in Chapter 2.2.6.2. Starch is expressed as Glc mg g^{-1} FW. JVP denotes the length of JVP in d. LI is expressed as mol m^{-2} d^{-1} PPFD in square brackets.

Starch metabolism shows a diurnal rhythm and concentrations of starch in leaves typically reach a maximum level at the end of the light period (Kemp and Blacklow, 1980; Zeeman and Ap Rees, 1999; Geiger *et al.*, 2000; Chia *et al.*, 2004; Zeeman *et al.*, 2007). To study further starch accumulation in leaves of juvenile and adult vegetative plants, diurnal changes in starch accumulation were investigated (Figure 4.6 A). For the estimation of developmental stages, a reciprocal transfer experiment was carried out (Chapter 3.2.1.1; Figure 3.3). Leaf material was collected from plants grown under constant SDs in 4 h intervals.

Starch, in both juvenile and adult vegetative phases, followed a linear manner of accumulation (Figure 4.6A). Compared to plants in JVP, adult plants accumulated more leaf starch at the end of light period (Figure 4.6 B). The average rate of starch synthesis (Figure 4.6 C) was estimated by dividing the difference between the starch content at the beginning and end of the day by the length in h of the light period. Plants in JVP synthesized less amounts of starch compared to average rate of starch synthesis of plants in AVP (Figure 4.5 A, B; Figure 4.6). Under dark conditions, starch was remobilized falling to lowest levels by the end of the dark period. However, at this point considerable amounts of leaf starch were remained in

adult plants, compared to leaf starch levels of juvenile plants (Figure 4.6 A). This is in a good agreement with the physiological and biochemical data in Chapter 5.

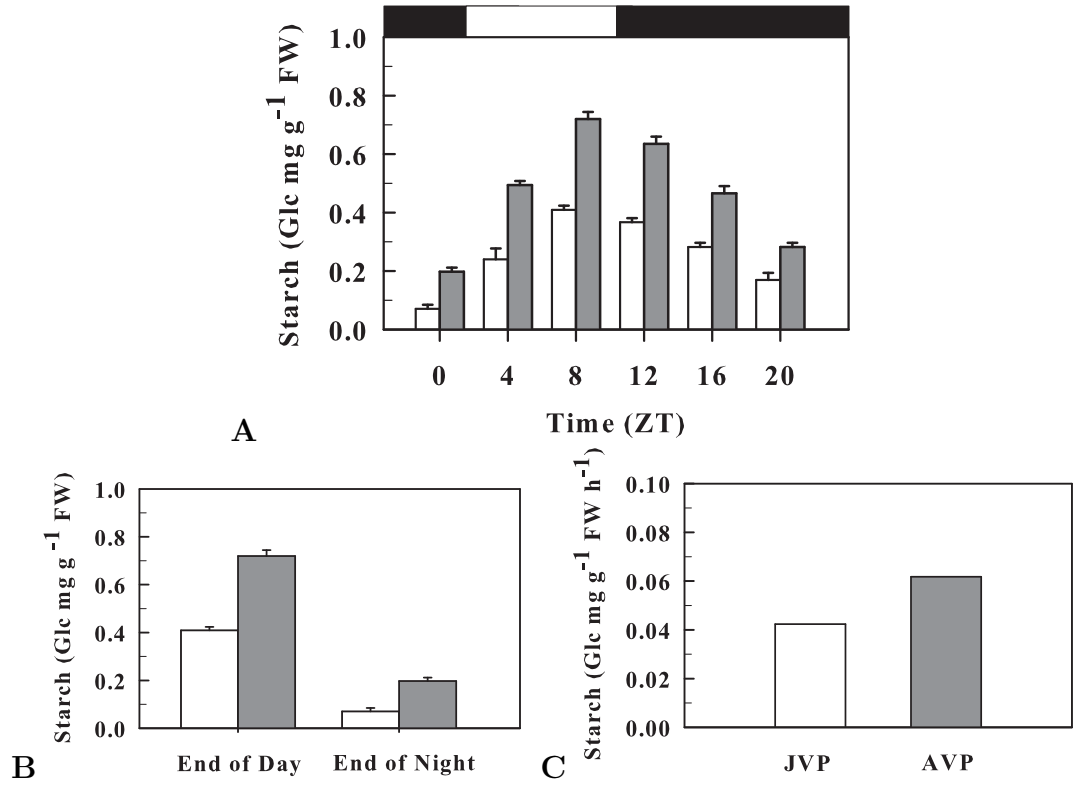

Figure 4.6: **Influence of Developmental Phase on Leaf Starch Diurnal Changes in *Antirrhinum***

(A) Influence of JVP (□) and AVP (■) on diurnal starch accumulation of *Antirrhinum* Bells F1 leaves in SD conditions [The x-axis starts at the end of night (0 h), followed by day and night, as indicated by the bar at top (white and black, respectively)]. (B) Influence of JVP (□) and AVP (■) on starch acumulation levels at the end of the day and the end of the night. (C) The rate of starch synthesis of plants in JVP (□) and AVP (■). It was calculated from the net difference in starch content at the beginning and end of the day, divided by the length of photoperiod (8 h). For the estimation of JVP length a reciprocal transfer experiment was carried out (Chapter 3.3.2; Figure 3.3). The starch content was determined as indicated in Chapter 2.2.6.2. Starch is expressed as Glc mg g^{-1} FW while the rate of starch synthesis as Glc mg g^{-1} FW h^{-1}. The results are means ± SEM (8 separate samples, each of four individual plants).

Several lines of evidence indicate that Mal and Glc are the two major forms of carbon exported from chloroplasts during starch degradation (Weber *et al.*, 2000; Servaites and Geiger, 2002; Weise *et al.*, 2004). Mal is produced by chloroplastic

isoforms of β-amylases (Scheidig *et al.*, 2002a; Kaplan and Guy, 2005; Fulton *et al.*, 2008) and is exported by the MEX-1 (Niittyla *et al.*, 2004). Glc can be produced via the metabolism of maltotriose or other malto-oligosaccharides by disproportionating enzyme (*DPE*; Lin and Preiss, 1988; Critchley *et al.*, 2001). HXK is known to act as a Glc sensor (Rolland *et al.*, 2002b).

The importance of temporal availability of starch in the regulation of plant growth and development, can be suported by observations in development of the *Arabidopsis* double mutants *dpe-1/mex-1* and *dpe-1/dpe-2*. Both mutants are very small and pale and under normal growth conditions, often fail to reach a mature developmental state. This severe phenotype can only partially be rescued by supplying both mutants with Suc (Messerli, 2007). Another hint is given by the observation that CAM and C_4-plants mainly grow during the day-time, when availability of carbohydrates is high due to the decarboxylation of malate, while C_3-plants show growth *maxima* at night-day transition phases, when metabolites derived from transitory starch (Ting, 1985; Geiger and Servaites, 1994; Cushman and Bohnert, 1999).

Another indication of how starch metabolism affects growth and development has been observed in *Arabidopsis* starch deficient mutants *pgm1*, *adg1* and *starch-free mutant1* (*stf1*; Caspar *et al.*, 1985, 1991; Wang *et al.*, 1998; Kofler *et al.*, 2000) that do not possess a relevant pool of transitory starch. These mutants show much lower growth rates than WT plants during the dark period, but are able to develop with comparable intensity to WT plants during the light period (Caspar *et al.*, 1985, 1991; Wang *et al.*, 1998; Wiese *et al.*, 2007).

It is possible that a sufficient starch accumulation level and/or the developmental stage ability to sustain a steady supply of several hexoses and Suc to be part of the mechanism, which is involved in the transition from JVP to AVP in *Antirrhinum*.

4.3.2.4 Effect of Developmental Stage on Phloem Carbohydrate Translocation in *Antirrhinum* RIL57

In order to investigate possible alteration in Suc translocation during the juvenile and adult phases of plant development, leaf phloem exudates were collected. For the selection of plants in JVP and AVP, a reciprocal transfer experiment was conducted (Chapter 3.3.3.1).

Enzymatic determinations in leaf exudates collected from plants grown under constant SD and LD conditions indicated that the most abundant carbohydrate in phloem exudates, for both juvenile and adult plants, was Suc while traces of the reducing carbohydrates Glc and Fru, were detectable (Figure 4.7).

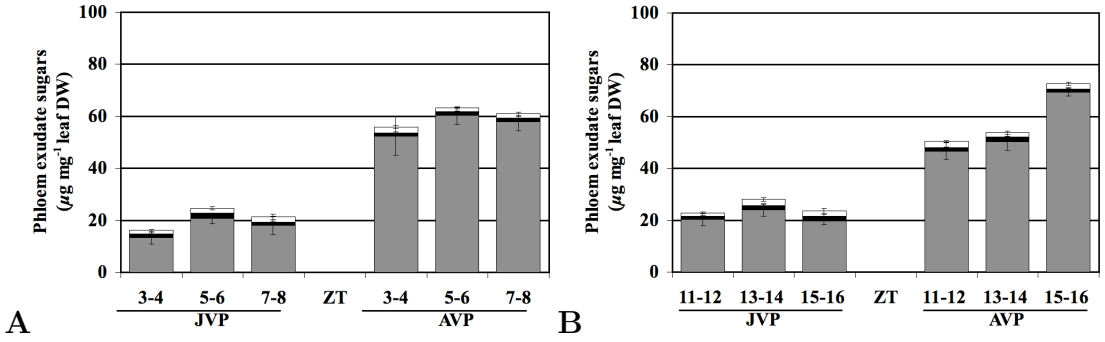

Figure 4.7: Effect of Developmental Phase on Phloem Carbohydrate Translocation in *Antirrhinum* RIL57

Soluble sugar determinations in phloem exudates collected from RIL 57 *Antirrhinum* plants during the JVP and AVP of plant development. Exudation of leaves took place in CE cabinets at 2 h intervals during the last 6 h of (A) SD (ZT 3 to ZT 8) and (B) LD (ZT 11 to ZT 16) conditions. For the estimation of JVP length a reciprocal transfer experiment was carried out (Chapter 3.3.3.1; Figure 3.4). Concentrations of Fru (□), Glc (■) and Suc (■) determined by enzymatic determinations (Chapter 2.2.6.2). The values are the SEM (n=3 separate determinations, each of 25 individual leaves) and represented in μg mg^{-1} of leaf DW.

Probably, the traces of Glc and Fru were due to Suc degradation, as Glc and Fru contents were very similar, which suggested their production by invertase Suc hydrolysis. However, such a carbohydrate composition would be expected for pure

phloem samples (Geigenberger *et al.*, 1993). The Glc plus Fru/Suc ratio never exceeded 0.1 in any of the samples analysed. In addition, no sign of absorption of the exudation medium by the plants was observed, nor of EDTA toxicity.

Sieve tube Suc exhibits diurnal changes with highest concentrations during the light period (Gerhardt *et al.*, 1987; Lejeune *et al.*, 1993). Whether phloem loading of Suc occurs via an apoplastic or symplastic mode is still somewhat controversial. However, both may operate in the same plant, usually at different times or locations (Turgeon *et al.*, 1993; Bel *et al.*, 1992; Turgeon and Wolf, 2009). In symplastic phloem loaders, such as *Antirrhinum* and close relatives, the members of the Lamiaceae and Scrophulariaceae families, carbohydrates are translocated in the form of raffinose and stachyose (Zimmermann and Ziegler, 1975). However, Suc is also still present in the phloem exudates at a very significant level (Turgeon *et al.*, 1993; Knop *et al.*, 2001).

Compared to plants in AVP, *Antirrhinum* juvenile plants had lower Suc translocation levels (Figure 4.7). This is likely due to the reduced net photosynthetic capacity of plants during the JVP or/ and the decreased export phloem capacity that results from the lesser total vein number and thus total number of plasmodesmata per unit of leaf area (Amiard *et al.*, 2005).

Comparing the effect of daylength on Suc translocation levels in juvenile and adult vegetative plants, no significant differences were observed (Figure 4.7). Under the particular light conditions, the 8 h d^{-1} extension in the LD treatment did not have any phototrophic effects on the Suc translocation levels. This was due to the quality of light extension provided in the LD treatment (Chapter 3.2.1.2).

Carbohydrate and dry matter accumulation into sink organs depends on phloem translocation of photoassimilate from source leaves. The sieve elements-companion cells complex is the functional entity responsible for the long-distance phloem translocation not only of photoassimilates, but also some small molecules, a variety of macromolecules, including mRNA, small RNA, hormones and proteins

(Kollmann *et al.*, 1970; Richardson *et al.*, 1982; Fisher *et al.*, 1992; Jorgensen *et al.*, 1998; Sasaki *et al.*, 1998; Lohaus *et al.*, 2000; Fiehn, 2003; Yoo *et al.*, 2004a).

Interestingly, it has been discovered that the FT protein (expressed in the leaf; Lifschitz and Eshed, 2006; Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Lin *et al.*, 2007; Mathieu *et al.*, 2007) and its paralog TSF (expressed in the stem; Yamaguchi *et al.*, 2005; Michaels *et al.*, 2005), act as the long distance floral stimulus or at least, they are part of it. FT-TSF complex transmitted through phloem sap to the SAM, where they act together with FD to activate transcription of floral meristem identity genes, resulting in floral initiation (Abe *et al.*, 2005; Wigge *et al.*, 2005).

It is possible that a total carbohydrate, or a particular carbohydrate level, may be required to reach a specific threshold, in order to sustain a steady supply of Suc for a sufficient bulk flow through the phloem from the leaves to the SAM to enable delivery of FT-TSF compex (Michaels *et al.*, 2005; Yamaguchi *et al.*, 2005; Lifschitz and Eshed, 2006; Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Lin *et al.*, 2007; Mathieu *et al.*, 2007), and possibly other molecules (Chailakhyan, 1936; Bernier *et al.*, 1993; Machackova *et al.*, 1993; Havelange *et al.*, 1996; Thomas and Vince-Prue, 1997; Lejeune *et al.*, 1988; King and Evans, 2003; Bernier and Perilleux, 2005) that render the SAM competent to flower.

4.3.3 Effect of [CO_2] on Carbohydrate Accumulation in *Antirrhinum* Bells F1

The findings that at limiting PPFD can reduce the length of the juvenile phase (Chapter 3.3.3.3) led to an investigation to determine whether the length of JVP could, at least in part, be controlled by photosynthetic assimilate availability. To test this hypothesis, plant material from clearly defined developmental growth stages were collected from plants grown at ambient and elevated [CO_2], under 3 mol m^{-2} d^{-1} PPFD in CE chambers.

4.3.3.1 Effect of [CO_2] on Dry Weight Partitioning in *Antirrhinum* Bells F1

Remarkably, during the early stages of plant development CO_2 enrichment had very little effect on DW partitioning rates (Figure 4.8). Compared to plants grown in ambient [CO_2], an increase in DW under elevated [CO_2], was observed. However, this increase was not temporally correlated with the start of the AVP in *Antirrhinum*.

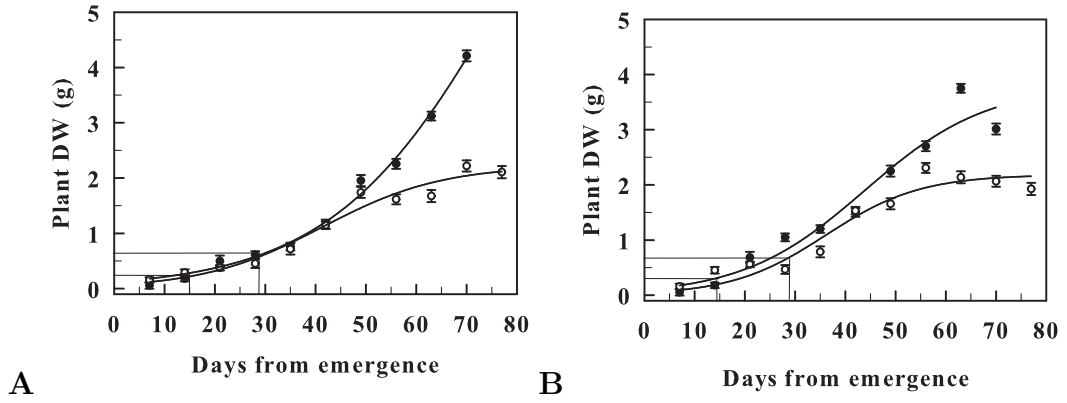

Figure 4.8: **Effect of [CO_2] on Plant Dry Weights of *Antirrhinum* Bells F1**

DW of *Antirrhinum* Bells F1 plants grown under ambient (○) and elevated (●) SD (A) and LD (B) conditions, respectively. The end of JVP is indicated by a barrier (|). Elevated and ambient [CO_2] were 1000 and ~385 vpm, respectively.

For a number of species, data have shown that photosynthesis is increased by elevated [CO_2] (Poorter and Navas, 2003; Long *et al.*, 2004). The degree of this increase depends on interaction with other factors such as temperature (Turnbull *et al.*, 2002) and nitrogen availability (Kruse *et al.*, 2003; Tocquin *et al.*, 2006; Stitt and Krapp, 1999). It has also been shown that carbohydrate assimilation is stimulated more strongly in younger than in mature leaves (Miller *et al.*, 1997; Wait *et al.*, 1999), which leads to increasing contents of starch, Suc and Glc (Poorter *et al.*, 1997).

However, if plants are exposed to long-term [CO_2] enrichment, acclimation is observed and assimilation decreases gradually (Stitt, 1991; Ainsworth *et al.*, 2003). Growth indicators such as DW usually react in a less-pronounced way towards elevated [CO_2], but there is an enormous amount of variability (Curtis and Wang, 1998; Poorter and Navas, 2003; Nowak *et al.*, 2004). For instance, in antithesis with the [CO_2] carbohydrate accumulation response (Bernier *et al.*, 1993; Corbesier *et al.*, 1996, 1998), it has been shown for several plant species that the effect of elevated [CO_2] on growth is poorly correlated (Marc and Gifford, 1984; Reekie and Bazzaz, 1991; Reekie *et al.*, 1994), compared with its dramatic effect on the transition to reproductive phase (Bernier *et al.*, 1993; Corbesier *et al.*, 1996, 1998). Therefore, it might be that DW and carbohydrate partitioning respond in opposite ways; in antithesis with the total carbohydrate accumulation (Chapter 4.3.3.2) the DW increasing rate levels occur after a long-term [CO_2] enrichment.

4.3.3.2 Effect of [CO_2] on Soluble Carbohydrate Accumulation in *Antirrhinum* Bells F1

Qualitative analysis of HPLC data (Figure 4.9) revealed the presence of seven different carbohydrates: Ery, Fru, Glc, MI, Mtl, Suc and Xyl. Compared to plants grown in ambient [CO_2], most of the soluble carbohydrates identified in plant tissue collected under elevated [CO_2], was rapidly and dramatically increased (Figure A.21 to Figure A.27). This could be explained by several lines of evidence that carbohydrate accumulation, plant growth and [CO_2] are positively correlated (Drake *et al.*, 1997).

However, at the end of JVP, total soluble carbohydrate accumulation in plants grown under elevated [CO_2] was similar to that of plants grown in ambient [CO_2] (Table 4.3; Figure 4.10). This observation is in good agreement with the hypothesis expressed in Chapter 4.3.2.2 that a total carbohydrate threshold level may be required before plants undergo the transition within the vegetative phase.

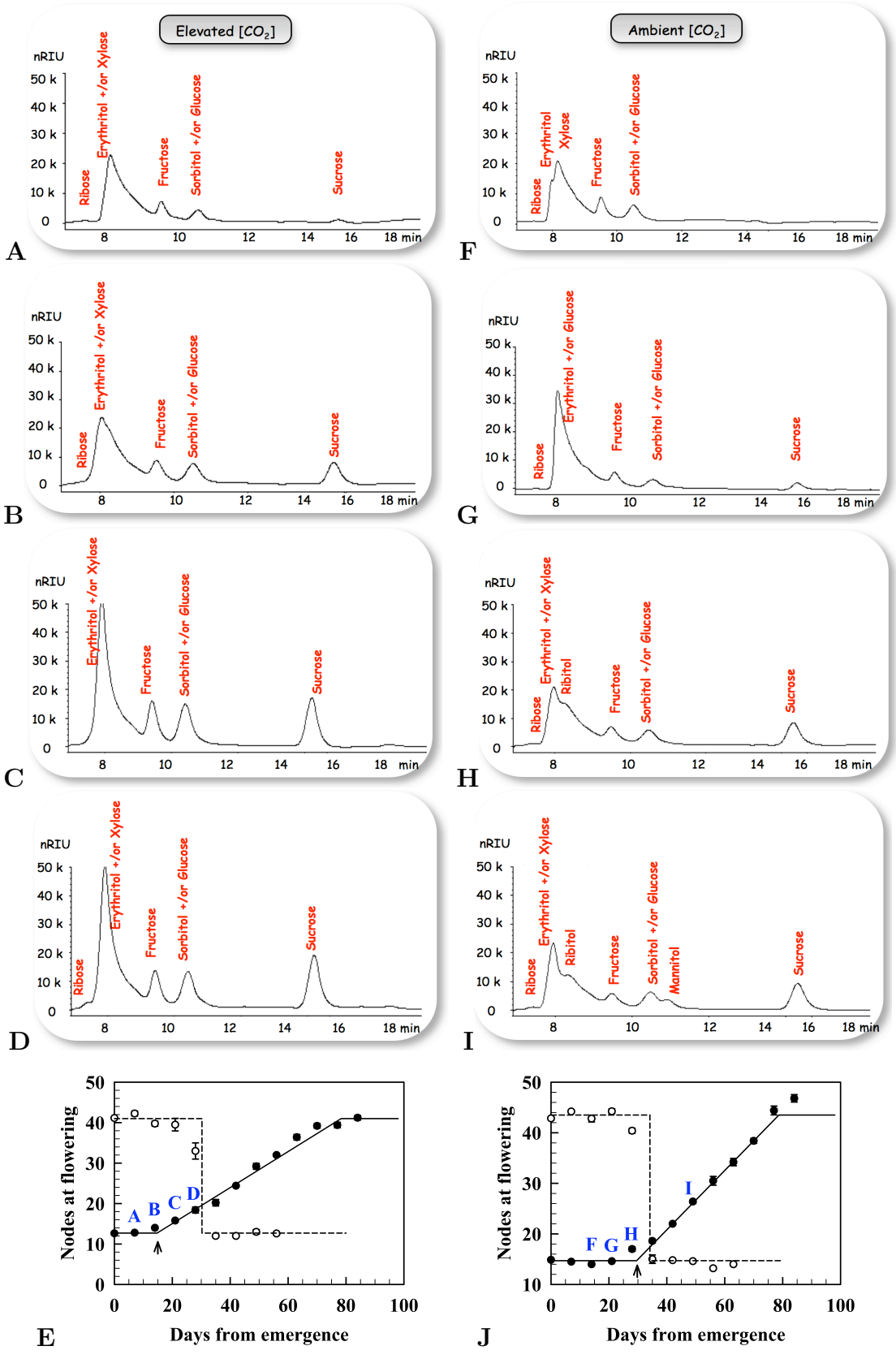
Elevated [CO2]
Ambient [CO2]
nRIU
50 k
40 k
30 k
20 k
10 k
0
8
10
12
14
16
18 min
Ribose
Erythritol +/or Xylose
Erythritol
Xylose
Fructose
Sorbitol +/or Glucose
Ribitol
Mannitol
Sucrose
A
B
C
D
E
F
G
H
I
J
Nodes at flowering
Days from emergence
0
20
40
60
80
100
10
30
50

Figure 4.9: **Carbohydrate Profiles at Selected Stages of Plant Development from Leaf Tissue Harvested from the Ambient and Elevated Treatments During the CO_2 Experiment**

Carbohydrate profiles at selected stages of plant development from leaf tissue harvested from the ambient and elevated CO_2 treatments. The corresponding stages of development represented by the carbohydrate profiles shown are indicated on the transfer experiment plots (E and J for the elevated and ambient [CO_2], respectively) by letters. Elevated and ambient [CO_2] were 1000 and ~385 vpm, respectively. The end of JVP in plots E and J is indicated by a black arrow.

Table 4.3: **Effect of [CO_2] on Soluble Carbohyrates Accumulation in *Antirrhinum* Bells F1**

	Soluble Carbohydrates								
Experimental Conditions	**Ery**	**Fru**	**Glc**	**MI**	**Mtl**	**Suc**	**Xyl**	**Total** (at the end of JVP)	**JVP**
[CO_2] \ LI									
Ambient [2.5]	0.37	1.58	1.15	0.07	0.90	0.47	18.8	23.14	28.8
Elevated [2.5]	1.48	1.36	1.49	0.09	0.78	2.8	12.5	20.15	14.7

Total sugars refer as the sum of Ery, Fru, Glc, MI, Mtl, Suc and Xyl. Carbohydrate accumulation is expressed as mg g^{-1} DW. The experimental conditions are indicated in Chapter 3.2.1.3. Sample preparation and determination described in Chapter 2.2.4 and Chapter 2.2.5, respectivelly. LI is expressed as mol m^{-2} d^{-1} PPFD in square brackets. JVP denotes the length of JVP in d.

It has already been demonstrated that elevated [CO_2] causes several developmental transitions. These developmental shifts correlate with reduced photosynthesis as resulted by [CO_2] acclimation and increased carbohydrate accumulation. Usuda and Shimogawara (1998) and Kauder *et al.* (2000) provide evidence that vegetative growth of *Raphanus sativus* and *Solanum tuberosum* plants was accelerated under elevated [CO_2]. During the period of rapid growth of *Raphanus sativus* and *Solanum tuberosum* under elevated [CO_2], carbohydrates formed by CO_2 assimilation were exported to sink tissues and used for accelerated shoot growth, earlier tuber induction and root formation, respectively.

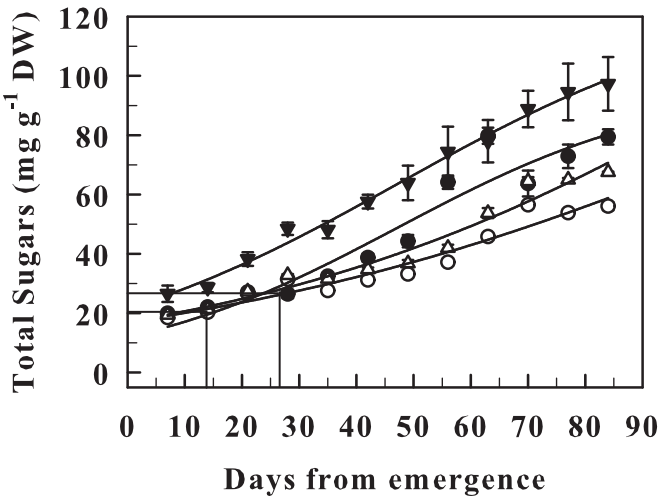

Figure 4.10: Effect of [CO_2] on Total Soluble Carbohydrates in *Antirrhinum* Bells F1

Total soluble carbohydrate accumulation across plant development under SD ambient (○), SD elevated (●), LD ambient (△) and LD elevated (▼) [CO_2]. For the estimation of JVP length a reciprocal transfer experiment was carried out (Chapter 3.3.3.3; Figure 3.5). Solid and broken lines show the fitted relationships. The horizontal and vertical lines indicate the total carbohydrate accumulation at the end of JVP. Total carbohydrates are refered as the sum of Ery, Fru, Glc, MI, Mtl, Suc and Xyl. Bars represent the SEM. They expressed as mg g^{-1} DW. Bars represent the SEM. Elevated and ambient [CO_2] were 1000 and ~385 vpm, respectively.

Moreover, it has been demonstrated that elevated [CO_2] accelerates leaf senescence and causes a developmental shift which correlates with reduced photosynthesis and increased carbohydrate accumulation (Woodrow and Grodzinski, 1993; Micallef *et al.*, 1995; Miller *et al.*, 1997; Ludewig and Sonnewald, 2000). However, the mechanism underlying this developmental transition, under elevated [CO_2], is unknown. It has been hypothesized that elevated levels of [CO_2] may accelerate senescence by increasing the soluble carbohydrate flow through HXK, as overexpression of HXK in transgenic *Lycopersicon esculentum, Nicotiana tabacum* and several cut-flower species leads to accelerated senescence (Dai *et al.*, 1999; Masclaux *et al.*, 2000; van Doorn, 2004).

4.3.3.3 Effects of [CO_2] on Insoluble Carbohydrate Accumulation in *Antirrhinum* Bells F1

The length of JVP, as revealed by the reciprocal transfer approach, was significantly shorter in plants grown under elevated [CO_2] (Chapter 3.3.3.3; Figure 3.5). The effects of elevated [CO_2] on plant development has been proved highly complex and diverse. Under the current experimental condition, starch accumulation in plants grown under both [CO_2], increased following germination (Figure 4.11).

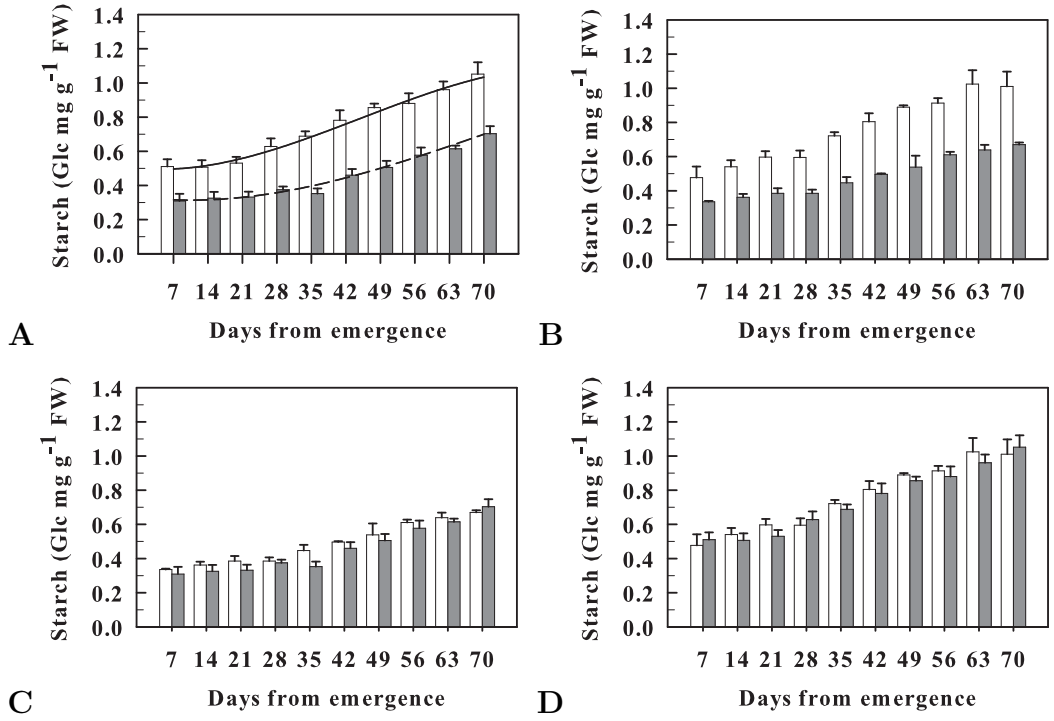

Figure 4.11: **Effect of [CO_2] on Starch Accumulation in *Antirrhinum* Bells F1**

(A, B) Starch accumulation across plant development of *Antirrhinum* Bells F1 under elevated (□) and ambient (■) [CO_2], in SD (A) and LD (B) photoperiods. (C, D) Starch accumulation across development in LD (□) and SD (■) photoperiods, under ambient (C) and elevated (D) [CO_2]. For the estimation of JVP length a reciprocal transfer experiment was carried out (Chapter 3.3.3.3; Figure 3.5). Starch is expressed as Glc mg g^{-1} FW. Elevated and ambient [CO_2] were 1000 and ~385 vpm, respectively. Solid lines in plot A show the fitted relationships. Bars represent the SEM.

However, the most rapid and effectual response to CO_2 was demonstrated by plants

grown under elevated [CO_2], in both light regimes. Furthermore, in agreement with previous data demonstrated, slight differences in starch accumulation in plants grown under SD and LD conditions were observed (Figure 4.11 C, D). This is due to the low intensity incandescent light provided in the 8 h^{-1} extension in the LD treatment. Furthermore, small differences in the starch contents between leaf tissue collected from plants grown under both [CO_2] at the end of JVP were observed (Table 4.4).

Table 4.4: **Effect [CO_2] on Starch Accmulation in *Antirrhinum* Bells F1**

Experimental Conditions		**Starch Accumulation** (at the End of JVP)	**JVP**
LI	**[CO_2]**		
2.5	Elevated	0.52	14.7
2.5	Ambient	0.36	28.8

The experimental conditions for the [CO_2] experiments are indicated in Chapter 3.2.1.3. Sample preparation and determination described in Chapter 2.2.6.2. Starch is expressed as Glc mg g^{-1} FW. JVP denotes the length of JVP in d. LI is expressed as mol m^{-2} d^{-1} PPFD. Elevated and ambient [CO_2] were 1000 vpm and ~385 vpm, respectively.

In Chapter 4.3.2.3, it was shown that starch contents of *Antirrhinum* leaves display a diurnal pattern (Zeeman and Ap Rees, 1999; Zeeman *et al.*, 2004, 2007), where starch is converted back to sugar during the night to sustain maintenance and growth. In addition, Figure 4.6 showed that starch content in juvenile plants is remobilized, falling to lower levels, compared to adult plants, by the end of the dark period (similar data have been obtained from the *Arabidopsis* physiological experiments in Chapter 5). Consequently, this means reduced sugar availability for growth, not only during the night but also for the first hours of the next light period for plants in JVP. This reduced carbohydrate availability might be responsible for several developmental responses (Zeeman and Ap Rees, 1999; Gibon *et al.*, 2004; Rasse and Tocquin, 2006), such as the prolonged JVP under shaded light conditions (low irradiance) and ambient [CO_2].

Grimmer and Komor (1999) reported that carbon export from leaves of *Ricinus communis* during the light period was identical for plants exposed to ambient and elevated [CO_2], while scotoperiodic exports of elevated [CO_2] exposed plants doubled those of their ambient [CO_2] exposed counterparts. According to this growth response, they presumed that *Ricinus* plants grown under ambient [CO_2] are sink-limited during light periods and source-limited in dark periods. Hence, if the main difference between ambient and elevated [CO_2] grown plants happens during the dark period, then the plant ability to regulate a steady flux of available carbohydrates during the dark appears pivotal to several developmental responses such as the transition within the vegetative phase.

4.4 Conclusions

The results in this Chapter are consistent with the hypothesis that *Antirrhinum* plants require a sufficient level of photosynthate assimilates to undergo the transition within the vegetative phase.

LI and elevated [CO_2] had a dramatic impact on the length of the juvenile phase, due to photosynthate assimilate availability, confirming the linkage of LI and [CO_2], length of JVP and assimilation availability in *Antirrhinum*. Under the current experimental conditions, ample evidence was provided that photosynthate assimilates may be a limiting factor during the juvenile phase, compared to the later developmental stages. Quantitative and qualitative analysis of HPLC data, obtained by the LI and [CO_2] approaches, show that a particular carbohydrate assimilate level might be required before plants undergo the transition from juvenile to adult phase of plant development. The present results support an earlier suggestion that there might be a delay in the transition within the vegetative phase with respect to the attainment of maximal carbohydrate accumulation (Tsai *et al.*, 1997).

The importance of temporal availability of carbohydrates in the transition within the vegetative phase can be supported by the seasonal and diurnal monitoring of starch contents. Starch metabolism is regulated by circadian rhythms and concentrations of starch in leaves typically reach *maxima* at the end of light period (Kemp and Blacklow, 1980; Zeeman and Ap Rees, 1999; Geiger *et al.*, 2000; Chia *et al.*, 2004; Zeeman *et al.*, 2007). Compared to *Antirrhinum* plants in JVP, adult plants accumulated more leaf starch at the end of light period. During the dark period, leaf starch is degraded with the carbohydrates released used to provide substrates for leaf respiration and for continued Suc synthesis and export (Zeeman and Ap Rees, 1999; Zeeman *et al.*, 2004, 2007). At the end of scotoperiod, considerable amounts of leaf starch remained in adult plants, compared to starch levels of juvenile plants. Furthermore, plants in JVP synthesized less amounts of starch, compared to average rate of starch synthesis of adult plants. It might be that a

sufficient starch accumulation level and/ or the developmental stage ability to sustain a steady supply of several hexoses and Suc during the AVP to be part of the mechanism, which is involved in the transition from JVP to AVP in *Antirrhinum*.

Moreover, phloem exudate studies show that Suc could be a limiting factor during *Antirrhinum* JVP. Compared to plants in AVP, *Antirrhinum* juvenile plants had lower Suc translocation rate levels. This is likely due to the reduced net photosynthetic capacity of plants during the JVP or/and the decreased export phloem capacity that results from the lesser total vein number, and thus total number of plasmodesmata per unit of leaf area (Amiard *et al.*, 2005).

It is possible that a total carbohydrate, or a particular carbohydrate level, may be required to reach a specific threshold, in order to sustain a steady supply of Suc for a sufficient bulk flow through the phloem from the leaves to the SAM to enable delivery of FT-TSF complex, and possibly other molecules that render the SAM competent to flower.

Chapter 5

Genetic Analysis of Juvenile Phase Length in *Arabidopsis*

5.1 Introduction

A number of physiological, biochemical and molecular approaches have been used to study aspects of plant growth and development. Mutant genotypes are among these important tools and have been used to dissect the physiological function of complex systems ranging from embryogenesis to senescence. Furthermore, they allow the investigation of functional interaction between genes involved in different genetic pathways revealing the complex cellular regulatory networks that control plant development.

The transition to flowering has been genetically explored in *Arabidopsis* and many loci have been cloned through the study of natural variation and induced mutations. This has led to the conclusion that multiple environmental and endogenous pathways promote and enable floral induction. The photoperiodic pathway is well documented for its promotive effect. Genetic and molecular approaches have identified genes that are responsible for the photoperiod response. Some of these genes encode proteins that specifically regulate flowering such as CO and FT, are involved in the regulation of light signal inputs e.g PHYs and CRYs or are components of the circadian clock e.g GI, CCA-1 and LHY (Jackson, 2009; Massiah, 2007; Thomas *et al.*, 2006; Thomas, 2006). Apart from photoperiod, pathways such as hormone biosynthesis and signaling, and light quality promote floral induction by activating the expression of a group of genes responsible for the floral transition, known as FPIs (Weigel and Meyerowitz, 1993; Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Blazquez and Weigel, 2000; Samach *et al.*, 2000). On the other hand, pathways that enable the floral transition regulate the expression of floral repressors. The pathways that regulate the floral repressor *FLC* are the most well characterized (Chapter 1.5.2.2). In addition, genetic analysis suggests that genes such as *TFL-1* (Bradley *et al.*, 1997), *LHP-1* (TFL-2; Gaudin *et al.*, 2001), *HST-1* (Telfer and Poethig, 1998) and *TEMPRANILLO* (*TEM*; Castillejo and Pelaz, 2008) extend the vegetative growth phase by repressing the FPIs.

Information concerning the carbohydrate status in plant tissues is of great importance during all developmental stages, as their availability triggers many metabolic and developmental responses. Several lines of evidence have implicated carbohydrates as playing a role in control of the vegetative to reproductive phase change (Ohto *et al.*, 2001; Dijken *et al.*, 2004; Wilson *et al.*, 2005; Bernier and Perilleux, 2005). Mutants are considered useful tools to facilitate the analysis of sugar and starch metabolism and floral initiation. Mutation in loci such as *PGM-1* (Caspar *et al.*, 1985, 1991), *ADG-1* (Lin *et al.*, 1988; Wang *et al.*, 1998), *SEX-1* (Yu *et al.*, 2001) and *BAM-3* (Lao *et al.*, 1999) alter the rate of starch synthesis, accumulation or mobilization causing late flowering phenotypes even under inductive LD conditions, suggesting their involvement in the transition to the reproductive phase. However, the wide-range effect of carbohydrates is clouded by their dyadic function as nutrients and as signaling molecules and by the interaction between carbohydrate signaling and hormonal systems. Characterization of the sugar-response mutants has revealed that many of them are also defective in phytohormone metabolism or response (Gibson, 2005; Rolland *et al.*, 2006; Ramon *et al.*, 2008), disclosing a tight interplay between sugar and hormone networks (Gibson, 2005; Rolland *et al.*, 2006; Ramon *et al.*, 2008).

Arabidopsis mutants offer a powerful means for the elucidation of the complex pathways that regulate plant development. Several mutants have been used extensively in developmental studies and different mutant screens have been developed. In this Chapter, a physiological assay has been developed in *Arabidopsis* that allows the length of the JVP to be measured. To determine the potential involvement of diferent genetic pathways in the juvenile to adult phase transition several *Arabidopsis* mutant genotypes were exploited.

5.2 Specific Materials and Methods

5.2.1 Photoperiod Experiments

The *Arabidopsis* experiments were set up as described in Chapter 2.2.2 by exploiting SD and LD conditions. When 50% of seedlings emerged, the trays with the seedlings were transferred into growth cabinets (Saxcil®, Chester, UK) and the daylength treatments initiated. Seven to nine replicate plants were transferred every day from SDs to LDs with the exception of plants grown in continuous SD and LD conditions where 16 replicate plants were used.

5.2.1.1 Light Sources and Spectral Measurements

SD conditions (100 μmol m^2 s^{-1} PAR) were achieved using a combination of fluorescent (General Electric 60W, HU) and incandescent (Philips 32W, NL) light tubes. LD conditions consisted of a combination of fluorescent (General Electric 60W, HU) and incandescent (Philips 32W, NL) light for the first 8 h d^{-1} (94 μmol m^2 s^{-1} PAR) and low intensity (6 μmol m^2 s^{-1} PAR) incandescent (Philips 32W, NL) light for the 8 h d^{-1} extension. Light quality and quantity were measured with an EPP 2000 Fiber Optic Spectrometer (StellarNet Inc. USA).

5.2.2 Estimation of Juvenile Phase Length

The phases of photoperiod sensitivity were estimated counting the number of rosette leaves and number of days from 50% of seedling emergence at the appearance of the floral bolt at 1 cm height. Flowering time data were fitted in a non-linear regression analysis model by using the NON-LINEAR REGRESSION ANALYSIS directive of Sigma Plot 11® (Systat Software, Chicago, USA).

5.3 Results and Discussion

5.3.1 Defining the Juvenile Phase Length in *Arabidopsis* Reference Accessions

In order to estimate the length of JVP in *Arabidopsis* WTs three *Arabidopsis* accessions, Col-0, Ler-0 and Ws-4, comprising the most commonly used laboratory strains were selected. To determine the length of the JVP the photoperiod transfer approach was applied.

Part of the experimental assay was to grow control plants in constant SD and LD conditions until flowering (Table 5.1). The flowering response to daylength varied significantly among the three WT accessions. Under the experimental conditions applied, Col-0 was the latest flowering phenotype under SD conditions, whereas Ws-4 was the earliest flowering phenotype. However, assessing the number of leaves at flowering under LDs, no significant differences were observed.

Table 5.1: Flowering Time Profiles of *Arabidopsis* WT Accessions

	Number of leaves		Number of days		
Strain	SD	LD	SD	LD	**JVP**
Col-0	33.9 (±0.7) a	5.7 (±0.3) a	65.2 (±5.7) a	29.8 (±0.2) a	2-3
Ler-0	25.0 (±0.2) b	4.2 (±0.1) a	62.5 (±3.9) a	24.7 (±0.2) b	5-6
Ws-4	14.7 (±0.2) c	4.0 (±0.3) a	40.7 (±3.5) b	19.3 (±0.1) c	7-8

Data are represented by analysis of 16 plants for each photoperiod treatment. Flowering time is expressed as the number of rosette leaves and number of days from 50% of seedling emergence to the appearance of the floral bolt at 1 cm height. JVP is expressed as number of days from 50% of seedling emergence. SEM indicated in parenthesis. Values followed by the same letter within a column are not significantly different according to the Student's t test at a 0.05 level of significance.

The experiment was performed at least four times, in the same CE chambers with identical results. As revealed by the transfer approach (Figure 5.1; Table 5.1), the

three *Arabidopsis* accessions displayed differences in JVP length. Specifically, Ws-4, Ler-0 and Col-0 seedlings exposed to seven to eight, five to six and two to three SDs (number of days in SD conditions before they transferred in LD conditions), respectively following seedling emergence initiated flowering not different to those grown continuously in LDs, indicating for these durations photoperiod insensitivity and plant being juvenile. A linear increase in leaf number and days to flower with successive transfer date can be seen for all the accessions transferred following the end of JVP (Figure 5.1). This illustrates the delay in inflorescence initiation caused by extended time spent in non-inductive SD conditions.

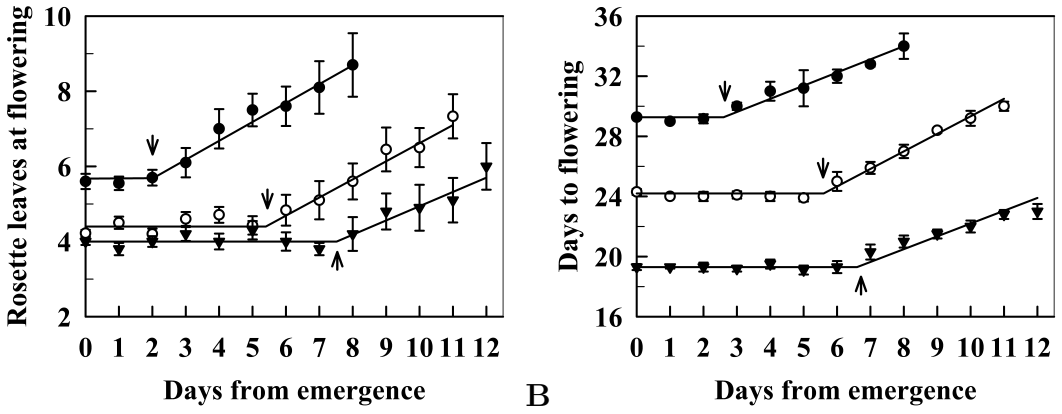

Figure 5.1: **Estimation of the Juvenile Phase Length in WT Accessions of *Arabidopsis***

Estimation of the length of the JVP of Col-0 (●), Ler-0 (○) and Ws-4 (▼). (A) number of rosette leaves at flowering; (B) number of days to flowering. Plants were transferred from SDs to LDs at time intervals shown in x-axes. Arrows indicate the end of the JVP. Points represent the mean and SE of leaves and days to flower of replicate plants transferred on each occasion.

Data in the literature regarding the estimation of JVP length in *Arabidopsis* in response to flowering are limited. However, Mozley and Thomas (1995) and Bradley *et al.* (1997) by transfering Ler seedlings from SDs to LDs and LDs to SDs, respectively estimated a JVP length similar to that indicated in Figure 5.1.

WT *Arabidopsis* accessions are geographically delimited genetic variants and valuable models for the study of plant phenotypes that result from genotypic and

environmental influences. Although they are intra-species variants, there are many differences between them, including morphological and physiological responses. It has been shown that *Arabidopsis* accessions may differ in height, leaf size, and flower shape (Purugganan and Suddith, 1998) as well as in agronomically important traits such as resistance or susceptibility to pathogens (Nam *et al.*, 1997), seed size and flowering time (Alonso-Blanco *et al.*, 1999).

However, the JVP length does not appear to be the only factor contributing to flowering time. Other components such as the duration of AVP may also contribute and these may differ in the accessions. For instance, despite the hastened JVP of Col-0, its late flowering phenotype might be attributed a prolonged photoperiod sensitive phase. Similarly in Ws-4, despite its prolonged JVP compared to the other two WT accessions, the early flowering phenotype might be due to a shortened photoperiod sensitive phase. This indicates that both developmental phases are subject to modification.

5.3.1.1 Juvenile to Adult Phase Transition and Carbohydrate Relationships in the Ws-4 Accession

In Chapter 4 it was hypothesized that in species such as *Antirrhinum* the ability to regulate a steady flux of available carbohydrates appears pivotal to the transition within the vegetative phase. To determine whether a relationship exists in *Arabidopsis* between the transition within the vegetative phase and carbohydrate accumulation, the diurnal metabolite changes in juvenile and adult Ws-4 plants were monitored (Figure 5.2).

The selection of Ws-4 WT was based on its prolonged JVP and consequently, the increased biomass for plant material collection. Juvenile and adult vegetative Ws-4 plants showed a steady rate of starch accumulation during the light period, and almost complete degradation of the starch during the dark period (Figure 5.2 A).

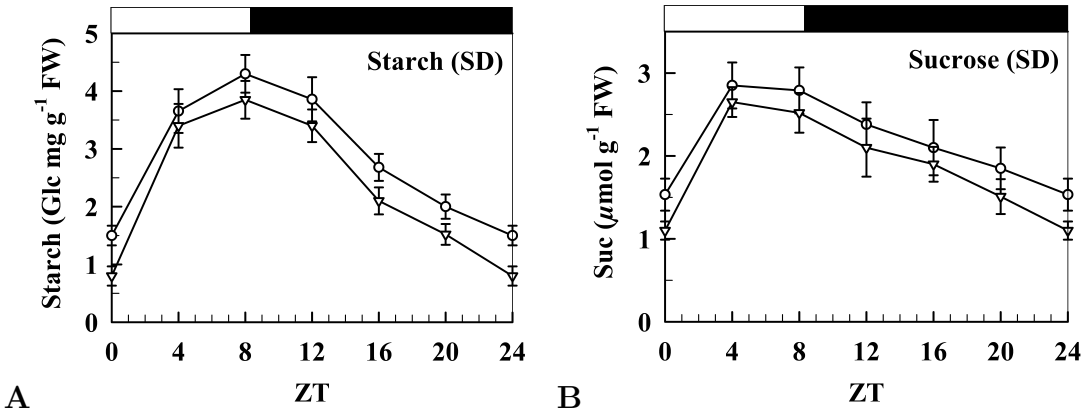

Figure 5.2: **Diurnal Changes of Starch and Suc Contents in Ws-4 Accession of *Arabidopsis***

Diurnal changes of starch (A) and Suc (B) contents in juvenile ($\bigtriangledown$) and adult ($\bigcirc$) vegetative Ws-4 seedlings. Plant material was collected under SDs on day 6 and day 11 from emergence for the juvenile and adult phases respectively, at time intervals shown in x-axes. All results are the mean and SE of three technical replicates. White and black bars on the top are subjective day and night. ZT 0 and 24 are values from the same data set.

Compared to plants in JVP, starch accumulation in adult plants was slightly higher at the end of light period. However, at the end of the dark period more leaf starch remained in adult Ws-4 plants, relative to leaf starch levels of juvenile plants. This is in a good agreement with the starch accumulation pattern observed in juvenile and adult vegetative *Antirrhinum* plants (Chapter 4.3.2.3).

Suc is synthesised at the same time as transitory starch during photosynthesis. Its accumulation in both juvenile and adult vegetative plants rose early in the first 4 h of light period, stabilized for the remainder of the light period and fell to the lowest level at the end of dark period (Figure 5.2 B). However, slight differences in Suc accumulation at the end of dark period in juvenile and adult vegetative Ws-4 plants were observed.

5.3.2 Defining the Juvenile Phase Length in Starch Deficient Mutants

In Chapter 4 it was shown that a sufficient starch accumulation level and/or the developmental stage ability to sustain a steady supply of hexoses and Suc might be part of a mechanism involved in the transition within the vegetative phase in *Antirrhinum.* To determine whether mutations that affect starch metabolism and catabolism are related to events involved in the transition within the JVP in *Arabidopsis,* starch deficient mutants were tested in photoperiod transfer experiments. The nuclear-encoded, recessive mutants *adg-1* (Wang *et al.*, 1998) and *pgm-1* (Caspar *et al.*, 1985), which have dramatically low starch accumulation contents were examined.

The developmental differences in WT and starch deficient mutant plants of the same chronological age are presented in Figure 5.3, showing the importance of transitory starch for normal plant growth and development.

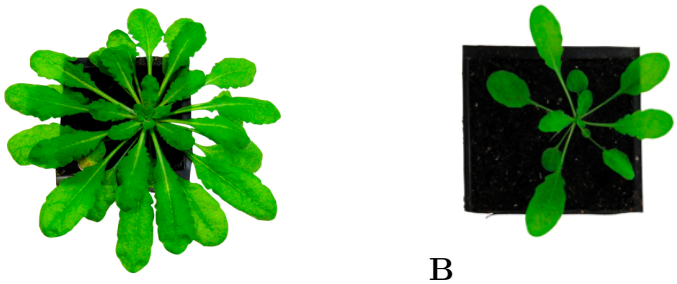

Figure 5.3: **Phenotypes of Col-0 and *adg-1* Genotypes of *Arabidopsis***

Phenotypes of Col-0 WT (A) and *adg-1* (B) starch deficient mutant in LDs, at the same chronological age.

The *adg-1* mutant has no detectable alpha-D-Glc-1-phosphate adenyl transferase (AGP) activity as it is deficient in the small subunit protein *ADP GLC PYROPHOSPHORYLASE* (*APS*; Lin *et al.*, 1988; Wang *et al.*, 1998), whereas the *pgm-1* is unable to synthesize starch due to inactivation of the chloroplastic isozyme of the phosphoglucomutase, which converts the Glc-6-phosphate into Glc-1-phosphate

(Caspar *et al.*, 1985, 1991). With very low starch levels the rate of growth and net photosynthesis of both mutants and WT are indistinguishable when the genotypes are grown in continuous light conditions. However, under SD conditions the growth of *adg1* and *pgm-1* is impaired and flowering is significantly delayed compared to WT, both in terms of the number of days and number of rosette leaves (Table 5.2; Figure 5.4). Under LD conditions the starch deficient mutants have a flowering time similar or slightly later to that of the WT.

Table 5.2: Flowering Time Profiles of WT and Starch Deficient Mutants of *Arabidopsis*

	Number of leaves		Number of days		
Strain	SD	LD	SD	LD	**JVP**
Col-0	33.9 (±0.7) a	5.7 (±0.3) a	65.2 (±0.4) a	29.8 (±0.2) a	2-3
adg-1	48.4 (±0.8) b	12.1 (±0.6) b	87.6 (±1.1) b	42.6 (±0.9) b	6-7
pgm-1	42.8 (±1.5) b	8.9 (±0.6) ab	83.0 (±1.8) b	39.2 (±0.5) b	5-6

Data are represented by analysis of 14 plants for each photoperiod treatment. Flowering time is expressed as the number of rosette leaves and number of days from 50% of seedling emergence to the appearance of the floral bolt at 1 cm height. JVP is expressed as number of days from 50% of seedling emergence. SEM indicated in parenthesis. Values followed by the same letter within a column are not significantly different according to the Student's t test at a 0.05 level of significance.

However, it has already been indicated (Bernier *et al.*, 1993; Eimert *et al.*, 1995) that vernalization completely suppresses the late flowering phenotype of *pgm-1* suggesting that the late-flowering phenotype observed in starch deficient mutants is not due to the defect in starch accumulation and slow growth rates, but more to the inability to mobilize the stored carbohydrates during the dark period. It has been demonstrated (Weber *et al.*, 2000; Servaites and Geiger, 2002; Weise *et al.*, 2004) that Mal and Glc are the two major forms of carbon exported from chloroplasts during scotoperiod, as a result of the starch degradation with Mal being exported by MEX-1 (Niittyla *et al.*, 2004), whereas HXK operates as a Glc sensor (Rolland *et al.*, 2002b). It is possible that these carbohydrates may effect the juvenile to adult vegetative phase transition in *Arabidopsis*.

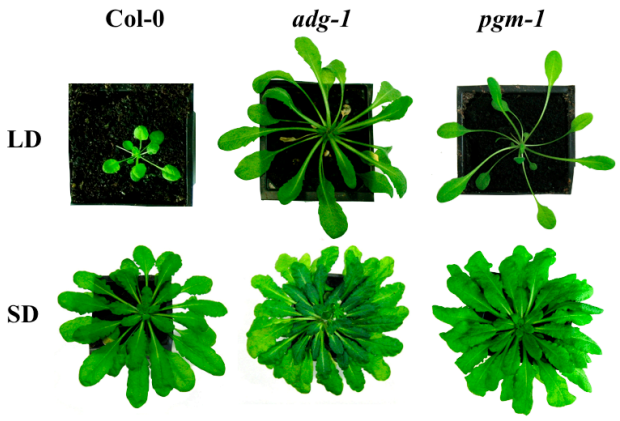

Figure 5.4: **Phenotypes of WT and Starch Deficient Mutants *adg-1* and *pgm-1* of *Arabidopsis***
Flowering time phenotypes of WT and starch deficient mutants *adg-1* and *pgm-1* of *Arabidopsis* grown under SD and LD conditions.

The transfer experiment was performed three times, in the same CE chambers with similar results (Figure 5.5).

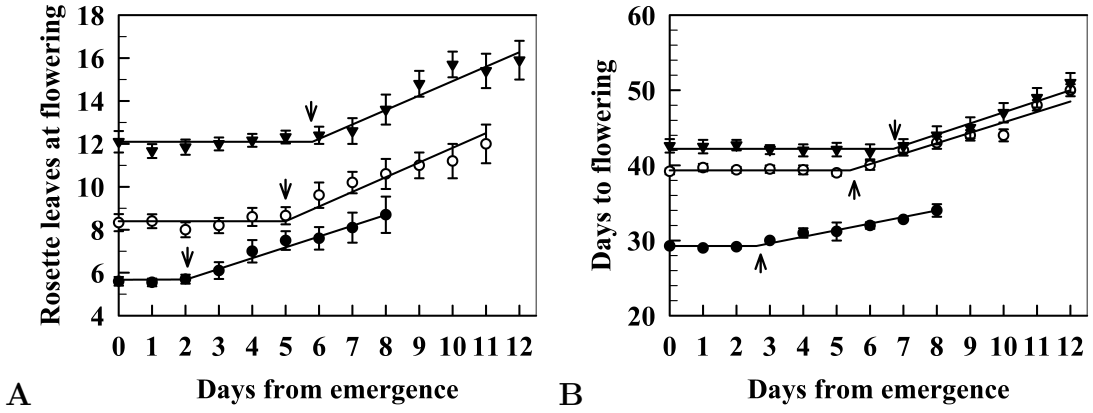

Figure 5.5: **Estimation of the Juvenile Phase Length of Starch Deficient Mutants of *Arabidopsis***

Estimation of the juvenile phase length of Col-0 (●), *pgm-1* (○) and *adg-1* (▼) genotypes. (A) number of rosette leaves; (B) number of days to flowering. Plants were transferred from SDs to LDs at time intervals shown in x-axes. Arrows indicate the end of the JVP. Points represent the mean and SE of leaves and days to flower of replicate plants transferred on each occasion.

By applying the photoperiod transfer approach it was revealed that both mutants

were insensitive to photoperiod for longer periods after their emergence than the WT, which signify longer JVP lengths (Figure 5.5; Table 5.2). The *adg-1* and *pgm-1* mutant seedlings have JVP lengths of approximately 5-6 and 6-7 d., respectively. Comparison to the JVP length of the Col-0 WT, which is estimated 2 d., provides evidence for the involvement of starch metabolism and catabolism related events in the transition within the vegetative phase in *Arabidopsis*.

5.3.2.1 Juvenile to Adult Phase Transition and Carbohydrate Relationships in *adg-1* mutant

Information concerning the carbohydrate status in plants is critical as their availability or lack triggers many metabolic and developmental responses. It has already been suggested that lack of diurnal carbohydrate availability (Chapter 4.3.2.3; Chapter 4.3.3.3) might be involved in the transition within the vegetative phase in *Antirrhinum*. To test this further, the diurnal metabolite changes in juvenile and adult vegetative *adg-1* and WT seedlings were assesed.

WT and *adg-1* mutant seedlings were collected under SDs and LDs on day 5 and day 9 from emergence for the juvenile and adult phases, respectively. Furthermore, to ensure *Arabidopsis* seedlings under LD conditions received similar LI to those grown under SDs, photoperiod was artificially increased without modifying the total quantity of light available for photosynthesis, by extending the SD treatment with low intensity incandescent light (Chapter 5.2.1.1).

In WT, photosynthate assimilates generated in excess of sink demand with effect on elevated starch accumulation levels at the end of light period (Figure 5.6 A; B). On the other hand, no starch accumulated in *adg-1* mutant seedlings, irrespective of daylength. During the night, reduced Suc (Figure 5.6 C; D) content triggers degradation of starch, which is almost fully remobilized by the end of the dark period.

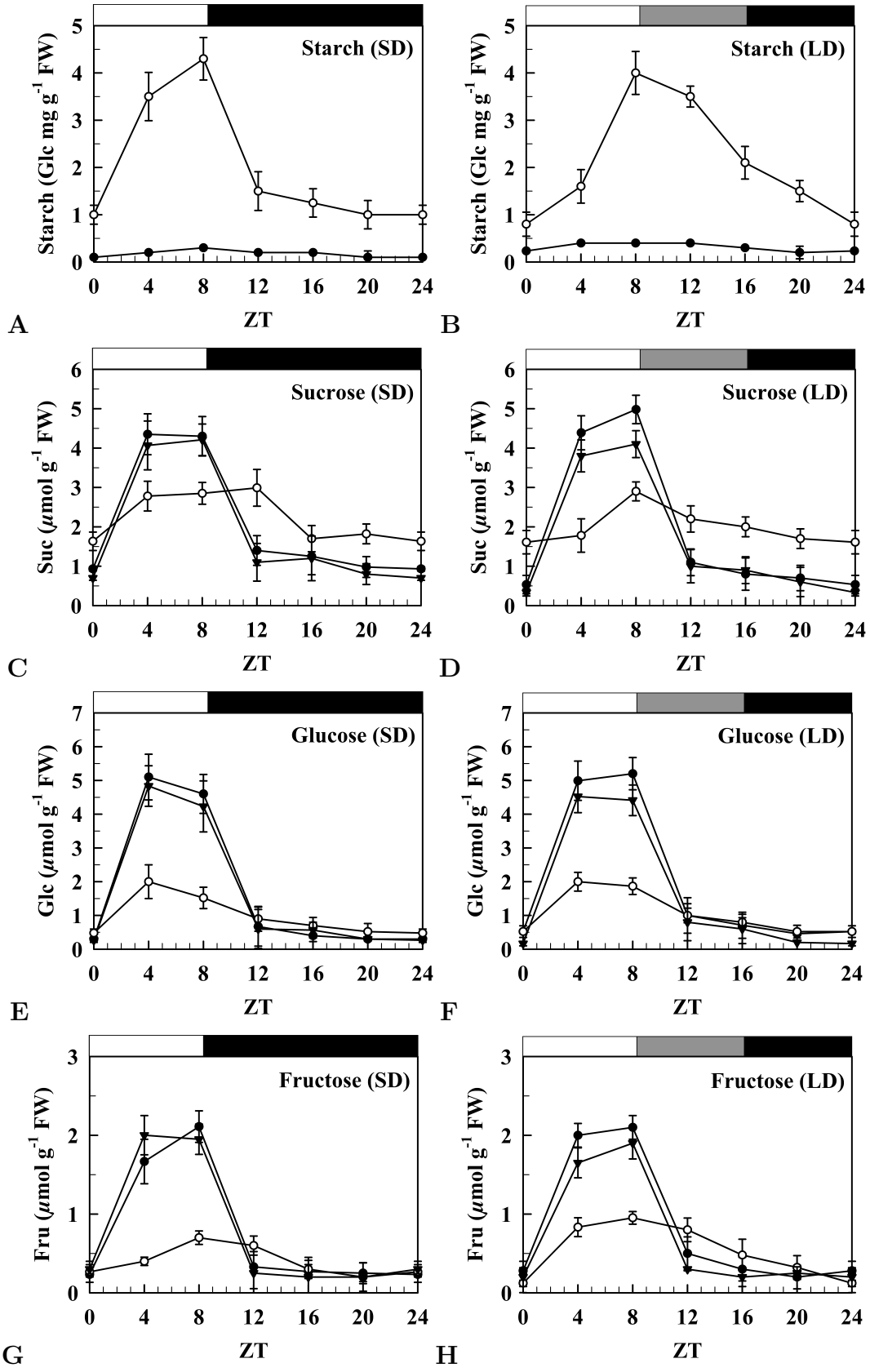
Starch (SD)
Starch (LD)
Starch (Glc mg g-1 FW)
Sucrose (SD)
Sucrose (LD)
Suc (µmol g-1 FW)
Glucose (SD)
Glucose (LD)
Glc (µmol g-1 FW)
Fructose (SD)
Fructose (LD)
Fru (µmol g-1 FW)
ZT
A
B
C
D
E
F
G
H

Figure 5.6: **Diurnal Metabolite Changes in Col-0 WT and *adg-1* Starch Deficient Mutant Genotypes**

Diurnal changes of starch (A, B), Suc (C, D), Glc (E, F) and Fru (G, E) in Col-0 and *adg-1* genotypes. Adult Col-0 (○), adult *adg-1* (●) and juvenile *adg-1* (▼) seedlings were collected under SDs (A, C, E, G) and LDs (B, D, F, H) at time intervals shown in x-axes. All results are the SEM of three biological replicates. White and black bars on the top are subjective day and night, whereas the grey bar in the LD treatment indicates the photoperiod extension with low intensity incandescent light. ZT 0 and 24 are values from the same data set.

Compared to WT, *adg-1* accumulated considerable amounts of Suc (Figure 5.6 C; D), Glc (Figure 5.6 E; F) and Fru (Figure 5.6 G; H) during the day, rather than being used for biosynthesis and growth. Under dark conditions, soluble carbohydrates were depleted in *adg-1* possibly due to a period of rapid respiration (Gibon *et al.*, 2004). Noticeably, at the end of night lesser amounts of Suc remained in juvenile and adult *adg-1* mutant under both photoperiods, compared to Suc levels of adult WT plants.

It is possible that a trophic pathway, via starch catabolism related events during the night and in the beginning of the following light period might be involved in the prolonged JVP length in starch deficient mutants. It has been proposed that the inhibition of growth in starch deficient mutants is primarily caused by a disturbance of metabolism and growth, which is triggered by a transient period of sugar depletion during the dark (Bernier *et al.*, 1993; Eimert *et al.*, 1995; Trethewey and Smith, 2000; Gibon *et al.*, 2004). Furthermore, it has been demonstrated that the expression of hundreds of genes is altered in the *pgm-1* mutant at the end of the night period, compared with WT at the same time (Thimm *et al.*, 2004). This includes many genes that are required for nutrient assimilation, biosynthesis and growth. Remarkably, when the night was extended by 4–6 h, global gene expression analysis in WT resembled that in *pgm-1* at the end of the normal night (Thimm *et al.*, 2004). Taken together, these results could suggest that soluble carbohydrate depletion during the night leads to marked changes on gene expression stimulating

an inhibition of carbohydrate utilization with direct effects on vegetative phase change.

However, in addition to transitory starch providing a source of carbon for growth during the following night (Trethewey and Smith, 2000; Gibon *et al.*, 2004) and for the beginning of the next light period, it may also act as an overflow for newly assimilated carbon (Stitt and Quick, 1989), when assimilation exceeds the demand for Suc. This mechanism is inactivated in *adg-1* mutant, as demonstrated by elevated soluble carbohydrate levels (Figure 5.6), and hardly any starch at the end of the light period in both juvenile and adult *adg-1* mutant seedlings.

A number of physiological, biochemical and molecular approaches have shown that early growth and development in *Arabidopsis* seedlings can be arrested in the presence of high Glc and Suc levels. Characteristics such as shoot development, leaf formation, cotyledon expansion, greening, and hypocotyl and root elongation are among the postgerminative processes that are subject to high level soluble carbohydrate repression (Jang *et al.*, 1997; Dijkwel *et al.*, 1997; Kurata and Yamamoto, 1998; Arenas-Huertero *et al.*, 2000; Gibson, 2000; Gazzarrini and McCourt, 2001; Eastmond and Graham, 2001). The physiological rationale for soluble carbohydrate repression during the early developmental phase could be that elevated soluble carbohydrate accumulation levels reflect suboptimal growth conditions (Lopez-Molina *et al.*, 2001), at a crucial developmental stage such as the first weeks of plant development. Therefore, inhibition of developmental programs such as the transition within the vegetative phase in starch deficient mutants may result from the activation of the soluble carbohydrate repression events; a protective mechanism during the early phase of plant development. Based on this repressing growth response, a series of *glucose insensitive* (*gin*) mutants have been isolated (Zhou *et al.*, 1998; Moore *et al.*, 2003). Characterization of these mutants regarding the length of JVP and time to flowering (Chapter 5.3.4), further supports this hypothesis.

5.3.3 Defining the Juvenile Phase Length in Starch Excess Mutants

Starch is degraded by phosphorylating enzymes to maltodextrin, which is then converted to Mal and Glc by *BAM-3* and *DPE-1* in the chloroplast for scotoperiodic export (Lao *et al.*, 1999; Critchley *et al.*, 2001; Scheidig *et al.*, 2002a,b). Mutants that are unable to degrade starch provide a valuable tool to study the scotoperiodic effects of carbon exported from chloroplasts on plant development. These genotypes retain a high starch content, even after prolonged scotoperiod by the impaired function of the key participating phosphorylating enzymes. Reduced activity of GWD (Yu *et al.*, 2001; Kotting *et al.*, 2005) and SEX-4 (Niittyla *et al.*, 2006; Kotting *et al.*, 2009) lead to a reduced rate of starch breakdown and in the accumulation of high levels of starch in *sex-1* and *sex-4*, respectively. The *bam-3* mutant, which carries a point mutation in At4g17090 locus (McCallum *et al.*, 2000) leads also to a SEX phenotype (Lao *et al.*, 1999; Kaplan and Guy, 2005). In this mutant maltodextrin conversion to Mal and Glc in the chloroplasts is blocked (Lao *et al.*, 1999). To test whether mutations affecting starch mobilization are affected in JVP length, the *sex-1*, *sex-4* and *bam-3* mutants were utilized in photoperiod transfer experiments.

The photoperiod experiments were performed three times in the same CE chambers with similar results. Mutants with SEX phenotypes were late flowering compared to WT, flowering later in SDs than they do in LDs (Figure 5.7; Figure 5.8; Table 5.3). This is due to their inability to mobilize the stored carbohydrates during the night. *sex-1*, *sex-4* (data not shown) and *bam-3* mutants exposed to approximately 4-7 SDs following seedling emergence initiated flowering not different to those grown continuously in inductive LDs, signifying longer JVP lengths than the Col-0 WT. Using number of days to bolting and total number of rosette leaves to measure flowering time in order to estimate JVP length generated different estimates for JVP length (Figure 5.7). This might be due to the fact that both indicators are not surrogates of each other and might be correspond to differentially regulated

temporal components of plant ontogeny (Pouteau *et al.*, 2006).

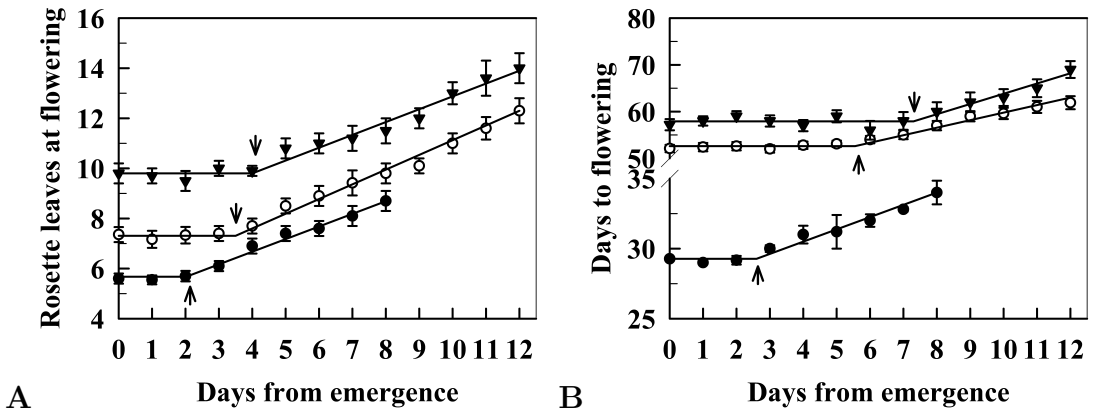

Figure 5.7: **Estimation of the JVP Length of *Arabidopsis* Starch Excess Mutants**

Estimation of the juvenile phase length of Col-0 (●), *sex-1* (○) and *bam-3* (▼) genotypes. (A) number of rosette leaves; (B) number of days to flowering. Plants were transferred from SDs to LDs at time intervals shown in x-axes. Arrows indicate the end of the JVP. Points represent the mean and SE of leaves and days to flower of replicate plants transferred on each occasion. Note the y-axis break and change of values in plot B.

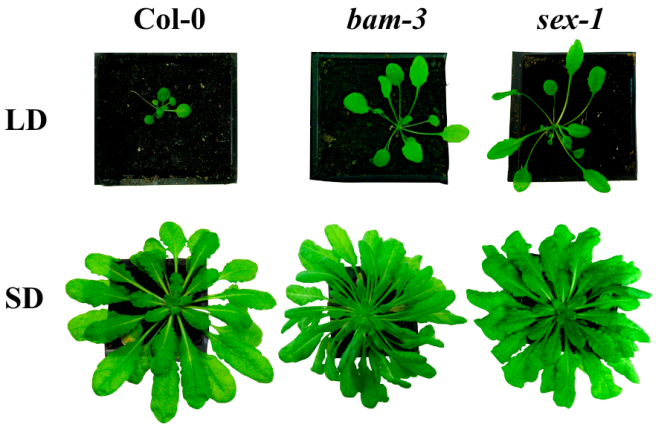

Figure 5.8: **Flowering Time Phenotypes of WT and Starch Excess Mutants *bam-3* and *sex-1* of *Arabidopsis***

Table 5.3: Flowering Time Profiles of Starch Excess Mutants of *Arabidopsis*

Strain	Number of leaves		Number of days		JVP
	SD	LD	SD	LD	
Col-0	33.9 (±0.7) a	5.7 (±0.3) a	65.2 (±1.7) a	29.8 (±0.2) a	2-3
bam-3	49.3 (±0.9) b	11.3 (±0.3) b	84.2 (±2.1) b	57.2 (±1.2) b	4-7
sex-1	45.0 (±1.4) b	7.3 (±0.3) a	74.7 (±1.4) b	52.1 (±0.8) b	3-6
sex-4	45.2 (±0.8) b	8.1 (±0.4) a	76.3 (±1.1) b	50.2 (±0.6) b	4-6

Data are represented by analysis of 14 plants for each photoperiod treatment. Flowering time is expressed as the number of rosette leaves and number of days from 50% of seedling emergence to the appearance of the floral bolt at 1 cm height. JVP is expressed as number of days from 50% of seedling emergence. SEM indicated in parenthesis. Values followed by the same letter within a column are not significantly different according to the Student's t test at a 0.05 level of significance.

However, the longer JVP length of mutants with SEX phenotypes, as compared to Col-0 WT provides a further piece of evidence for the involvement of events related to starch degradation in the transition within the vegetative phase in *Arabidopsis*. Furthermore, in a similar manner with starch deficient mutants, it can be hypothesized that plants in JVP may require starch accumulation to reach a particular level, in order to sustain a steady supply of Glc, Mal and/or Suc during the scotoperiod to undergo smoothly the transition from juvenile to adult phase of plant development.

5.3.3.1 Juvenile to Adult Phase Transition and Carbohydrate Relationships in the *sex-1* Mutant

The *sex-1* mutant is known for its impaired ability to degrade starch (Caspar *et al.*, 1991; Yu *et al.*, 2001), whereas it has been demonstrated that its growth rate and plant biomass accumulation are hampered because part of the fixed carbon is not available for scotoperiodic export (Zeeman *et al.*, 1998). To determine whether the impaired ability of *sex-1* affects carbohydrate accumulation at the end of night,

diurnal metabolite changes in juvenile and adult vegetative *sex-1* seedlings were determined.

Starch is progressively accumulated in both WT and *sex-1* mutant as the seedlings age (Figure 5.9).

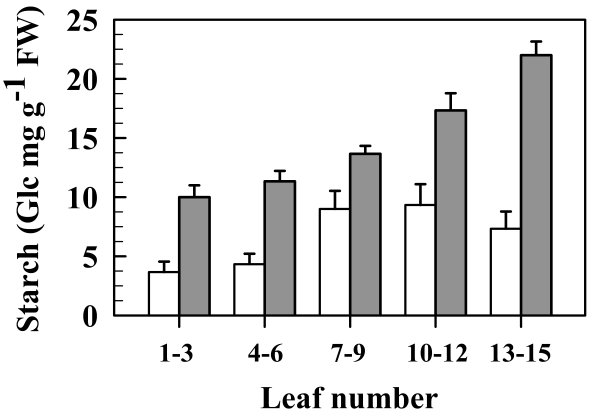

Figure 5.9: **Starch Content of Leaves of Different Ages of Col-0 WT and *sex-1* Mutant**

Starch content of leaves of different ages from Col-0 WT (□) and *sex-1* (■) mutant of *Arabidopsis*. Material was collected at ZT 7 under SD conditions, at developmental stages shown in x-axis. Leaf 1 and leaf 15 denote the youngest and oldest leaves, respectively. All results are the SEM of three biological replicates.

However, compared to WT, *sex-1* mutant showed high starch content throughout the day/night cycle and less diurnal variation under both SDs and LDs (Figure 5.10 A; B). *sex-1* seedlings in AVP had slightly more starch than seedlings in JVP. The decreased impact of the different photoperiods on the rate of starch accumulation and degradation in Col-0 WT is due to the light extension used in the LD treatment. Thus, by the end of the day, WT seedlings growing under both photoperiods accumulated similar starch levels. At night, seedlings growing in SDs had slightly faster rate of starch degradation than plants growing in LD. However, at the end of night, similar starch contents were determined in WT grown in both photoperiods.

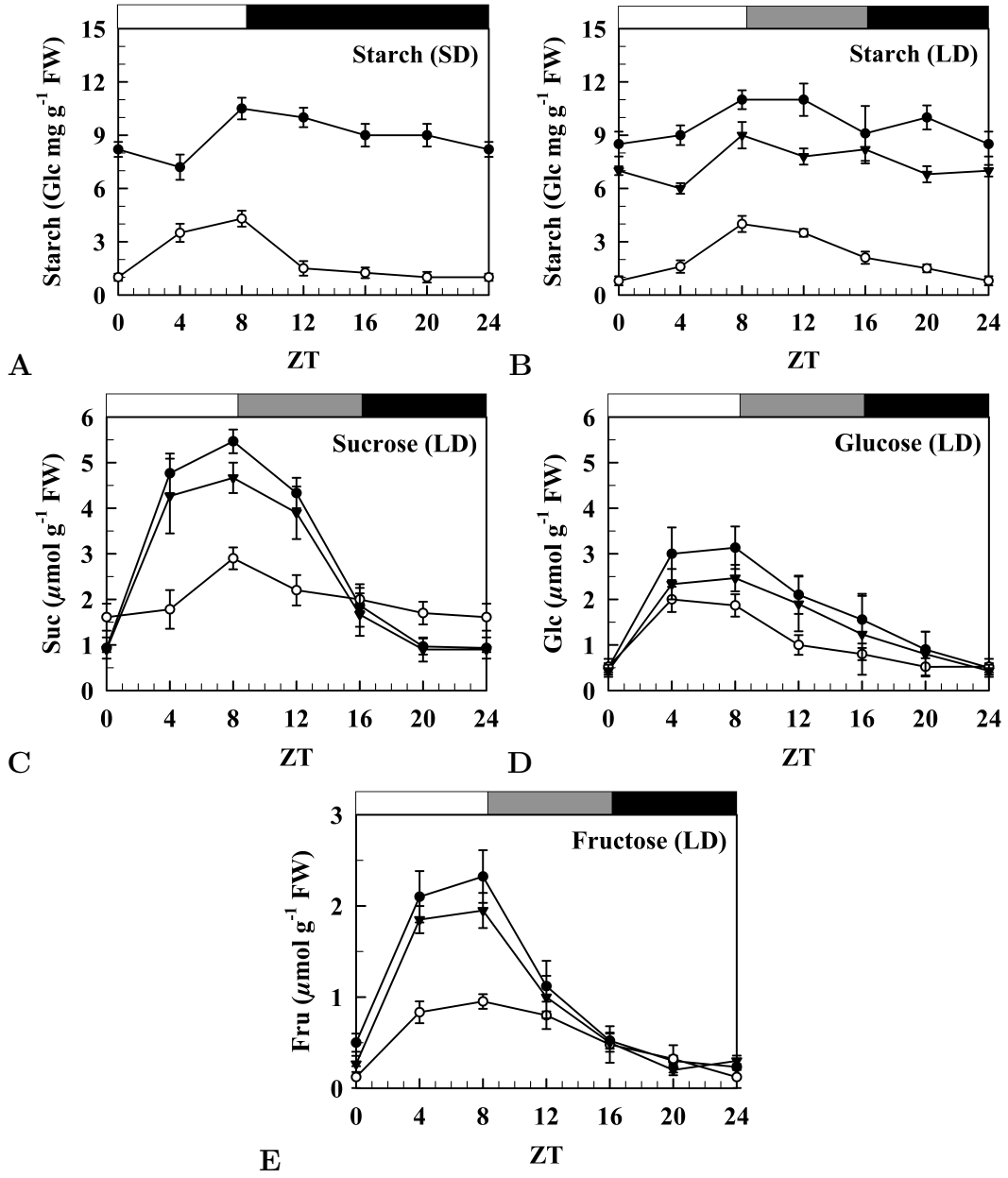

Figure 5.10: **Diurnal Metabolite Changes in Col-0 WT and *sex-1* Mutant Genotypes**

Diurnal changes of starch (A, B), Suc (C), Glc (D) and Fru (E) in Col-0 and *sex-1* genotypes. Adult Col-0 (○), adult *sex-1* (●) and juvenile *sex-1* (▼) seedlings were collected under SDs (A) and LDs (B, C, D, E) on day 5 and day 9 from emergence, for the juvenile and adult phases respectively, at time intervals shown in x-axes. All results are the SEM of three biological replicates. White and black bars on the top are subjective day and night, while the grey bar in the LD treatment indicates the photoperiod extension with low intensity incandescent light. ZT 0 and 24 are values from the same data set.

Determination of soluble carbohydrates (Figure 5.10 C; D; E) extracted from juvenile and adult *sex-1* mutant seedlings showed that Suc and Fru accumulated in large amounts during the day, relative to WT. In dark conditions, soluble carbohydrates were depleted in *sex-1,* in a pattern roughly similar to that of *adg-1* (Figure 5.6). Suc accumulation levels in both juvenile and adult *sex-1* mutant seedlings, were slightly reduced compared to WT Suc levels at the end of dark period (Figure 5.10 C).

The lack of starch turnover in *sex-1* (Figure 5.10 A; B) has an influence on general carbohydrate availability, reducing the amount of Suc (Figure 5.10 C) and Mal (Chia *et al.*, 2004; Niittyla *et al.*, 2004) contents at the end of night. The importance of temporal availability of Mal in the regulation of plant growth can be suported by observations in development of the *Arabidopsis* double mutant *mex-1/dpe-1.* This mutant is very small and pale, and under normal growth conditions often fail to reach a mature developmental state (Alison Smith, personal communication). Furthermore, despite the fact that the *sex-1* and *adg-1* mutants being impaired in different genetic pathways, their metabolism and growth inhibition might be triggered by a transient period of soluble carbohydrate depletion during the dark period. It is possible that both mutants function in the same physiological pathway controlling the length of JVP. As with *adg-1,* it is possible that in *sex-1* carbohydrate depletion during the night leads to critical changes on gene expression stimulating an inhibition of carbohydrate utilization with direct effects on JVP length.

5.3.4 Defining the Juvenile Phase Length in Glucose Insensitive Mutants

Mutants showing sugar insensitive phenotypes represent a valuable tool in unravelling sugar-response pathways affecting plant development. To determine whether mutations affecting carbohydrate sensitivity are involved in the transition within the vegetative phase, the *gin-1* (*aba-2*), *gin-2* (*hxk-1*) and *gin-6* (*abi-4*) mutants were analysed.

Plants use HXK as a Glc sensor to integrate nutrient, light intensity, and hormone signaling systems for controlling plant development in response to environmental conditions (Moore *et al.*, 2003). GIN-1 (ABA-2) encodes a unique short-chain dehydrogenase/reductase that is required for ABA synthesis. It acts downstream of HXK and ETR-1 that is also involved in the Glc-signaling pathway (Zhou *et al.*, 1998). GIN-2 (HXK-1) encodes an HXK that functions as a sugar sensor with both signaling and metabolic functions in plants (Jang and Sheen, 1994; Dai *et al.*, 1999)

Noticeably, it has been shown that in addition to GIN-6, mutations insensitive to Suc which include the *sucrose uncoupled-6* (*sun-6*), *sugar insensitive-5* (*sis-5*) and *impaired sucrose induction-3* (*isi-3*) are allelic to ABI-4 (Huijser *et al.*, 2001; Rook *et al.*, 2001). ABA is regarded as an inhibitor in several developmental processes with the transition to the reproductive phase among them (Bernier, 1988). Phenotypic analyses in *gin-1* (*aba-2*), *gin-2 (hxk-1*) and *gin-6* (*abi-4*) mutants indicated that they flower at the same time with their respective WT in LDs but slightly earlier in SD conditions (Table 5.4; Figure 5.11).

Table 5.4: Flowering Time Profiles of *Arabidopsis* Glucose Insensitive Mutants

Strain	Number of leaves		Number of days		JVP
	SD	LD	SD	LD	
Ler-0	25.0 (±0.2) a	4.2 (±0.1) a	62.5 (±3.9) a	24.7 (±0.2) a	5-6
gin-1	19.1 (±0.5) b	4.1 (±0.1) a	49.5 (±0.5) b	28.6 (±0.2) a	3-4
gin-2	18.3 (±0.6) b	3.8 (±0.1) a	59.4 (±1.6) b	22.0 (±0.5) a	2-3
Col-0	33.9 (±0.7) c	5.7 (±0.3) a	65.2 (±5.7) a	29.8 (±0.2) a	2-3
gin-6	30.4 (±0.8) c	5.9 (±0.3) a	49.5 (±4.9) b	31.7 (±0.7) a	1-2

Data are represented by analysis of 14 plants for each photoperiod treatment. Flowering time is expressed as the number of rosette leaves and number of days from 50% of seedling emergence to the appearance of the floral bolt at 1 cm height. JVP is expressed as number of days from 50% of seedling emergence. SEM indicated in parenthesis. Values followed by the same letter within a column are not significantly different according to the Student's t test at a 0.05 level of significance.

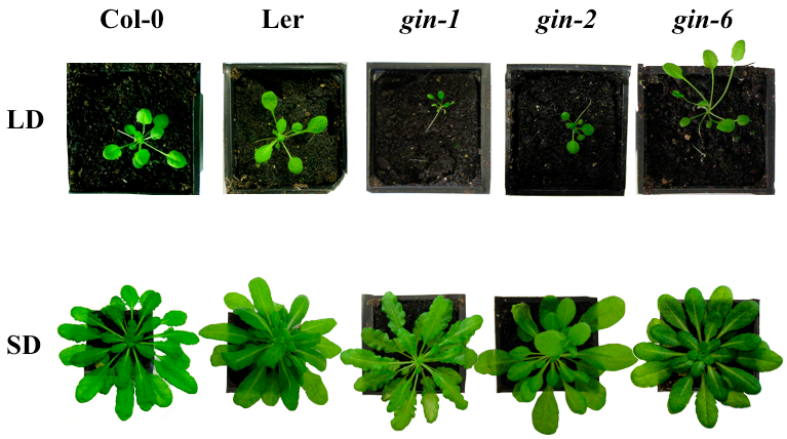

Figure 5.11: **Flowering Time Phenotypes of WT and Glucose Insensitive Mutants of *Arabidopsis***

Flowering time phenotypes of Col-0, Ler-0, *gin-1* (*aba-2*), *gin-2* (*hxk-1*) and *gin-6* (*abi-4*) genotypes in LD and SD conditions. The photo-morphogenetic response to daylength was assayed by providing the additional hours of light in the LD treatment with low intensity incandescent light.

To estimate juvenility the photoperiod transfer experiment was performed three times, in the same CE chambers with identical results (Figure 5.12).

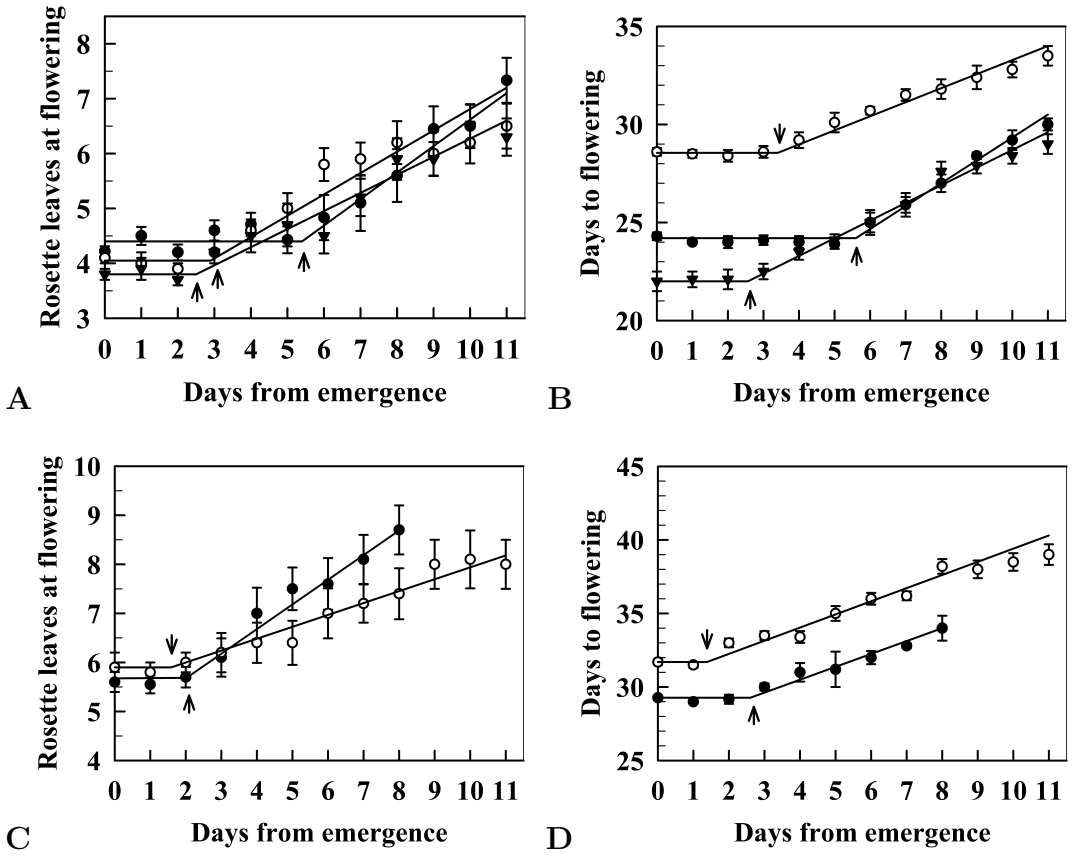

Figure 5.12: **Estimation of the Juvenile Phase Length of Glucose Insensitive Mutants of *Arabidopsis***

(A, B) estimation of the JVP length of Ler-0 (●), *gin-1* (○) and *gin-2* (▼). (C, D) estimation of the JVP length of Col-0 (●) and *gin-6* (○). (A, C) rosette leaves to flowering; (B, D) days to flowering. Plants were transferred from SDs to LDs at time intervals shown in x-axes. Arrows indicate the end of the JVP. Points represent the mean and SE of leaves and days to flower of plants transferred on each occasion.

Examination of the JVP lengths of the *gin* mutants relative to their WTs revealed significant differences (Figure 5.12; Table 5.4). More specifically, photoperiod transfer experiments demonstrated that *gin-1* (*aba-2*) and *gin-2* (*hxk-1*) mutants hastened their JVP by several days compared to Ler-0 WT. This indicates the significance of these loci in the mechanism that controls the short JVP in *Arabidopsis*.

The *gin-6* (*abi-4*) mutant contains a T-DNA insertion in the promoter of the ABI-4

locus (Arenas-Huertero *et al.*, 2000), which encodes an APETALA-2 domain transcription factor (Finkelstein *et al.*, 1998) in Col-0 background. The JVP length in Col-0 has already been shown that is hastened compared to the other two reference accessions of *Arabidopsis.* However, in a similar pattern with the other *gin* mutants, *gin-6* (*abi-4*) seedlings have a shortened JVP length, which is approximately 1-2 d from seedling emergence. However, due to the short JVP length of Col-0 WT, it is obvious that only marginal differences can be observed in mutations affecting juvenility in this background. Hence, it would be better to study the effect of mutations such as *gin-6* (*abi-4*) on juvenility in backgrounds other than Col-0 such as Ler-0 and Ws-4, in which their JVP is prolonged or to grow the plants under LI levels that would prolong JVP.

In higher plants, carbohydrates affect growth and development throughout the life cycle, from germination to senescence (Rolland *et al.*, 2006). The exploitation of *gin* mutants of *Arabidopsis* in photoperiod transfer experiments revealed their altered JVP length and time to flowering compared to WTs. It can be speculated that the hastened JVP length and time to flowering in *gin* mutants might be caused by their inability to sense the carbohydrate accumulation levels. It is possible that during this early developmental stage in *Arabidopsis* carbohydrates may reflect suboptimal growth conditions with direct effects in the transition within the vegetative phase.

Functional analysis of the *gin-6* (*abi-4*) mutant has shown that it is also defective in phytohormone metabolism or response, disclosing a tight interplay between sugar and hormones pathways, particularly for ABA. This hormone is regarded as an inhibitor of several developmental processes such as flowering (Bernier, 1988). Razem *et al.* (2006) showed that ABA may influence flowering by regulation of the FLC mRNA levels. FLC, a MADS box transcription factor is regarded as a repressor of the floral transition (Michaels and Amasino, 1999). It might be that the JVP length in *Arabidopsis* is affected by the same regulatory pathway that is involved in floral transition.

5.3.5 Defining the Juvenile Phase Length in the *ethylene overproducer-1* Mutant of *Arabidopsis*

Ethylene regulates several developmental transitions throughout plant growth and development, which includes the transition to flowering. The effect on flowering might be caused by changes either in floral development after flower bud differentiation, in the timing of the transition from vegetative to reproductive growth or during the vegetative phase change. To determine whether this plant growth regulator is involved in underlying mechanism controlling the juvenile to adult phase change, the *ethylene overproducer-1* (*eto-1*) mutant of *Arabidopsis* was utilized.

The *eto-1* mutation is recessive that results in approximately 10-fold overproduction of ethylene compared to Col-0 WT (Guzman and Ecker, 1990), which is caused by increased stability of ACS5, an isoform of the rate-limiting enzyme 1-Aminocyclopropane-1-Carboxylate Synthase (ACS or ACC synthase; Chae *et al.*, 2003). The large amount of ethylene in *eto-1* may flow into the ethylene signaling pathway causing several developmental effects.

Flowering times in *eto-1* and Col-0 WT were assesed and the mutant flowered slightly earlier than WT under LD conditions (Table 5.5; Figure 5.13)

Table 5.5: Flowering Time Profile of *ethylene overproducer-1* Mutant of *Arabidopsis*

	Number of leaves		Number of days		
Strain	SD	LD	SD	LD	**JVP**
Col-0	33.9 (±0.7) a	5.7 (±0.3) a	65.2 (±5.7) a	29.8 (±0.2) a	2-3
eto-1	41.6 (±0.9) b	5.1 (±0.2) a	93.8 (±1.7) b	25.8 (±0.4) a	1-2

Data are represented by analysis of 14 plants for each photoperiod treatment. Flowering time is expressed as the number of rosette leaves and number of days from 50% of seedling emergence to the appearance of the floral bolt at 1 cm height. JVP is expressed as number of days from 50% of seedling emergence. SEM indicated in parenthesis. Values followed by the same letter within a column are not significantly different according to the Student's t test at a 0.05 level of significance.

However, SD conditions conferred late flowering to *eto-1* compared to Col-0 providing evidence that ethylene may be a factor in accounting for the differences in flowering time observed between the WT and *eto-1* mutant. The promotive effect of ethylene on time to flowering can also be supported by the late flowering phenotype, even under inductive LDs (Bleecker *et al.*, 1988) of *ethylene receptor-1* (*etr-1*); a Glc hypersensitive (Zhou *et al.*, 1998) and ethylene insensitive (Bleecker *et al.*, 1988) mutant of *Arabidopsis*.

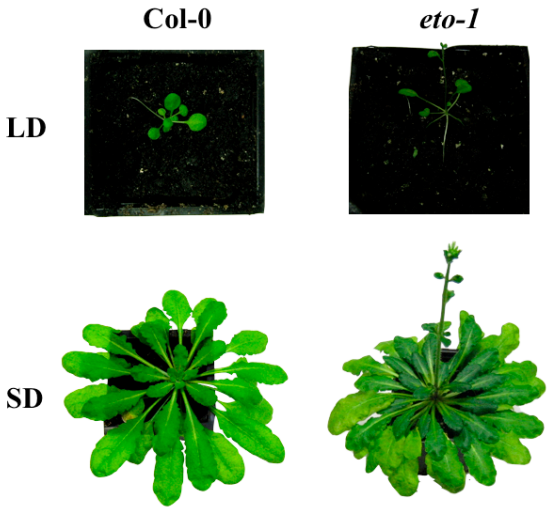

Figure 5.13: **Flowering Time Phenotypes of Col-0 WT and *ethylene overproducer-1* Mutant of *Arabidopsis* under LD and SD Conditions**

The photoperiod experiment was performed twice, in the same CE chambers with identical results. By using the photoperiod transfer approach in *eto-1* slight differences in JVP length compared to WT were revealed (Figure 5.14). Both of the assayed flowering indicators revealed that *eto-1* mutant was insensitive to photoperiod for marginally shorter period of time, compared to WT.

Ethylene controls various processes in the plant life cycle, including seed germination, root hair development, root nodulation, flower senescence, abscission and fruit ripening (Johnson and Ecker, 1998). Studies in *Arabidopsis* have revealed a universally conserved set of components in the ethylene signaling pathway.

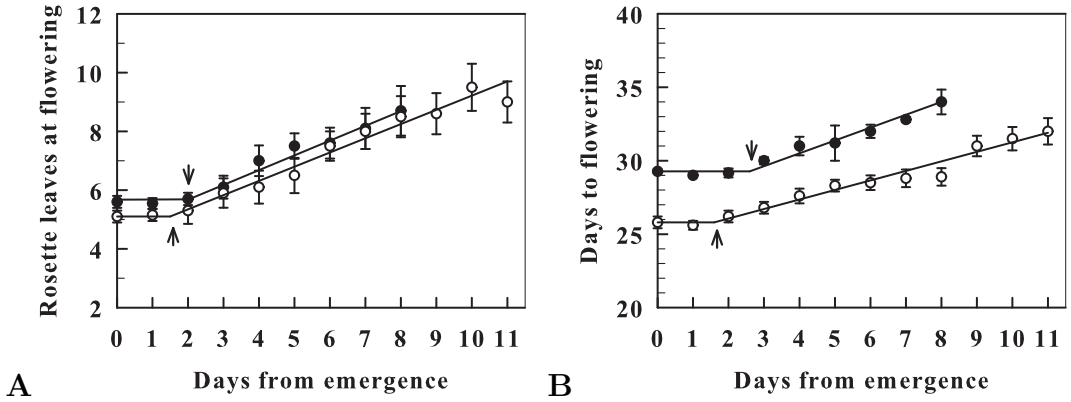

Figure 5.14: **Estimation of the Juvenile Phase Length in *ethylene overproducer-1* Mutant of *Arabidopsis***

Estimation of the juvenile phase length of Col-0 (●), and *eto-1* (○) genotypes. (A) rosette leaves to flowering; (B) number of days to flowering. Plants were transferred from SDs to LDs at time intervals shown in x-axes. Arrows indicate the end of the JVP. Points represent the mean and SE of leaves and days to flower of replicate plants transferred on each occasion.

It has been shown that it is perceived by the ethylene receptor family ETR-1 Chang *et al.*, 1993), ETR-2 (Hua and Meyerowitz, 1998), ETHYLENE INSENSITIVE-4 (EIN-4; Hua *et al.*, 1998), ETHYLENE RESPONSE SENSOR-1 (ERS-1; Hua *et al.*, 1995, 1998), and ETHYLENE RESPONSE SENSOR-2 (ERS-2; Hua *et al.*, 1995, 1998), whereas the signal is further transduced by the signaling pathway components CONSTITUTIVE TRIPLE RESPONSE-1 (CTR-1; Kieber *et al.*, 1993; Clark *et al.*, 1998), EIN-2 (Alonso *et al.*, 1999), EIN-3 (Chao *et al.*, 1997) and ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR-1 (ERF-1; Solano *et al.*, 1998).

It has also been demonstrated that ethylene clearly interacts with carbohydrate signal(s) in controlling seedling growth and development. This was suggested by the finding that the ACC prevents inhibition of cotyledon greening and expansion at high concentrations of Glc in WT (Zhou *et al.*, 1998; Gibson *et al.*, 2001). In addition, the ethylene insensitive mutants *ein-2*, *ein-3* and *etr-1* display Glc hypersensitivity (Zhou *et al.*, 1998; Cheng *et al.*, 2002; Yanagisawa *et al.*, 2003),

whereas the *eto-1* and *ctr-1* (also known as *gin-4* and *sis-1*) Glc insensitivity (Zhou *et al.*, 1998).

However, due to the short JVP length of Col-0 WT, it is estimable that only marginal differences can be observed in mutations affecting juvenility in this background. Hence, in a similar way as *gin-6* it would be better to study the effect of mutations such as *eto-1* on juvenility in backgrounds other than Col-0 such as Ler-0 and Ws-4, or to grow the plants under LI levels that would prolong juvenility.

5.3.6 Estimation of Juvenile Phase Length in Floral Repressors

Floral incompetence during the JVP has led to the speculation that the underlying mechanism of juvenility may involve activities of strong floral repressors at the SAM. To test whether genes acting as repressors of the floral transition affect the JVP length in *Arabidopsis,* the transfer approach was applied to *tfl1-2*, *lhp-1* (*tfl-2*) and *hst-1* mutants.

Genetic and molecular approaches have identified the functions of TFL-1, HST-1 and LHP-1 (TFL-2). Transcripts of these genes are detected in all plant tissues, including roots, seedlings, leaves, inflorescences and siliques (Schmid *et al.*, 2005; `http://bar.utoronto.ca/efp`). However, it has been shown that they mainly act at the SAM regulating shoot floral competence, acting on the floral meristem identity genes. In the case of TFL-1 and HST-1, it has been demonstrated that they function as signals to coordinate shoot meristem identity by regulating LFY and AP-1 (Shannon and Meeks-Wagner, 1993; Ratcliffe *et al.*, 1999; Bradley *et al.*, 1997; Telfer and Poethig, 1998; Conti and Bradley, 2007). HST-1 encodes an ortholog of the EXPORTIN-5 gene of yeast (Bollman *et al.*, 2003). Exportin proteins export a variety of proteins, including both phosphorylated forms of several transcription factors (Boustany and Cyert, 2002) and double-stranded RNA-binding proteins (Brownawell and Macara, 2002). Given that expression of the HST-1 gene is not

specific to the juvenile or the adult phases of plant development, a possible mode of function for HST-1 in phase change might be indirect. For instance, it has been shown that miRNA precursors are efficiently transported by EXPORTIN-5 to the cytoplasm with several developmental effects (Yi *et al.*, 2003). LHP-1 (TFL-2) is the only HETEROCHROMATIN PROTEIN-1-like (HP-1) homologue encoded by the *Arabidopsis* genome (Gaudin *et al.*, 2001; Kotake *et al.*, 2003; Takada and Goto, 2003). It has been demonstrated that it functions as a negative regulator during the transition to the reproductive phase by repressing the expression of FT, but with no effect on expression of the other FPIs (Gaudin *et al.*, 2001; Kotake *et al.*, 2003; Takada and Goto, 2003; Nakahigashi *et al.*, 2005).

hst-1 and *tfl-1* mutants were early flowering under SDs compared to WT, both in terms of the number of days and number of rosette leaves to bolting (Table 5.6; Figure 5.15). Under LD conditions they flowered at the same time with Ler WT. The *lhp-1* mutant, which is in Ws-4 background flowered at the same time with WT under LDs but earlier under SD conditions.

Table 5.6: Flowering Time Profiles of Floral Repressor Genotypes of *Arabidopsis*

	Number of leaves		Number of days		
Strain	SD	LD	SD	LD	**JVP**
Ler-0	25.0 (±0.2) a	4.2 (±0.1) a	62.5 (±1.2) a	24.7 (±0.2) a	5-6
hst-1	7.5 (±0.4) b	2.2 (±0.1) a	39.5 (±0.6) b	20.8 (±0.2) a	<1
tfl-1	14.6 (±0.8) c	2.1 (±0.1) a	49.5 (±1.0) c	20.5 (±0.3) a	1-2
Ws-4	14.7 (±0.2) c	4.0 (±0.3) a	40.7 (±3.5) b	19.3 (±0.1) a	7-8
lhp-1	9.5 (±0.5) b	2.4 (±0.3) a	23.5 (±1.3) d	18.2 (±0.5) a	1-2

Data are represented by analysis of 14 plants for each photoperiod treatment. Flowering time is expressed as the number of rosette leaves and number of days from 50% of seedling emergence to the appearance of the floral bolt at 1 cm height. JVP is expressed as number of days from 50% of seedling emergence. SEM indicated in parenthesis. Values followed by the same letter within a column are not significantly different according to the Student's t test at a 0.05 level of significance.

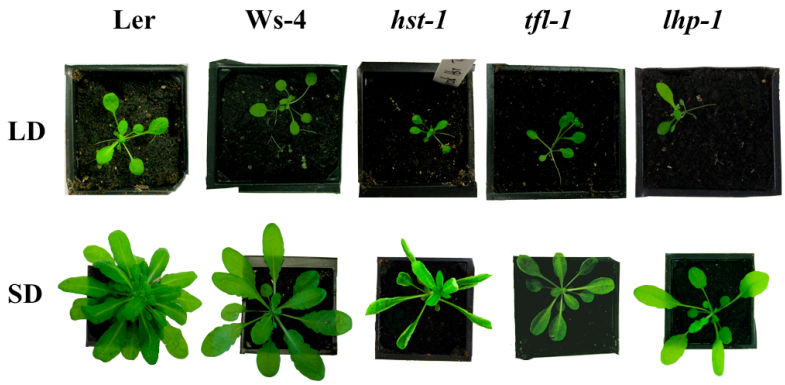

Figure 5.15: **Flowering Time Phenotypes of WTs and Floral Repressors Genotypes of *Arabidopsis***

Flowering time phenotypes of Ler-0, Ws-4, *hst-1*, *tfl-1* and *lhp-1* (*tfl-2*) genotypes in LD and SD conditions. The photo-morphogenetic response to daylength was assayed by providing the additional hours of light in the LD treatment with low intensity incandescent light.

The transfer experiment was performed three times, in the same CE chambers with identical results. By assessing juvenility in *tfl-1, hst-1 and lhp-1* (*tfl-2*) seedlings a hastened JVP length was revealed (Figure 5.16). Comparison with the JVP length of the WT accessions, which is significantly longer provided evidence for the involvement of these three loci in the transition within the vegetative phase of *Arabidopsis*. Further support for the involvement of TFL-1 in the vegetative phase change come from the study of Bradley *et al.* (1997) on inflorescence commitment in *Arabidopsis.* By applying LDs to SDs seedling transfers, they demonstrated a shortened JVP length of *tfl-1* compared to Ler WT.

Many TFL-1 homologs are known, and their functions and interactions are all well described. Pillitteri *et al.* (2004) proposed that CsTFL-1, a citrus TFL-1 homolog may play a role in the transition within the vegetative phase as CsTFL-1 was expressed at much higher levels in juvenile than adult *Citrus* plants. Furthermore, evidence for a role of TFL-1 homologs in juvenility derived also by analyzing the expression and function of MdTFL-1, a *Malus domestica* TFL-1 homolog. MdTFL-1 was preferentially expressed in vegetative tissues in *Malus* species and reduction

of its expression in juvenile plants by RNA interference (RNAi) caused an extremely early flowering phenotype (Kotoda and Wada, 2005; Kotoda *et al.*, 2006).

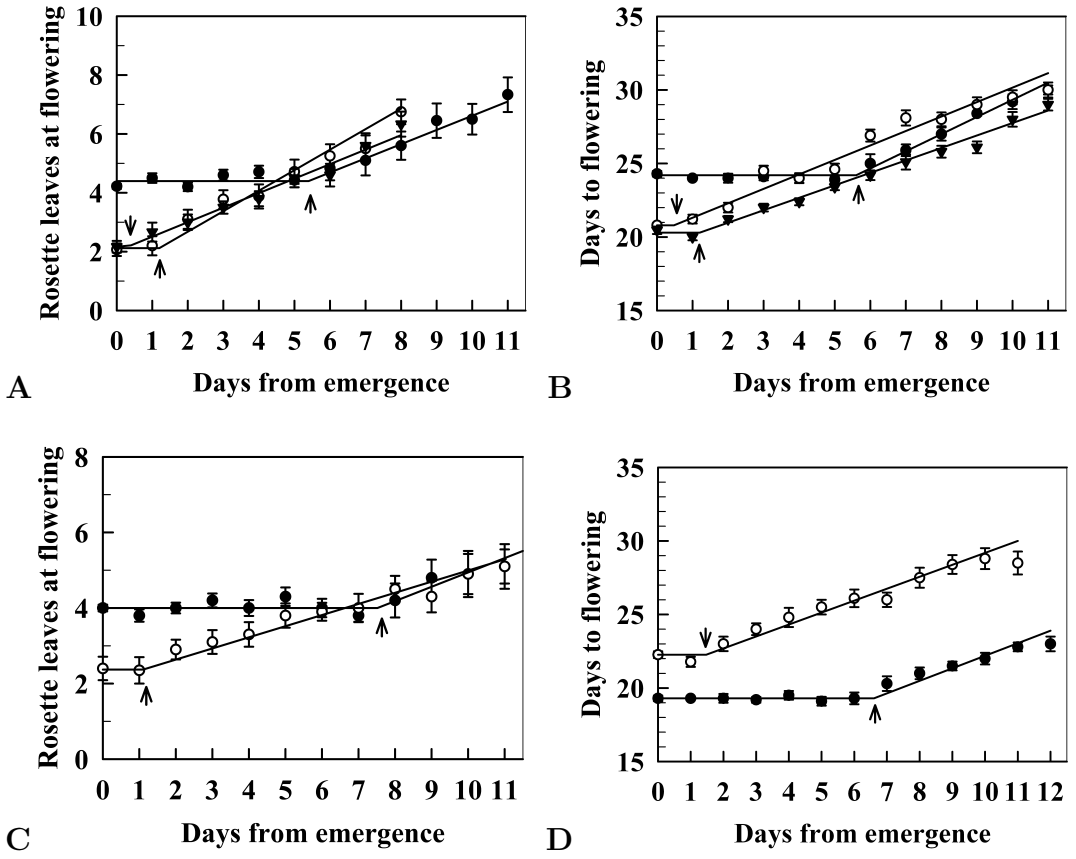

Figure 5.16: **Estimation of the Juvenile Phase Length of Floral Repressors of *Arabidopsis***

(A, B) length of JVP of Ler (●) , *hst-1* (○) and *tfl-1* (▼) genotypes, as revealed by the transfer approach. (C, D) length of JVP of Ws-4 (●) and *lhp-1* (*tfl-2*) (○) genotypes. (A, C) number of rosette leaves; (B, D) days to flowering. Plants were transferred from SDs to LDs at time intervals shown in x-axes. Arrows indicate the end of the JVP. Points represent the mean and SE of leaves and days to flower of replicate plants transferred on each occasion.

HST-1 was isolated in a screen for mutations that accelerated vegetative phase change in respect to morphological traits. It is the *Arabidopsis* ortholog of the miRNA nuclear export receptor Exportin-5 (Bollman *et al.*, 2003). Molecular studies on the juvenile to adult phase change in *hst* mutant revealed a role for miR156 in prolonging the JVP length of *Arabidopsis* (Wu and Poethig, 2006; Chuck *et al.*,

2007). Furthermore, expression of another microRNA, miR172 hasten the JVP length by repressing AP2-like repressors of FT (Aukerman and Sakai, 2003; Mathieu *et al.*, 2007; Jung *et al.*, 2007). It has been shown that the levels of miR156 and miR172 exhibit contrasting expression patterns (Chuck *et al.*, 2007; Wu *et al.*, 2009). As age proceeds, the decline of miR156 levels, and the increase in levels of miR172 and certain SPL genes, leads to the activation of FT in leaves, whereas the increase in SPLs in the meristem leads to the activation of FPIs and FMIs genes that promote the transition to reproductive phase (Yamaguchi *et al.*, 2009; Wang *et al.*, 2009).

As revealed by the transfer approach the length of JVP in *lhp-1* (*tfl-2*) mutant is estimated 2 d from seedling emergence (or 5-6 d from sowing). It has been demonstrated that LHP-1 functions as a negative regulator during the transition to the reproductive phase by repressing the expression of FT, but with no effect on the expression of the other FPIs (Gaudin *et al.*, 2001; Kotake *et al.*, 2003; Takada and Goto, 2003; Nakahigashi *et al.*, 2005). Kotake *et al.* (2003) measured the time course of expression of FT in *tfl-2* (*lhp-1*) mutant and found that FT was extensively upregulated. Ectopic expression of FT induces early flowering irrespective of daylength, while the abundance of *FT* mRNA in WT is very low during the early developmental stages (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). However, expression of FT started to increase in the *tfl-2* mutants as early as 5 d after sowing and reached a maximum level that was more than 100-fold higher than that of WT (Kotake *et al.*, 2003). The accumulation of FT mRNA was also observed in SD conditions, but its rate of increase was 1–2 d slower than in LD conditions. Interestingly, the upregulation of FT in *tfl-2* (*lhp-1*) mutant coincides with the end of JVP in *lhp-1,* as revealed by the photoperiod transfer experiments in this study. It is plausible that not only the activators but also the repressors are required for the precise regulation of developmental changes such as the transition within the vegetative phase. It has been demonstrated that the HP-1, with which LHP-1 shares homology maintains genes in a transcriptionally inactive state by remodeling the chromatin structure in the heterochromatin region

(Nakahigashi *et al.*, 2005). The known function of HP1 corresponds in this kind of regulation, making LHP-1 a potential HP1-like repressor of FT during the JVP in *Arabidopsis*.

Taken together, these results suggest that regulation of the JVP length might be through repression of FPIs transcription by repressors such as TFL-1, HST-1 (miR156) and LHP-1 (TFL-2).

5.3.7 Defining the Juvenile Phase Length in Photoreceptor Mutants of *Arabidopsis*

Light input is mediated by photoreceptors, namely the PHYs, CRYs and PHOTs that detect different aspects of light such as intensity, quality, duration and direction (Thomas, 2006). Through these variables, flowering is regulated by light-dependent photoperiodic and non-photoperiodic regulatory pathways (Thomas, 2006). In order to determine the potential role of photoreceptors in the juvenile to adult phase transition by carrying out photoperiod transfer experiments, the *phy-B* and *fha-1* (*cry-2*) mutant genotypes of *Arabidopsis* were employed.

PHY-B functions as a red/far-red photoreversible sensor in the low fluence range (Halliday *et al.*, 1994; Whitelam and Devlin, 1997), whilst FHA-1 (CRY-2) together with CRY-1 are photoreceptors for blue-light responses (Guo *et al.*, 1998). Analysis of flowering time of the *phy-B* and *fha-1* (*cry-2*) mutants has already elucidated their functions in the regulation of flowering time (Table 5.7). In agreement with previous reports (Goto *et al.*, 1991; Reed *et al.*, 1993; Endo *et al.*, 2005), *Arabidopsis* mutants deficient in PHY-B flowered earlier than WT under both SD and LD conditions (Table 5.7) indicating that PHY-B is a repressor of flowering. In contrast to *phy-B, fha-1* (*cry-2*) flowered late in both SDs and LDs demonstrating the promotive effect of FHA-1 (CRY-2) on time to flowering.

Table 5.7: Flowering Time Profiles of Photoreceptor Mutants of *Arabidopsis*

Strain	Number of leaves		Number of days		JVP
	SD	LD	SD	LD	
Ler-0	25.0 (±0.2) a	4.2 (±0.1) a	62.5 (±3.9) a	24.7 (±1.2) a	5-6
fha-1	29.2 (±0.5) b	12.1 (±0.3) b	70.2 (±3.5) b	33.5 (±0.6) b	7-8
phyB	7.1 (±0.6) c	3.3 (±0.2) a	48.2 (±2.7) c	16.5 (±0.4) c	1-2

Data are represented by analysis of 14 plants for each photoperiod treatment. Flowering time is expressed as the number of rosette leaves and number of days from 50% of seedling emergence to the appearance of the floral bolt at 1 cm height. JVP is expressed as number of days from 50% of seedling emergence. SEM indicated in parenthesis. Values followed by the same letter within a column are not significantly different according to the Student's t test at a 0.05 level of significance.

The photoperiod experiment was performed twice with identical results. As revealed by the photoperiod transfer approach, the JVP length of *phy-B* mutant is estimated to approximately less than 2 d from seedling emergence (Figure 5.16), which is significantly shorter than the JVP length of Ler WT. The hastened JVP of *phy-B* mutant compared to WT has already been reported by estimation of morphological characters such as the appearance of abaxial trichomes (Scott *et al.*, 1999); the appearance in *phy-b* mutant is significantly earlier than the WT.

In contrast to *phy-B, fha-1* (*cry-2*) mutant seedlings were demonstrated to be insensitive to photoperiod for longer period than the WT (Figure 5.17). *fha-1* (*cry-2*) mutant seedlings exposed to approximately 7-8 SDs following seedling emergence, flowered simultaneously with those grown continuously in LD conditions, indicating a prolonged JVP compared to WT.

By genetic analysis of multiple *phy* and *cry* mutants their interactions have been revealed (Lin, 2000). PHY-A and CRY-2 (FHA-1) promote floral induction by suppressing the effect of the inhibitory PHYs and by regulating FT expression via stabilization of CO in LDs (Thomas, 2006).

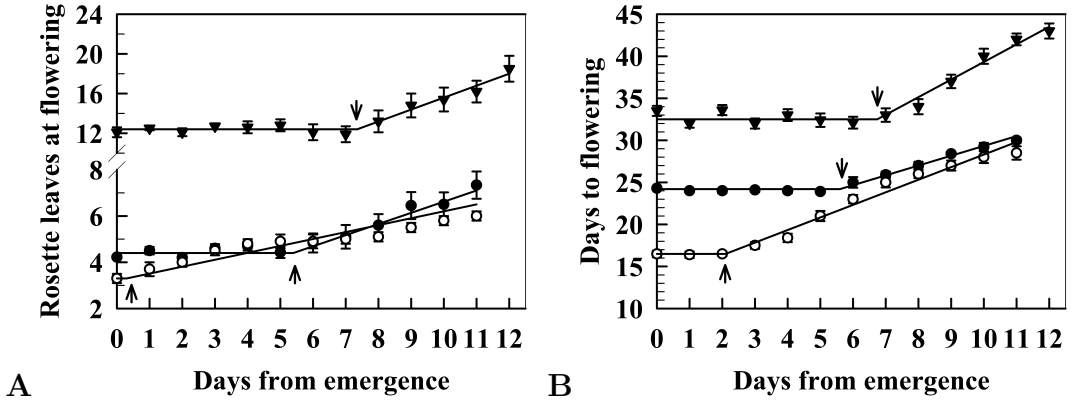

Figure 5.17: **Estimation of the Juvenile Phase Length of Photoreceptor mutants of *Arabidopsis***

Estimation of the juvenile phase length of Col-0 (●), *phyb* (○) and *fha-1* (*cry-2*) (▼) genotypes. (A) rosette leaves to flowering; (B) days to flowering. Plants were transferred from SDs to LDs at time intervals shown in x-axes. Arrows indicate the end of JVP. Points represent the mean and SE of leaves and days to flower of replicate plants transferred on each occasion. Note the y-axis break and change of values in plot A.

Furthermore, it has been shown that PHY-B has a general inhibitory effect on flowering in both LDPs and SDPs via inhibition of CO and FT (Yanovsky and Kay, 2002; Cerdan and Chory, 2003; Endo *et al.*, 2005). Endo *et al.* (2005) examined FT expression during the first week of seedling development in the WT, *phy-B* and a PHY-B:GFP overexpressing mutant line. Expression of FT was found to be lower in WT and PHY-B:GFP than the *phy-B* mutant, whereas the temporal increase of FT expression in *phy-B* coincided with the start of AVP phase, as chronologically and developmentally revealed by the photoperiod transfer experiments in this study. Furthermore, in a similar pattern with the function of LHP-1 (TFL-2) repressor, it is possible that PHY-B may act during the JVP to reduce the expression of FT transcription via CO, prolonging the photoperiod insensitive phase in *Arabidopsis*.

5.4 Conclusions

Arabidopsis mutants are valuable means to model the physiological function of genetic pathways controlling plant development. Their phenotypic characterization offer a powerful tool for the elucidation of the complex pathways that regulate the juvenile to adult vegetative phase change. In this Chapter, a physiological assay has been applied to *Arabidopsis* that allows the length of the JVP to be measured.

It has been shown that *Arabidopsis* WT accessions differ in several morphological and agronomical traits. By assessing juvenility in three *Arabidopsis* WTs important differences regarding their JVP length were revealed. The latest flowering ecotype Col-0 has the shortest JVP length, whilst the earliest flowering Ws-4 has the longest JVP length.

To determine whether a relationship between the transition within the vegetative phase and starch accumulation in a particular WT exists, the diurnal metabolite changes in juvenile and adult vegetative Ws-4 plants were analysed. In agreement with data presented in Chapter 4, more starch remained in adult plants, relative to starch content of juvenile plants at the end of dark period. This observation confirms previous hypothesis about the pivotal role of scotoperiodic carbohydrate availability in the transition within the vegetative phase.

Further support of this hypothesis was provided by assessing the JVP length of starch deficient and starch excess mutants of *Arabidopsis.* Both type of mutants were found to have longer JVP length compared to WT. Furthermore, diurnal metabolites changes observed in juvenile and adult *adg-1* and *sex-1* mutant plants suggest that the lack of starch turnover in both types of mutants influence general carbohydrate availability, reducing the amount of Suc contents at the end of night compared to WT. Moreover, despite being impaired in different genetic pathways, their metabolism and growth inhibition might be triggered in both, by a transient period of soluble carbohydrate depletion during the night. By studying both types of mutants, it can be postulated that Mal, as a product of starch degradation,

and/ or Suc depletion during the night lead to critical changes on gene expression, stimulating an inhibition of carbohydrate utilization with direct effects on JVP length.

Mutants showing sugar insensitive phenotypes represent valuable means to unravel sugar-response pathways affecting plant development. By assessing juvenility in four glucose insensitive mutants of *Arabidopsis*: *gin-1* (*aba-2*), *gin-2* (*hxk-1*), *gin-6* (*abi-4*) and *eto-1,* a shortened JVP length compared to WT were revealed. However, in antithesis with the promotive effect of carbohydrates in juvenile to adult phase change in *Antirrhinum,* the hastened JVP length of *gin* mutants demonstrates that soluble sugars reflect suboptimal growth conditions during this early phase change in *Arabidopsis.* This might be due to the developmental differences in two species; *Antirrhinum,* a species with an approximate generation time of four months has significantly more extensive and demanding sink tissues than *Arabidopsis* at the end of JVP. Furthermore, functional analysis of the *gin* mutants has revealed that many of them are also defective in phytohormone metabolism or response, disclosing a tight interplay between sugar and hormones pathways, particularly for ABA and ethylene. Hence, it is possible that carbohydrate involvement in the juvenile to adult phase change might be through their function as nutrients, signaling molecules, and by their interaction with hormonal networks.

The transition to flowering has been genetically explored in *Arabidopsis* and many loci have been cloned. This has led to the conclusion that multiple environmental and endogenous networks promote and enable floral induction. FT is the core protein of the pathway that promotes the transition to reproductive phase in response to LDs. In *Arabidopsis*, over-expression of the FT accelerates flowering time, and over-expressing mutant lines flower with only two rosette leaves, suggesting alterations in JVP and AVP lengths compared to WT. Classic photoperiod pathway mutants such as *gi, co* and *ft* have been defined as "blind" to photoperiod because these mutants can not respond to inductive photoperiod conditions; they exhibit similar leaf numbers and bolting time at flowering in both LDs and SDs (data

not presented). FHA-1 (CRY-2) also acts in photoperiod pathway but it retains a response to photoperiod. Assessing juvenility in *fha-1* (*cry-2*) mutant a prolonged JVP compared to WT was revealed, confirming the promotive role of CRY-2 (FHA-1). It has been demonstrated that CRY-2 (FHA-1) promotes floral induction by suppressing the effect of the inhibitory PHYs, and by regulating FT expression via stabilization of CO in LDs.

However, floral incompetence during the JVP has led to the speculation that the underlying mechanism of juvenility may involve activities of strong floral repressors such as TFL-1, HST-1, LHP-1 (TFL-2) and PHY-B. Assessment of the JVP in the four mutant lines, a significant shortened JVP length was revealed, compared to WT. It can be inferred from the current study that regulation of FT transcription and other FPIs by strong floral repressors such as TFL-1, HST-1, LHP-1 (TFL-2) and PHY-B could be the underlying mechanism of floral incompetence during the JVP.

Chapter 6

General Conclusions

6.1 Irradiance and Juvenile Phase Relationships in *Antirrhinum*

Light through the variables of duration, quality, direction and intensity constitutes a critical environmental plant growth indicator (Thomas, 2006). *Antirrhinum* is a photoperiod sensitive facultative LD species that flowers under both LDs and SDs, but sooner under LD conditions. However, little is known about the factors that regulate the temporal response to photoperiod as the plants are not equally sensitive throughout development. In addition, there is no ample evidence on how environmental conditions such as LI and [CO_2] affect such phases of sensitivity. The effects of LI on the length of photoperiod insensitive juvenile phase was investigated by using reciprocal transfer experiments between LDs and SDs. To ensure plant growth and development under LD conditions received similar LI to those grown under SDs, daylength was artificially increased without modifying the total quantity of light available for photosynthesis, by extending the SD treatment with low intensity incandescent light. The effect of LI on the length of the JVP was investigated under natural differences in LI in experiments carried out during summer, winter and spring light conditions (Chapter 3.2.1.1). Additionally, LI was manipulated within each experiment by imposing a shaded treatment. The estimation of the phases of photoperiod sensitivity revealed a relationship between LI and JVP length as reduced LI levels extended JVP and delayed floral initiation.

More specifically, estimates regarding the length of JVP in the three conducted experiments (Chapter 3.2.1.1) have been summarized in the following figures and tables: Figure 3.1; Figure 3.2; Figure 3.3; Table 3.2. For all experiments, the exposure to SD conditions during the JVP had no effect on the time to flowering or on the node number, as *Antirrhinum* plants were effectively insensitive to photoperiod during this period. However, after the end of the JVP, SDs delayed flowering and increased the number of nodes in all experiments carried out. Similarly, when plants were transferred from LDs to SDs, exposure to LDs during the JVP had no

effect on the time to flowering or on the number of nodes. A hastening of flowering time and decrease in the node number was seen once sufficient LD cycles had been received for flower commitment following the end of the JVP.

Reducing LI levels in all experiments by shading caused a significant increase in the length of JVP (Table 3.2). This increase was more dramatic in winter experiment (Figure 3.2; Table 3.2), which is probably due to season's reduced ambient LI levels, followed by the spring (Figure 3.1) and summer (Figure 3.3) experiments. In addition to the prolonged JVP under shaded conditions, a modification of the length AVP was also observed (Table 3.2). Plants grown under shaded conditions in summer, winter and spring experiments remained photoperiod sensitive for longer period than plants grown under unshaded conditions, indicating that both developmental phases are subject to modification.

6.2 [CO_2] and JVP Relationships in *Antirrhinum*

Throughout the last decades, much work was done in the field of environmental control of plant growth and development by conducting research on the effect of elevated [CO_2] on photosynthesis (Gunderson and Wullschleger, 1994; Norby *et al.*, 2002; Long *et al.*, 2004; Rogers *et al.*, 2004), plant growth (Ferris *et al.*, 2001; Taylor *et al.*, 2003; Hattenschwiler *et al.*, 2002), and carbohydrate accumulation (Masle *et al.*, 1993; Kehr *et al.*, 2001). It has been concluded that the effects of elevated [CO_2] on plant growth and development can be divided in two groups: in primary and secondary effects. Primary effects comprise increasing net photosynthesis (Stitt, 1991; Long and Drake, 1992), decreasing stomatal conductance (Mousseau and Saugier, 1992; Gonzalez-Meler *et al.*, 1996) and influence on dark respiration (Curtis and Wang, 1998; Davey *et al.*, 2004). Secondary effects include alterations in more complicated characteristics such as anatomy, morphology, physiology and development (Ceulemans and Mousseau, 1994).

It has long been shown that the removal of CO_2 during the dark period of the day/night cycle of plant growth can inhibit the transition to flowering (Langston and Leopold, 1954). Some studies have shown that the effect of elevated [CO_2] on time to flowering can be entirely explained by its effect upon growth (He and Bazzaz, 2003), whereas others have found that the effect of elevated [CO_2] on growth is poorly correlated with its effect on time to flowering (Marc and Gifford, 1984; Reekie and Bazzaz, 1991; Reekie *et al.*, 1994). However, it has been demonstrated that the presence of CO_2 is required by the photoperiod sensitive species for their temporal response to photoperiod (Bassi *et al.*, 1975).

Data regarding the effects of elevated [CO_2] on the length of JVP are limited. However, as increased LI levels hastened juvenility and time to flowering in *Antirrhinum*, possibly due to photosynthate availability, it was hypothesized that elevated [CO_2] might affect time to flowering by having a direct impact on the pre-flowering developmental phases such as the JVP. Hence, this hypothesis was tested by carring out CE experiments investigating the impact of [CO_2] enrichment on the growth phases of photoperiod sensitivity under low LI levels (2.5 mol m^{-2} d^{-1} PPFD). It has been demonstrated that LI below a particular compensation point limits photosynthesis and thus net carbon accumulation and plant growth. Therefore, it was further hypothesized that the effect of [CO_2] enrichment might be more pronounced at low PPFD, as it is likely at this particular compensation point photosynthates would be a limiting factor.

Elevated [CO_2] hastened the end of juvenility under 2.5 mol m^{-2} d^{-1} PPFD by approximately 14.1 d, compared to JVP of plants grown in ambient [CO_2] conditions (Figure 3.5). Previous studies (Adams and Jackson, 2004) quantifying the effect of elevated [CO_2] on the length of JVP under 8 mol m^{-2} d^{-1} PPFD revealed an effect of elevated [CO_2] on prolonging the JVP length by 5-6 d. However, the extent of this response was rather small, possibly due to the saturated LI level of 8 mol m^{-2} d^{-1} PPFD used in this experiments. In experiments described (Chapter 3.3.3.3), elevated [CO_2] levels under reduced PPFD (2.5 mol m^{-2} d^{-1}) had a dramatic im-

pact on JVP length and time to flowering. Such a response indicated that elevated [CO_2] may accentuate the effects of limited LI conditions on JVP length and time to flowering. It has been demonstrated that photosynthesis of plants utilizing the C_3 photosynthetic pathway is promoted by elevated [CO_2] due to the insufficient saturation of Rubisco by ambient [CO_2] (Long and Drake, 1992; Drake *et al.*, 1997). However, interactions with other environmental factors such as temperature (Turnbull *et al.*, 2002) and nutrient availability such as nitrogen (Kruse *et al.*, 2003) can alter the degree of photosynthetic promotion.

Collectively, the prolonged JVP length and time to flowering of plants grown under decreased LI and ambient [CO_2] might be linked with an insufficient supply of photosynthates produced under these conditions. This is a hypothesis that was tested in plant material generated in known growth phases revealing the relationships of assimilate levels and the transition within the vegetative phase in *Antirrhinu*m.

6.3 Juvenility and Carbohydrate Relationships in *Antirrhinum*

The core hypothesis of this project was that the prolonged JVP length observed in plants grown under decreased LI and ambient [CO_2] is linked with an insufficient supply of photosynthates produced under these conditions.

According to the data presented in Chapter 4, LI and elevated [CO_2] had a dramatic impact on JVP length due to photosynthate assimilate availability confirming the linkage of LI, [CO_2], length of JVP and assimilation availability. Under the experimental conditions applied, a correlation between low levels of photosynthetic assimilates and transition within the vegetative phase was revealed. Quantitative and qualitative analysis of HPLC data obtained by the LI and [CO_2] assays have shown that a particular carbohydrate assimilate level might be required before plants undergo the transition from juvenile to adult phase of plant development.

These results confirmed an earlier broad suggestion that there might be a delay in the transition within the vegetative phase with respect to the attainment of maximal carbohydrate accumulation (Tsai *et al.*, 1997).

Irradiance and elevated [CO_2] have profound effects on plant growth and development. Irradiance is known to have spatial and temporal impacts such as ontogenetic modifications in unshaded and shaded leaves (Sack *et al.*, 2003; Terashima *et al.*, 2006), acclimation responses to plant canopy architecture (Niinemets *et al.*, 2004b,a) and crucial modification to flowering time (Adams *et al.*, 1999, 2001, 2003; Mattson and Erwin, 2005). Enrichment with CO_2 in the phyllosphere often causes a reduction in stomatal density and a higher rate of CO_2 assimilation (Woodward and Kelly, 1995; Drake *et al.*, 1997; Woodward *et al.*, 2002). Furthermore, for a number of species data have shown that photosynthesis is increased by elevated [CO_2] (Poorter and Navas, 2003; Long *et al.*, 2004). In plants grown at ambient [CO_2], especially those utilizing the C_3 photosynthetic pathway, photosynthesis is limited by the competition between CO_2 and O_2 at the active site of Rubisco, which is involved in both photosynthesis and photorespiration.

Irradiance is captured by the chloroplasts, and the absorbed energy is converted into photosynthate assimilates via a photochemical reaction and the reductive pentose phosphate cycle (Nobel, 2009). Therefore, any increase in LI should thus lead to increasing levels of ATP and $NADPH^+$ created by the light dependent reactions, increasing the contents of starch, Suc and Glc in plant tissues (Bjorkman, 1981; Poorter *et al.*, 1997; Nobel, 2009). Regarding CO_2 effects, it is commonly accepted that carbohydrate accumulation, plant growth and [CO_2] are positively correlated (Drake *et al.*, 1997; Pritchard *et al.*, 1999; Tocquin *et al.*, 2006).

Starch metabolism is regulated by circadian rhythms (Lu *et al.*, 2005), and its concentrations in leaves typically reach maximum at the end of the light period (Kemp and Blacklow, 1980; Zeeman and Ap Rees, 1999; Geiger *et al.*, 2000; Chia *et al.*, 2004; Zeeman *et al.*, 2007). The importance of temporal availability of carbohydrates in the transition within the vegetative phase was further supported

by data obtained from the seasonal and diurnal monitoring of starch content in *Antirrhinum* plants grown under different LI and [CO_2]. Starch determinations in material collected from the LI (Figure 4.5; Figure 4.6) and [CO_2] assays showed that plants in AVP accumulated elevated leaf starch levels compared to leaf starch levels of plants in JVP. During the dark, leaf starch is degraded and the carbohydrates released are used to provide substrates for leaf respiration and for continued Suc synthesis and export (Zeeman and Ap Rees, 1999; Zeeman *et al.*, 2004, 2007). At the end of dark period, it was shown that considerable amounts of leaf starch levels remained in adult plants, compared to starch levels of juvenile plants. Furthermore, plants in JVP synthesized less amounts of starch compared to average rate of starch synthesis of adult plants. Hence, it is hypothesized that a specific starch accumulation level and/or the developmental stage ability to sustain a steady supply of several hexoses and Suc during the AVP might be part of a phototrophic mechanism controlling the transition from JVP to AVP in *Antirrhinum*.

Moreover, phloem exudate studies demonstrated that Suc could possibly be a limiting factor during the JVP. Compared to plants in AVP, *Antirrhinum* juvenile plants had lower Suc translocation rates. This is likely due to the reduced net photosynthetic capacity of plants during the JVP. However, the lower rate of phloem export could also result from the lower total vein number and thus total number of plasmodesmata per unit of leaf area (Amiard *et al.*, 2005).

It has been speculated that total carbohydrate, or a particular carbohydrate level may be required to reach a specific threshold in order to sustain a steady supply of sufficient bulk flow through the phloem from the leaves to the SAM to enable delivery of FT-TSF complex (Michaels *et al.*, 2005; Yamaguchi *et al.*, 2005; Lifschitz and Eshed, 2006; Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Lin *et al.*, 2007; Mathieu *et al.*, 2007), and possibly other molecules (Chailakhyan, 1936; Bernier *et al.*, 1993; Machackova *et al.*, 1993; Havelange *et al.*, 1996; Thomas and Vince-Prue, 1997; Lejeune *et al.*, 1988; King and Evans, 2003; Bernier and Perilleux, 2005) ; This would be necessary to render the SAM competent to flower.

6.4 Genetic Analysis of JVP in *Arabidopsis*

In Chapter 5, several *Arabidopsis* WTs and defined mutants defected in different genetic pathways were employed in order to gain a better understanding of the genetic basis underlying juvenility.

Col-0, Ler and Ws-4 WTs were shown to have different JVP lengths and time to flowering. These results suggested that apart from characteristics such as height, leaf size, flower shape, time to flowering and resistance or susceptibility to pathogens (Nam *et al.*, 1997; Purugganan and Suddith, 1998; Alonso-Blanco *et al.*, 1999), *Arabidopsis* WTs also differ in the temporal response to photoperiod.

Estimation of JVP length of a number of mutants acting in different genetic pathways has led to a model describing a simplified integrated network of pathways that quantitatively control the timing of the juvenile to adult phase transition. A version of this model, which divides the genetic pathways into those that enable the juvenile to adult phase change and those that promote it, is presented in Figure 6.1.

Study of starch assimilation in JVP and AVP plants was a main target in this project. Information concerning starch status in plant tissues is of great importance as its availability or lack stimulate many metabolic and developmental responses. Its importance is demonstrated by the late flowering phenotype of starch-deficient (*adg-1* and *pgm-1*) and starch-excess mutants (*bam-3, sex-1* and *sex-4*), and their reduced growth and photosynthetic rates compared to WT plants under inductive conditions (Caspar *et al.*, 1985; Schulze *et al.*, 1991; Geiger *et al.*, 1995). By applying the physiological assay to both starch deficient (Figure 5.5) and *sex* mutants (Figure 5.7), the involvement of starch assimilation or its mobilization products in regulation of juvenility was revealed.

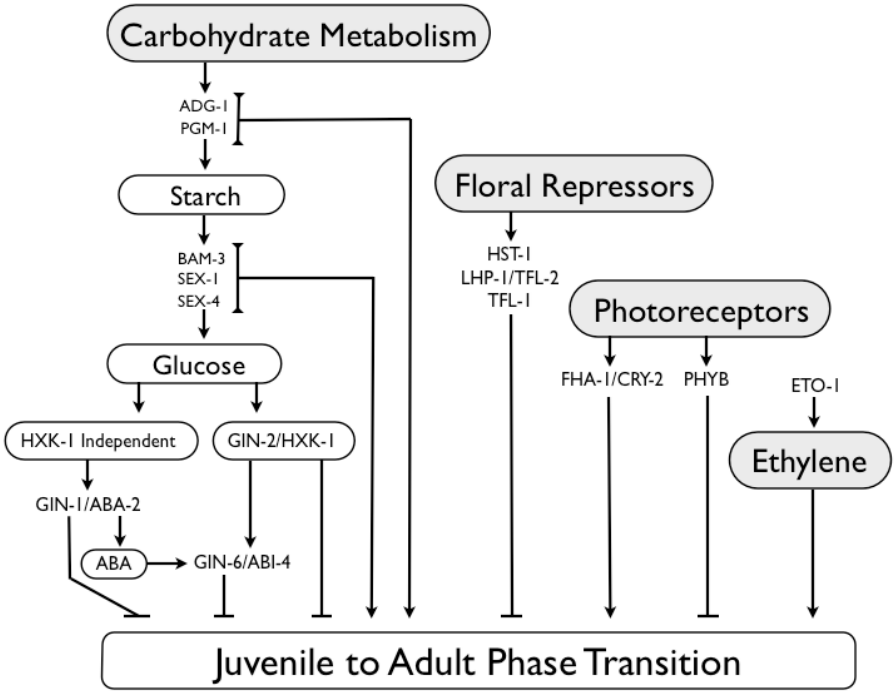

Figure 6.1: **Pathways that Promote or Enable the Juvenile to Adult Phase Transition in *Arabidopsis***

Mutations in different genetic pathways are grouped into those that promote (↓) and those that repress (⊥) the juvenile to adult phase transition. The enabling pathways regulate the ability of the leaf and meristem to respond to floral promotive signals from different environmental and endogenous cues.

More specifically, both type of mutants were found to be insensitive to photoperiod for longer periods compared to Col-0 WT, suggesting a prolonged JVP length. Mal and Glc are the final products of starch mobilization and the major forms of carbon exported from chloroplasts under dark conditions (Weber *et al.*, 2000; Servaites and Geiger, 2002; Weise *et al.*, 2004). Mal is exported by MEX-1 (Niittyla *et al.*, 2004), whereas HXK functions as a Glc sensor with both signaling and metabolic functions in plants (Jang and Sheen, 1994; Dai *et al.*, 1999).

Assessment of juvenility in four *Arabidopsis gin* mutants revealed altered JVP lengths and time to flowering in the mutants, compared to WTs. This suggests the involvement of these loci in the mechanism controlling the transition within the vegetative phase in *Arabidopsis*. Interestingly, functional analysis of the *gin* mu-

tants has shown that many of them are also defective in phytohormone metabolism or response, disclosing an interplay between sugar and hormone pathways, particularly ABA and ethylene.

The response to photoperiod is a prerequisite for the estimation of JVP in response to flowering. Classic photoperiod pathway mutants do not respond to inductive photoperiod conditions in any phase of their development. However FHA-1 (CRY-2), which also acts in photoperiod pathway maintains a response to photoperiod. Assessing juvenility in *fha-1* (*cry-2*) mutant revealed a prolonged JVP compared to WT, suggesting a promotive role for CRY-2 (FHA-1) similar to that in the transition to reproductive phase. It has been proposed that CRY-2 (FHA-1) promote floral induction by suppressing the effect of the inhibitory PHYs, and by regulating FT expression via stabilization of CO in LDs.

Floral incompetence during the JVP has also led to the speculation that the underlying mechanism of juvenility may involve activities of strong floral repressors such as TFL-1, HST-1, LHP-1 (TFL-2) and PHYB. A shorter JVP length observed in the mutants, compared to WTs. This suggests that JVP might act to reduce the expression of FT transcription and the other FPIs via repressors such as TFL-1, HST-1, LHP-1 (TFL-2) and PHYB.

6.5 Juvenility and Carbohydrate Relationships in *Arabidopsis*

The hypothesis that a trophic pathway, via starch catabolism related events might be involved in the regulation of JVP length in *Arabidopsis* was tested. The approach of monitoring the diurnal metabolite changes in WTs and mutant seedlings was followed. In agreement with data presented in Chapter 4 for *Antirrhinum*, considerable amounts of leaf starch levels remained in adult Ws-4 WT plants at the end of dark period, relative to leaf starch levels of juvenile plants. It is possible

that carbohydrate depletion during the night to leads to critical changes on gene expression, stimulating an inhibition of carbohydrate utilization with direct effects on JVP length. Furthermore, it was speculated that plants in JVP may require starch accumulation to reach a particular level, in order to sustain a steady supply of Glc, Mal and/or Suc during the scotoperiod to undergo the transition from juvenile to adult phase of plant development.

Further support on this hypothesis was provided by assessing the JVP length of starch deficient and SEX mutants of *Arabidopsis.* In addition, diurnal metabolites changes observed in *adg-1* and *sex-1* mutant plants suggest that the lack of starch turnover in both types of mutants influence on general carbohydrate availability, reducing significantly the amount of soluble carbohydrate levels at the end of night period. Moreover, despite the fact that they are impaired in different genetic pathways, their metabolism and growth inhibition might be triggered in both, by a transient period of soluble carbohydrate depletion during the night. It has been proposed that the inhibition of growth in starch deficient mutants such as *adg-1* and *pgm-1* is primarily caused by a disturbance of metabolism and growth, which is triggered by a transient period of sugar depletion during the dark. It has been indicated that the scotoperiodic sugar depletion was responsible for the alter expression of genes associated with nutrient assimilation, biosynthesis and growth in the *pgm-1* at the end of the night period, compared with WT at the same time (Thimm *et al.*, 2004). It has been demonstrated that when the night was extended by 4–6 h, global gene expression analysis in WT resembled that in *pgm-1* at the end of the normal night (Thimm *et al.*, 2004).

By monitoring the diurnal metabolite changes in *adg-1* and *sex-1* have shown that *adg-1* and, in part *sex-1,* accumulate elevated levels of soluble sugars during the day compared to WT. Physiological, biochemical and molecular approaches have shown that the early growth and development in *Arabidopsis* seedlings can be arrested in presence of high Glc and Suc levels. Characteristics such as shoot development, leaf formation, cotyledon expansion, greening, and hypocotyl elongation are among

the post-germinative processes that are subject to high levels soluble carbohydrate repression (Jang *et al.*, 1997; Dijkwel *et al.*, 1997; Kurata and Yamamoto, 1998; Arenas-Huertero *et al.*, 2000; Gibson, 2000; Gazzarrini and McCourt, 2001; Eastmond and Graham, 2001). It is possible that apart from starch providing a source of carbon for growth and development during the night (Trethewey and Smith, 2000; Gibon *et al.*, 2004), it may also act as an overflow for newly assimilated carbon (Stitt and Quick, 1989) when assimilation exceeds the demand for Suc. Therefore, it is proposed that the prolonged JVP length in starch deficient and SEX mutants might be caused by the elevated soluble carbohydrate accumulation levels, reflecting suboptimal growth conditions at a crucial developmental stage such as the first weeks of plant development.

Mutants lacking CRYs such as *FHA-1* (*CRY-2*), or having defects in their signaling pathway, show changes in chloroplast composition and disturbance of normal acclimation (Smith *et al.*, 1993; Walters *et al.*, 1999). These photoreceptors are either actually involved in perception of the light quantity with respect to photosynthetic acclimation, or their action is essential for the optimum function of the photosynthetic apparatus. This could explain and link the prolonged JVP length in *fha-1* (*cry-2*) mutant, and its regulation by the photosynthate assimilates. *PHYB, CO* and, indirectly, *PHYA* are under the regulation of *FHA-1* (*CRY-2*; Valverde *et al.*, 2004; Thomas *et al.*, 2006; Thomas, 2006), thus any change on the *FHA-1* (*CRY-2*) transcription levels would also affect the other photoreceptors and *CO*, which act directly upstream of *FT*.

Suc supply to the aerial part of seedlings under *in vitro* conditions, almost completely suppresses the late-flowering phenotype of mutants, such as *gi, co* and *fca*, but is unable to rescue *ft* and *fwa* (Roldan *et al.*, 1999) suggesting a Suc interaction somewhere in between the LD and autonomous signalling pathways, but upstream of *FT*. Further support on photosynthate involvement upstream of *FT* is provided by a microarray study revealing the regulation of *FHA-1* (*CRY-2*) transcription levels by Glc (Li *et al.*, 2006), and by the interaction of elevated [CO_2] with pho-

toperiod pathway mutants through a yet unknown mechanism (Song *et al.*, 2008).

The differential responses of carbohydrates to juvenility in species such as *Arabidopsis* and *Antirrhinum,* might be related to the size and activity of sink organs at the end of JVP. *Arabidopsis* plants at the of JVP have significantly less active sink organs, compared to *Antirrhinum.*

6.6 Recommendations for Future Work

Juvenility has significant scientific and economic implications in plant biology. It has long attracted interest as an aspect of the fundamental topic of aging and also has practical implications, especially in growth and development of those species in which, it is striking and prolonged. The work presented in this thesis was undertaken to improve our understanding on the physiological and genetic factors, which control juvenility in plants. This was achieved by exploiting *Antirrhinum* and *Arabidopsis* as model systems. However, there is a further research, which would be useful to deepen our knowledge in this field by using several physiological and molecular genetic approaches. This could be achieved by the identification of genetic and physiological markers associated with the end of the photoperiod or vernalization-insensitive juvenile phase in different species.

Approaches here should seek to combine CE studies on whole plants with modern methods of proteomics, metabolomics, bioinformatics and integrative modelling. *Arabidopsis* mutants provide a powerful tool for the elucidation of complex pathways that regulate juvenility. In this thesis, several lines of evidence have shown that carbohydrates involvement in the juvenile to adult phase change might be through their function as nutrients, signaling molecules, and by their interaction with hormonal networks. This could further be investigated by using several other mutant genotypes impaired in signal transduction pathways between sugar sensors and genes involved in photoperiod pathway. It has been shown in yeast carbohydrate signaling that the Ser/Thr protein kinase sucrose non-fermenting1 (Snf1) is

a key regulator (Carlson, 1999) between carbohydrate sensors and several target genes. However, apart from yeast, Snf-related protein kinases (SnRKs) are found also in plants, which are involved in a large number of regulatory functions. Some plant SnRKs are activated by sugar availability, although the exact nature of their responses to carbohydrates remains to be clarified (Rolland *et al.*, 2002a).

Furthermore, several lines of evidence indicate the involvement of *cis* and *trans* elements in the final steps of plant sugar signaling. To date, five different types of *cis* factors have been identified in sugar-regulated gene promoters: sugar responsive elements (SURE; Sun *et al.*, 2003), SP8 (Ishiguro and Nakamura, 1994), TGGACGG (Maeo *et al.*, 2001), G box (Giuliano *et al.*, 1988) and B box (Zourelidou *et al.*, 2002) elements. Interestingly, by screening the *Arabidopsis* FT regulatory sequence upstream of the start codon in PLACE (data not presented; `http://www.dna.affrc.go.jp/PLACE/`), a database of motifs found in plant *cis*-acting regulatory DNA elements, several *cis*-acting elements associated with sugar-responsive promoter regions were identified together with the light-responsive regions. This could reveal a potential link between carbohydrates and FT, the major output of the photoperiod pathway.

In *Arabidopsis*, FT is an important floral activator integrating floral signals from multiple pathways (Massiah, 2007; Jackson, 2009). In this thesis it was hypothesized that plants are florally incompetent during the JVP due to inactivity of the photoperiodic floral induction pathway. Further evidence could be provided by comparison of the temporal expression of FT with the length of JVP identified in WTs and mutant lines exploited in this work. In *Antirrhinum*, a full length EST clone in the Dragon DB was identified as being a putative FT homologue based on its high homology to *Arabidopsis FT* and the tomato *SP3D* (A. Massiah, personal communication). Study of its expression pattern showed that plants in JVP have a reduced ability to express FT compared to the latter developmental stages (A. Massiah, unpublished data).

Results in this thesis indicate that the underlying mechanism of juvenility involve

activities of strong floral repressors in both, SAM and leaf. Hence, HST-1, TFL-1, LHP-1 (TFL-2) and TEM genes could be strong candidates for isolation and study of their expression pattern in juvenile and adult *Antirrhinum* plants. Current work in our group includes the identification of a full length *Antirrhinum* EST clone as being a putative TEM homologue, based on its high homology to *Arabidopsis* TEM (A. Massiah, B. Thomas, T. Sgamma, personal communication). To determine its abundance in juvenile and adult vegetative *Antirrhinum* plants, gene expression analysis is currently being undertaken.

A full understanding of physiological basis of juvenility is needed to optimise the aerial environment in protected cropping, and to improve the accuracy of flowering models and scheduling in commercial horticulture; an important sector of the global economy. Currently, the profitability of this sector increasingly depends on the reliable scheduling of flower and vegetable crops with minimal waste in order to meet the demanding retail needs for continuity of high quality product. Photoperiodism and vernalization are exploited to achieve crop scheduling in clonally-propagated species. However, retail demands are increasingly moving towards seed-raised crops, which many of these are photoperiodically or vernalization-regulated. Hence, the understanding of juvenility is a key element in determining success in achieving flower crops and vegetables schedules. Furthermore, unravelling the mechanisms underlying juvenility, it should greatly accelerated current plant breeding programs, and facilitate rapid marker-assisted breeding for producing species with new and better developmental traits capable of adapting to changing climates and growing at different latitudes.

6.7 Project Conclusions

The project conclusions are summarized in terms of the aims outlined in Chapter 1.8. The multiple interactions among the components involved in the juvenile to adult phase transition as identified in this thesis, are presented in Figure 6.2.

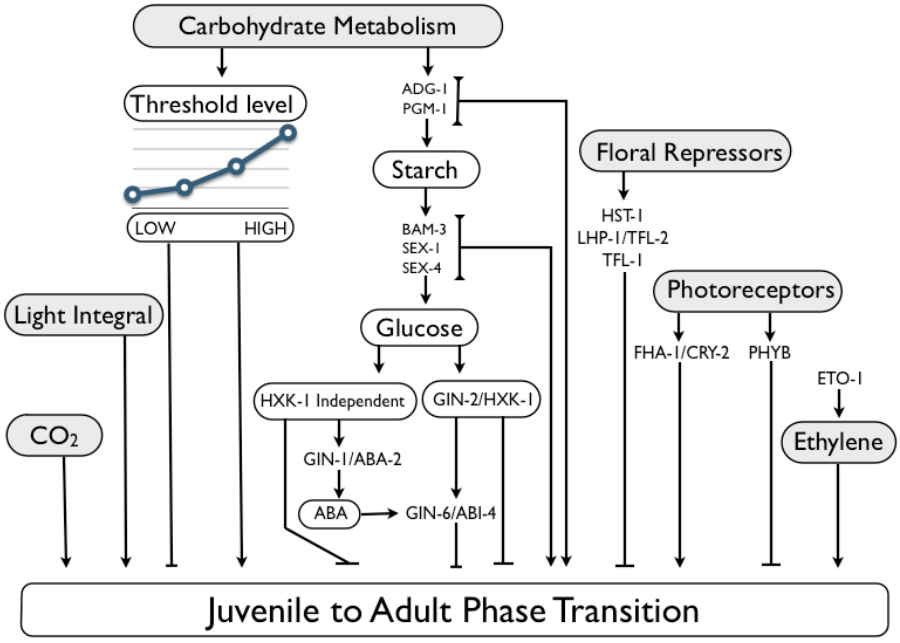

Figure 6.2: **Multiple Interactions Among the Components Involved in the Juvenile to Adult Phase Transition**

Components involved in the juvenile to adult phase transition are grouped into those that promote (↓) and those that enable (⊥) the phase change.

The first aim of this project was to study the effect of environmental factors such as LI and [CO_2] in assimilation of carbohydrates and their potential role in the transition within the vegetative phase in *Antirrhinum*. Estimation of the phases of photoperiod sensitivity by reciprocal transfer experiments revealed a relationship between LI , [CO_2] and JVP length. HPLC data analysis suggested the involvement of a phototrophic pathway controlling the juvenile to adult phase transition in *Antirrhinum*. Quantitative and qualitative carbohydrate analyses indicated that total carbohydrate, or a particular carbohydrate level may be required to reach

a specific threshold, in order to sustain a steady supply of Suc for sufficient bulk flow through the phloem from the leaves to the SAM to enable delivery of FT-TSF complex, and possibly other molecules that render the SAM competent to flower. This speculation is further supported by the phloem exudate studies and by the monitoring of the metabolite changes in *Antirrhinum* time course experiments.

A second aim of this project was to gain a better understanding of the genetic basis underlying juvenility using *Arabidopsis* defined mutants. Physiological analysis of the temporal response to an inductive photoperiod in mutants impaired in different genetic pathways have shown that multiple inputs influence the timing of the switch from juvenile to adult phase of plant development. It was demonstrated that carbohydrate involvement in the juvenile to adult phase change might be through their their function as nutrients, signaling molecules or by their interaction with hormones. Furthermore, physiological analysis of flowering time mutants have shown that a variety of environmental and endogenous signals act to promote and enable the juvenile to adult phase change by regulating the FPIs via floral activators and floral repressors, respectively.

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Appendix A

Additional Figures and Tables

A.1 Chapter 1

A.1.1 Tables

Table A.1: **List of *Arabidopsis* Genes that are Discussed in this Literature Review**

Gene name	**Abbreviation**	**Gene Identifier**	**Reference**
ADP GLUCOSE PYROPHOSPHORYLASE-1	ADG1	At5g48300	Lin *et al.*, 1988
APETALA1/AGAMOUS-LIKE-7	AP1/AGL7	At1g69120	Irish and Sussex, 1990
ALPHA-AMYLASE-1	AtAMY1	At4g25000	Yu *et al.*, 2005
ALPHA-AMYLASE-2	AtAMY2	At1g76130	Yu *et al.*, 2005
ALPHA-AMYLASE-3	AtAMY3	At1g69830	Yu *et al.*, 2005
BETA-AMYLASE-1	AtBMY1	At4g15210	Mita *et al.*, 1995
BETA-AMYLASE-3	AtBMY3/BAM3	At4g17090	McCallum *et al.*, 2000
Arabidopsis thaliana CENTRORADIALIS	ATC	At2g27550	Bradley *et al.*, 1997
Arabidopsis thaliana MYB33	AtMYB33	At5g06100	Gocal *et al.*, 2001b
Arabidopsis thaliana SUCROSE-PROTON SYMPORTER-9	AtSUC9	At5g06170	Sivitz *et al.*, 2007
BROTHER of FT and TFL-1	BFT	At5g62040	Hanzawa *et al.*, 2005
CAULIFLOWER	CAL	At1g26310	Purugganan *et al.*, 1995
CAM1	CAM1	N/A	Eimert *et al.*, 1995
CIRCADIAN CLOCK ASSOCIATED-1	CCA1	At2g46830	Wang and Tobin, 1998
CENTRORADIALIS	CEN	embCAC21564.1	Schwarz-Sommer *et al.*, 1992
CONSTANS	CO	At5g15840	Kobayashi *et al.*, 1999
CONSTITUTIVE PHOTOMORPHOGENIC-1	COP1	At2g32950	Wei and Deng, 1992
CRYPTOCHROME-1	CRY1	At4g08920	Ahmad *et al.*, 1995
CRYPTOCHROME-2	CRY2/FHA	At1g04400	Ahmad *et al.*, 1995
DISPROPORTIONATING ENZYME-2	DPE2	At2g40840	Chia *et al.*, 2004
EARLY FLOWERING-3	ELF3	At2g25930	Zagotta *et al.*, 1996
EARLY FLOWERING-4	ELF4	At2G40080	Doyle *et al.*, 2002
EARLINESS PER SE	EPS	N/A	Bullrich *et al.*, 2002

Gene name	Abbreviation	Gene Identifier	Reference
FCA	FCA	At4g16280	Reeves and Coupland, 2001
FD	FD	At4g35900	Abe *et al.*, 2005
FLAVIN-BINDING KELCH DOMAIN F BOX PROTEIN-1	FKF1	At1g68050	Nelson *et al.*, 2000
FLOWERING LOCUS C/AGAMOUS-LIKE 25	FLC/AGL25	At5g10140	Henderson *et al.*, 2003
FPA	FPA	At2g43410	Quesada *et al.*, 2005
FLOWERING PROMOTING FACTOR-1	FPF1	At5g24860	Xu *et al.*, 2005
FLOWERING LOCUS T	FT	At1g65480	Kobayashi *et al.*, 1999
FRUITFULL/AGAMOUS LIKE-8	FUL/AGL8	At5g60910	Mandel and Yanofsky, 1995
FVE	FVE	At2g19520	Ausin *et al.*, 2004
FWA	FWA	At4g25530	Soppe *et al.*, 2000
FY	FY	At5g13480	Simpson *et al.*, 2003
GA REQUIRING-1	GA1	At4g02780	Silverstone *et al.*, 1997
GA REQUIRING-4	GA4	At1g15550	Xu *et al.*, 1997
GA REQUIRING-5	GA5	At4g25420	Xu *et al.*, 1997
GIBBERELLIC ACID INSENSITIVE	GAI	At1g14920	Peng *et al.*, 1997
GIGANTEA	GI	At1g22770	Park *et al.*, 1999
GLUCOSE-INSENSITIVE-4/ABA DEFICIENT-2	GIN1/ABA2	At1g52340	Zhou *et al.*, 1998
GLOSSY-15	GL15	g115977625R	Moose and Sisco, 1994
HASTY	HST	AT3G05040	Bollman *et al.*, 2003
HEADING DATE-1	HD1	Os03g03164	Yano *et al.*, 2000
HEADING DATE-3 ALPHA	HD3A	Os06g0157700	Yano *et al.*, 2000
ELONGATED HYPOCOTYL-5	HY5	At5g11260	Koornneef *et al.*, 1980
LUMINIDEPENDENS	LD	At4g02560	Koornneef *et al.*, 1991

Gene name	Abbreviation	Gene Identifier	Reference
LEAFY	LFY	At5g61850	Weigel and Meyerowitz, 1993
LATE ELONGATED HYPOCOTYL	LHY	At1g01060	Schaffer *et al.*, 1998
LOV KELCH PROTEIN-2	LKP2	At2g18915	Schultz *et al.*, 2001
LUX ARRHYTHMO/PHYTOCLOCK-1	LUX/PCL1	At3g46640	Hazen *et al.*, 2005
MALTOSE EXCESS-1	MEX1	At5g17520	Lu *et al.*, 2006
MOTHER of FT and TFl-1	MFT	At1g18100	Yoo *et al.*, 2004b
MORI1	MORI1	N/A	Asai *et al.*, 2002
Nicotiana tobacco SUCROSE TRANSPORTER-1	NTSUT1	AM491605	Kuhn *et al.*, 1997
Oryza sativa GIGANTEA	OsG1	Os01g0182600	Hayama *et al.*, 2003
PHYTOCHROME-SIGNALING EARLY FLOWERING-1	PEF1	TAIR: 1005246161	Ahmad and Cashmore, 1996b
PHYTOCHROME-SIGNALING EARLY FLOWERING-2	PEF2	N/A	Ahmad and Cashmore, 1996b
PHYTOCHROME-SIGNALING EARLY FLOWERING-3	PEF3	N/A	Ahmad and Cashmore, 1996b
PHOSPHOGLUCOMUTASE	PGM	At5g51820	Yu *et al.*, 2001
PHOTOTROPIN-1	PHOT1	At3g45780	Jarillo *et al.*, 1998
PHOTOTROPIN-2	PHOT2	At5g58140	Kagawa *et al.*, 2004
PHYTOCHROME A	PHYA	At1g09570	Whitelam *et al.*, 1993
PHYTOCHROME B	PHYB	At2g18790	Koornneef *et al.*, 1980
PHYTOCHROME C	PHYC	At5g35840	Reed *et al.*, 1993
PHYTOCHROME D	PHYD	At4g16250	Reed *et al.*, 1993
PHYTOCHROME E	PHYE	At4g18130	Reed *et al.*, 1993
PHOTOPERIOD RESPONSE	PPD	N/A	Laurie *et al.*, 1995

Gene name	Abbreviation	Gene Identifier	Reference
PSEUDO-RESPONSE REGULATOR 5	PRR5	At5g24470	Ito *et al.*, 2007
PSEUDO-RESPONSE REGULATOR 7	PRR7	At5g02810	Harmer and Kay, 2005
PSEUDO-RESPONSE REGULATOR 9	PRR9	At2g46790	Harmer and Kay, 2005
RNA-DEPENDENT RNA POLYMERASE 6	RDR6	At3g49500	Elmayan *et al.*, 1998
SERRATE	SE	At2g27100	Prigge and Wagner, 2001
STARCH EXCESS-1	SEX1	At1g10760	Caspar *et al.*, 1991
SUPPRESSOR OF GENE SILENCING-3	SGS3	At5g23570	Mourrain *et al.*, 2000
SUPPRESSOR OF CONSTANS OVEREXPRESSION-1	SOC1	At2g45660	Lee *et al.*, 2000
SELF-PRUNING	SP	LEU84140	Pnueli *et al.*, 1998
SPINDLY-4	SPY4	At3g11540	Jacobsen and Olszewski, 1993
SQUINT	SQN	At2g15790	Berardini *et al.*, 2001
TEMPRANILLO	TEM	At1g25560	Castillejo and Pelaz, 2008
TEOPOD1	TP1	MaizeGDB ID:136479	Dudley and Poethig, 1993
TERMINAL FLOWER 1	TFL1	At5g03840	Ratcliffe *et al.*, 1999
TIMING OF CAB EXPRESSION-1	TOC1	At5g61380	Millar *et al.*, 1995
TWIN SISTER OF FT	TSF	At4g20370	Kobayashi *et al.*, 1999
TREHALOSE-6-PHOSPHATE SYNTHASE	TPS	At1g78580	Leyman *et al.*, 2001
UVB-RESISTANCE-8	UVR8	At5g63860	Kliebenstein *et al.*, 2002
REDUCED VERNALIZATION RESPONSE-1	VRN1	At3g18990	Levy *et al.*, 2002
REDUCED VERNALIZATION RESPONSE-2	VRN2	At4g16845	Chandler *et al.*, 1996
REDUCED VERNALIZATION RESPONSE-3	VRN3	AB361063	Yan *et al.*, 2006
ZIPPY/ARGONAUTE-7	ZIP/AGO-7	At1g69440	Hunter *et al.*, 2003
ZEITLUPE	ZTL	At5g57360	Somers, 1999

A.2 Chapter 3

A.2.1 Figures

Figure A.1: **Overview of *Antirrhinum* Growth Systems**

Overview of *Antirrhinum* growth systems. (A) Plants grown in automatic moveable trolleys in GH conditions. (B) Plants grown in growth chambers with temperature, CO_2, lighting and RH control.

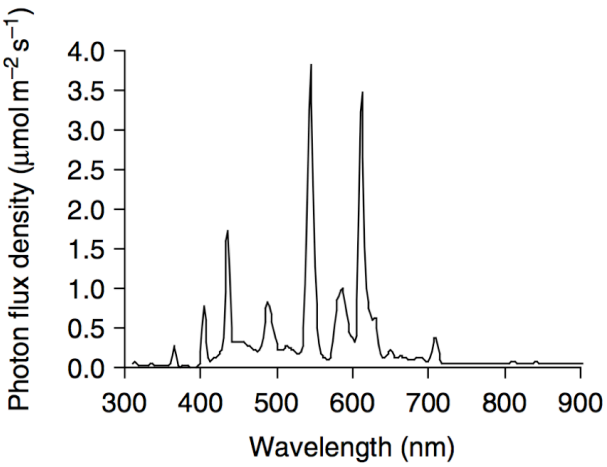

Figure A.2: **Spectra of Experimental Light in CE Cabinets During the *Antirrhinum* RIL57 Experiments**

The spectra of experimental light in CE cabinets (Sanyo Fitotron, Loughborough, UK) during RIL57 reciprocal transfer experiments.

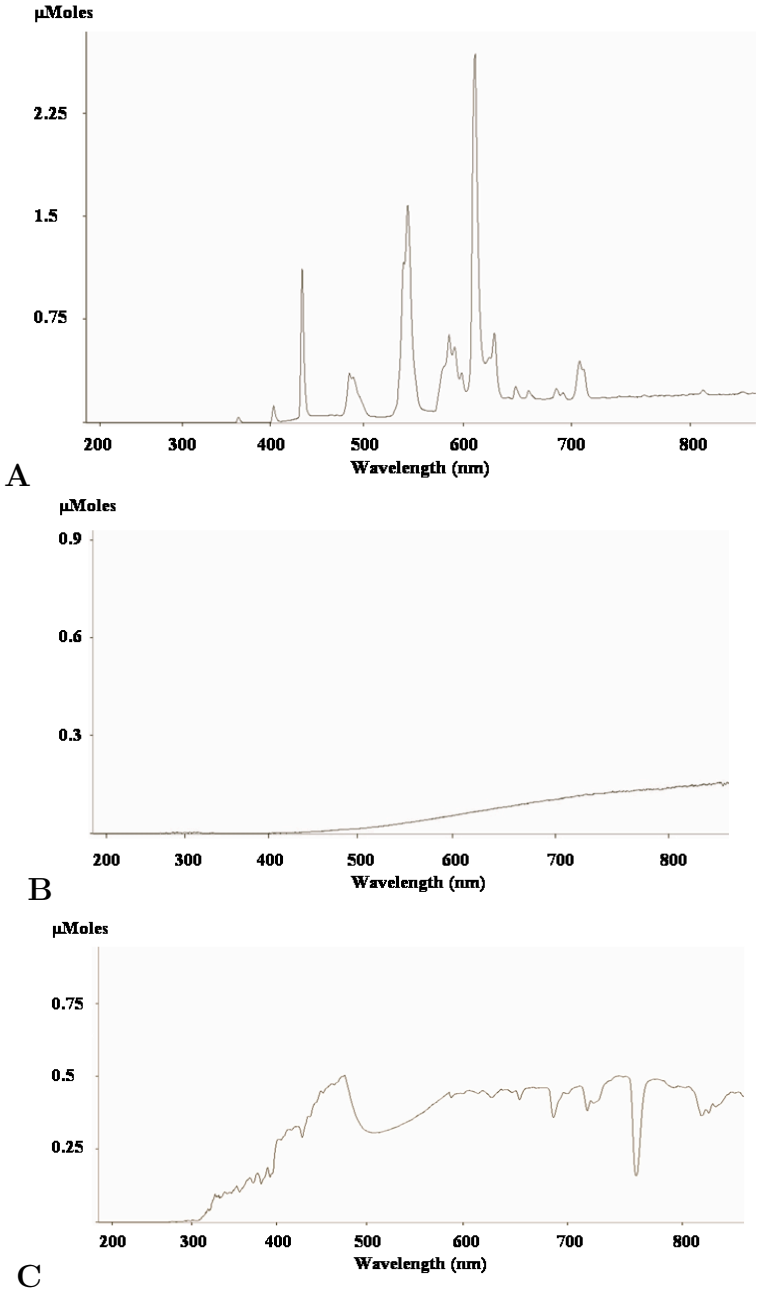

Figure A.3: **Spectra of Experimental Light Used in *Antirrhinum* CO_2 Transfer Experiments**

Spectra obtained by fluorescent (A) and low intensity incandescent (B) lighting in *Antirrhinum* CO_2 transfer experiments; they are shown as the relative spectral irradiance in the wavelength range of 300-800 nm. (C) direct sunlight; University of Warwick, Wellesbourne campus on Januray 30, 2007, at 15:30 GMT.

Figure A.4: **Effect of Plant Reciprocal Transfers on Time to Flowering During the Summer Experiment in *Antirrhinum* Bells F1**

The effect of plant reciprocal transfers from SDs to LDs (A) and LDs to SDs (B) on time to flowering of *Antirrhinum* Bells F1 during the summer experiment. Numbers in A and B indicate the time in weeks that plants spent in SDs and LDs before they transferred in LDs and SDs, respectively. Images taken by V. Valdes.

Figure A.5: **Effect of Plant Reciprocal Transfers on *Antirrhinum* Flowering Time Under Elevated [CO_2]**

Effect of reciprocal transfers from LDs to SDs (A) and SDs to LDs (B) on flowering of *Antirrhinum* Bells F1 under elevated [CO_2]. The delay to flowering with successive transfer illustrates the delay in inflorescence initiation caused by extended time spent in non inductive SD conditions.

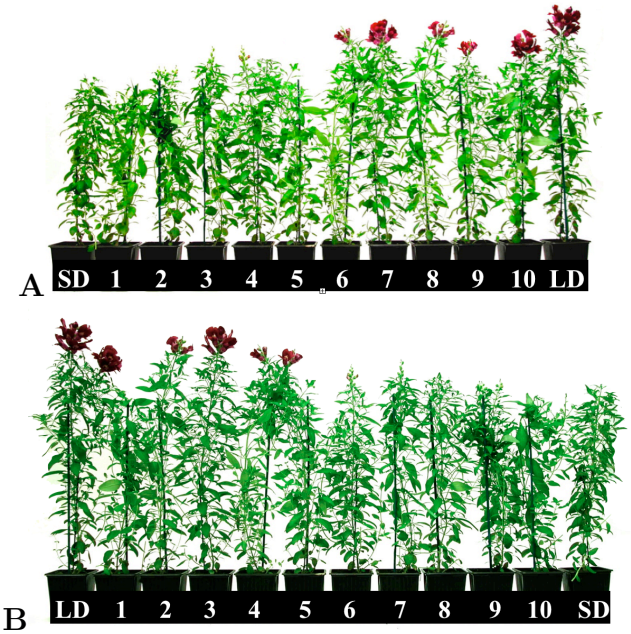

Figure A.6: **Effect of Plant Reciprocal Transfers on *Antirrhinum* Flowering Time Under Ambient [CO_2]**

Effect of plant reciprocal transfers from LDs to SDs (A) and SDs to LDs (B) on flowering of *Antirrhinum* Bells F1 under ambient [CO_2]. The delay to flowering with successive transfer illustrates the delay in inflorescence initiation caused by extended time spent in non inductive SD conditions.

A.3 Chapter 4

A.3.1 Figures

A.3.1.1 Effect of LI on Soluble Sugar Accumulation in *Antirrhinum*

Summer Experiment

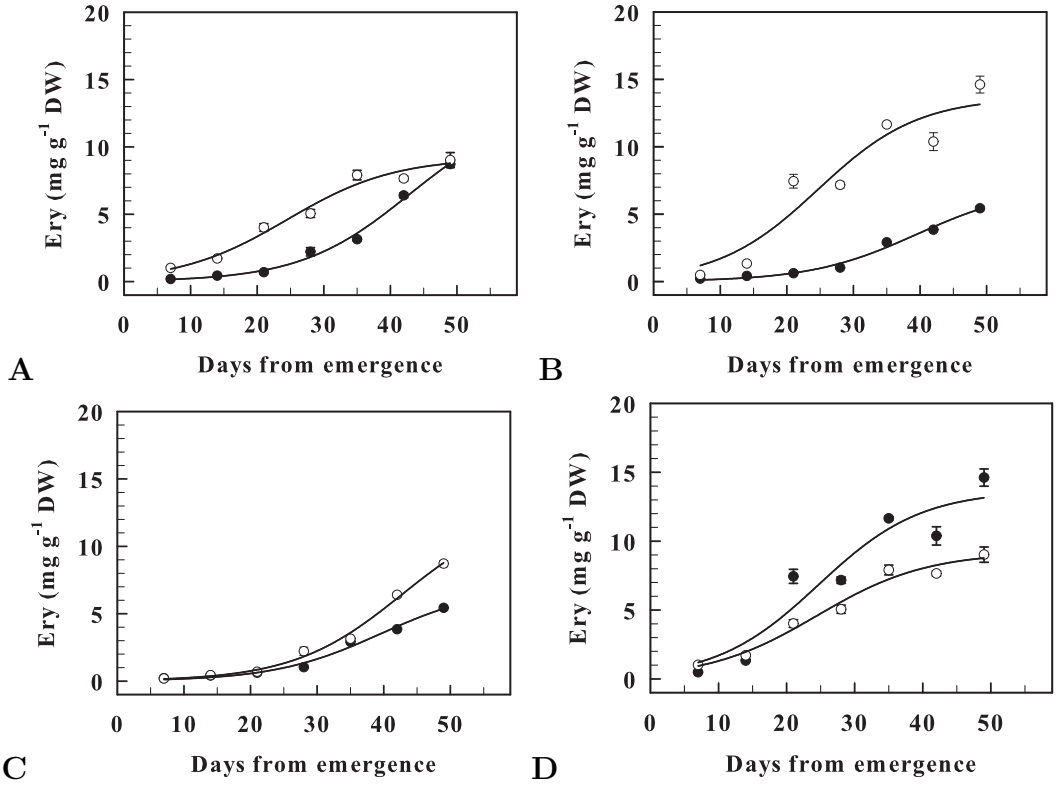

Figure A.7: **Changes in Ery Contents in *Antirrhinum* Leaves During Development in the Summer Experiment**

Non linear regression (–) of Ery contents in *Antirrhinum* leaves during the summer experiment. (A, B) Ery contents in SDs (A) and LDs (B) under unshaded (○) and shaded conditions (●). (C, D) Ery contents in shaded (C) and unshaded (D) SD (○) and LD (●) conditions. SD shaded: r^2 0.99; SD unshaded: r^2 0.97; LD shaded: r^2 0.98; LD unshaded: r^2 0.90. Error bars indicate ± SEM.

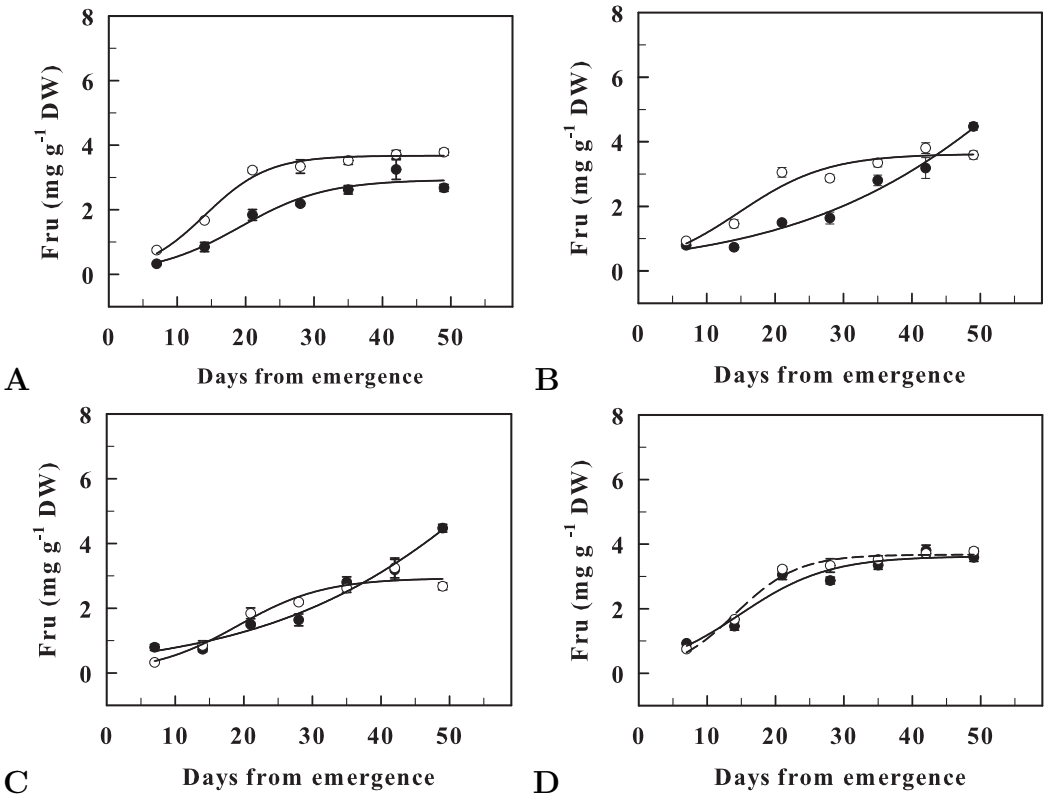

Figure A.8: **Changes in Fru Contents in *Antirrhinum* Leaves During Development in the Summer Experiment**

Non linear regression (–) of Fru contents in *Antirrhinum* leaves during the summer experiment. (A, B) Fru contents in SDs (A) and LDs (B) under unshaded (○) and shaded (●) conditions. (C, D) Fru contents in shaded (C) and unshaded (D) SD (○) and LD (●) conditions. SD shaded: r^2 0.95; SD unshaded: r^2 0.98; LD shaded: r^2 0.97; LD unshaded: r^2 0.93. Error bars indicate ± SEM.

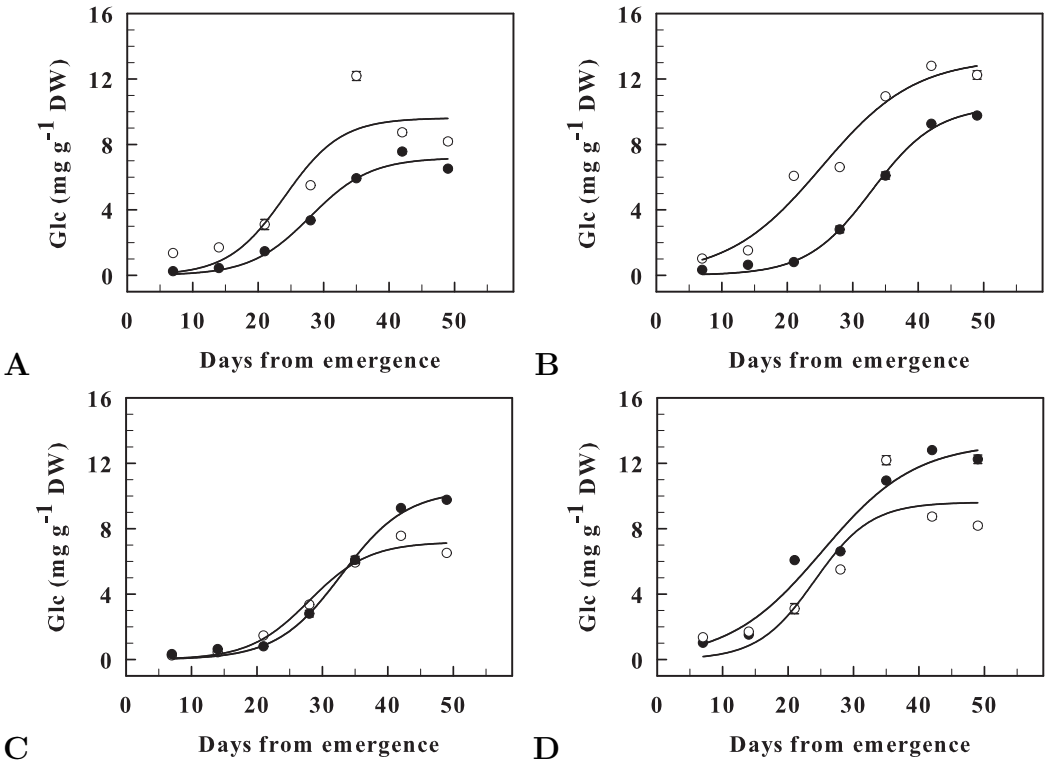

Figure A.9: **Changes in Glc Contents in *Antirrhinum* Leaves During Development in the Summer Experiment**

Non linear regression (–) of Glc contents in *Antirrhinum* leaves during the summer experiment. (A, B) Glc contents in SDs (A) and LDs (B) under unshaded (○) and shaded (●) conditions. (C, D) Glc contents in shaded (C) and unshaded (D) SD (○) and LD (●) conditions. SD shaded: r^2 0.98; SD unshaded: r^2 0.82; LD shaded: r^2 0.99; LD unshaded: r^2 0.96. Error bars indicate ± SEM.

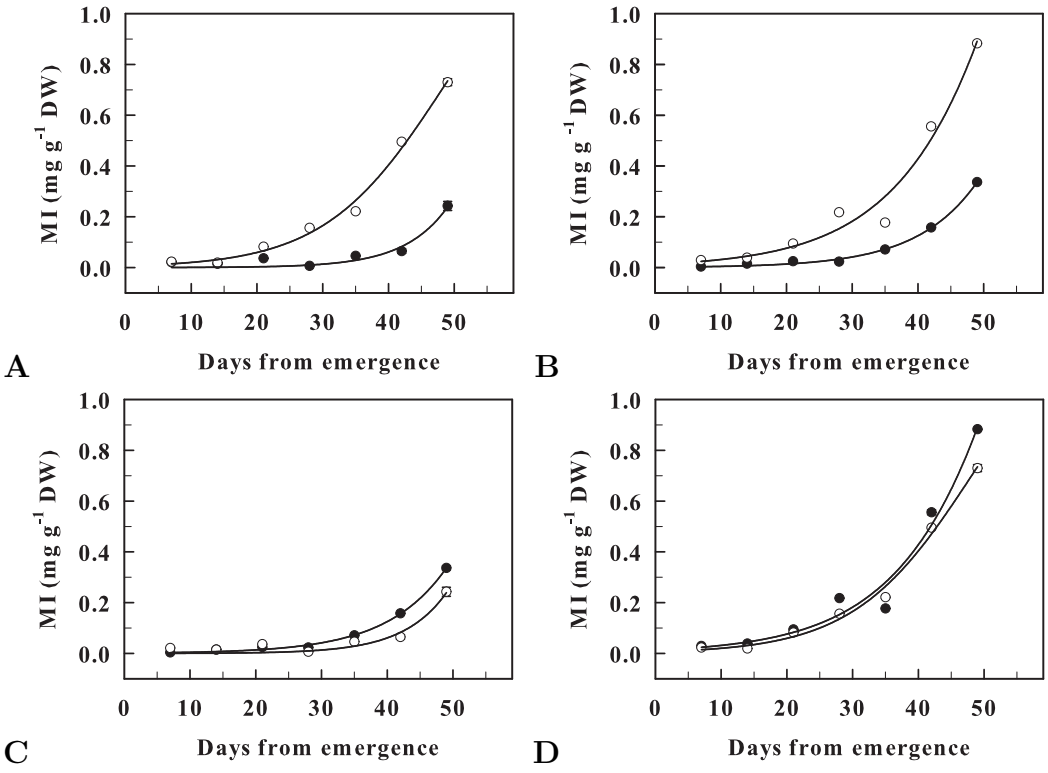

Figure A.10: **Changes in MI Contents in *Antirrhinum* Leaves During Development in the Summer Experiment**

Non linear regression (–) of MI contents in *Antirrhinum* leaves during the summer experiment. (A, B) MI contents in SDs (A) and LDs (B) under unshaded (○) and shaded (●) conditions; (C, D) MI contents in shaded (C) and unshaded (D) SD (○) and LD (●) conditions. SD shaded: r^2 0.94; SD unshaded: r^2 0.99; LD shaded: r^2 0.99; LD unshaded: r^2 0.97. Error bars indicate ± SEM.

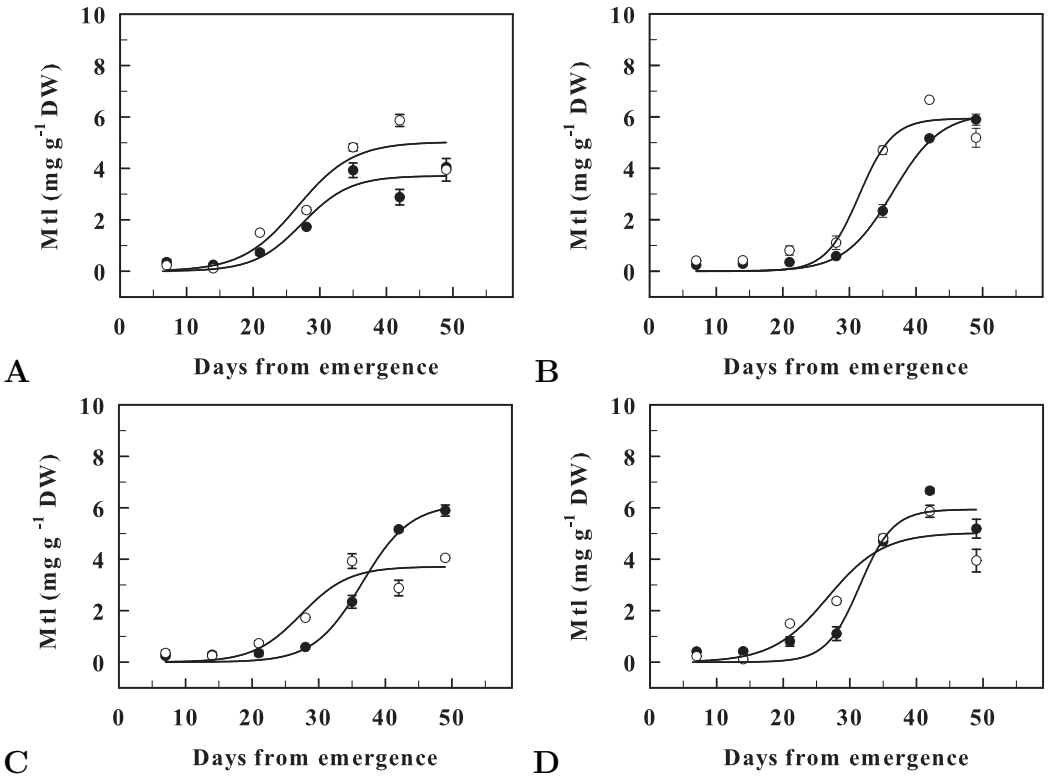

Figure A.11: **Changes in Mtl Contents in *Antirrhinum* Leaves During Development in the Summer Experiment**

Non linear regression (–) of Mtl contents in *Antirrhinum* leaves during the summer experiment. (A, B) Mtl contents in SDs (A) and LDs (B) under unshaded (○) and shaded (●) conditions. (C, D) Mtl contents in shaded (C) and unshaded (D) SD (○) and LD (●) conditions. SD shaded: r^2 0.91; SD unshaded: r^2 0.90; LD shaded: r^2 0.99; LD unshaded: r^2 0.97. Error bars indicate ± SEM.

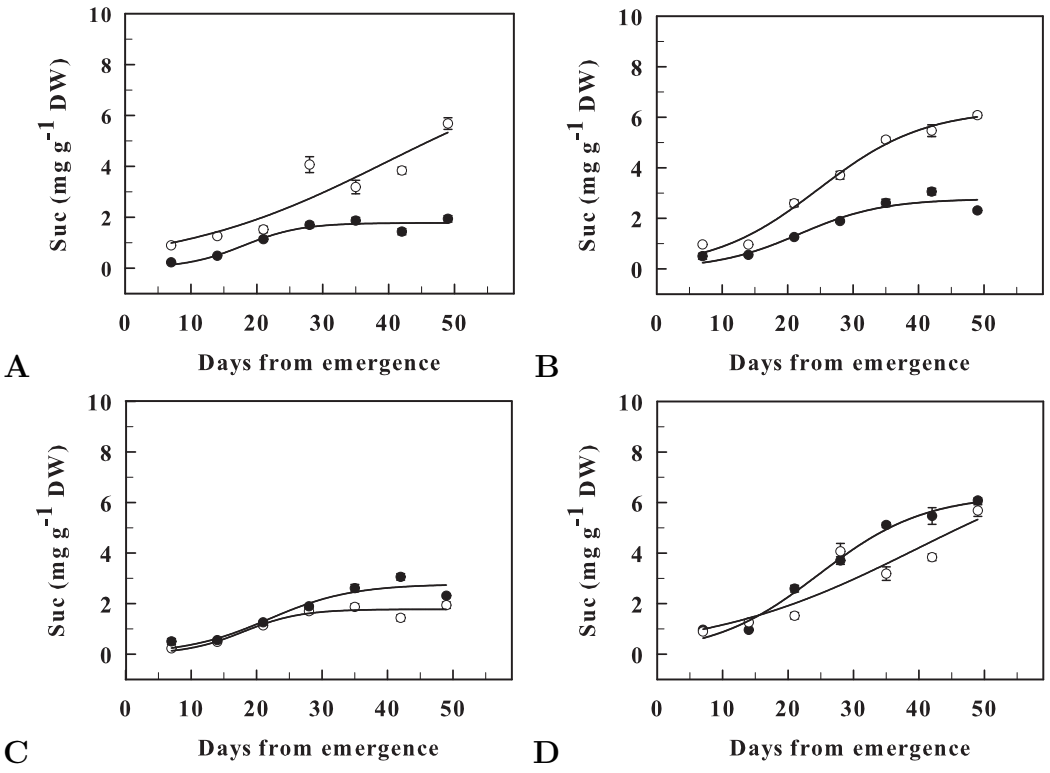

Figure A.12: **Changes in Suc Contents in *Antirrhinum* Leaves During Development in the Summer Experiment**

Non linear regression (–) of Suc contents in *Antirrhinum* leaves during the summer experiment. (A, B) Suc contents in SDs (A) and LDs (B) under unshaded (○) and shaded (●) conditions. (C, D) Suc contents in shaded (C) and unshaded (D) SD (○) and LD (●) conditions. SD shaded: r^2 0.93; SD unshaded: r^2 0.85; LD shaded: r^2 0.92; LD unshaded: r^2 0.98. Error bars indicate ± SEM.

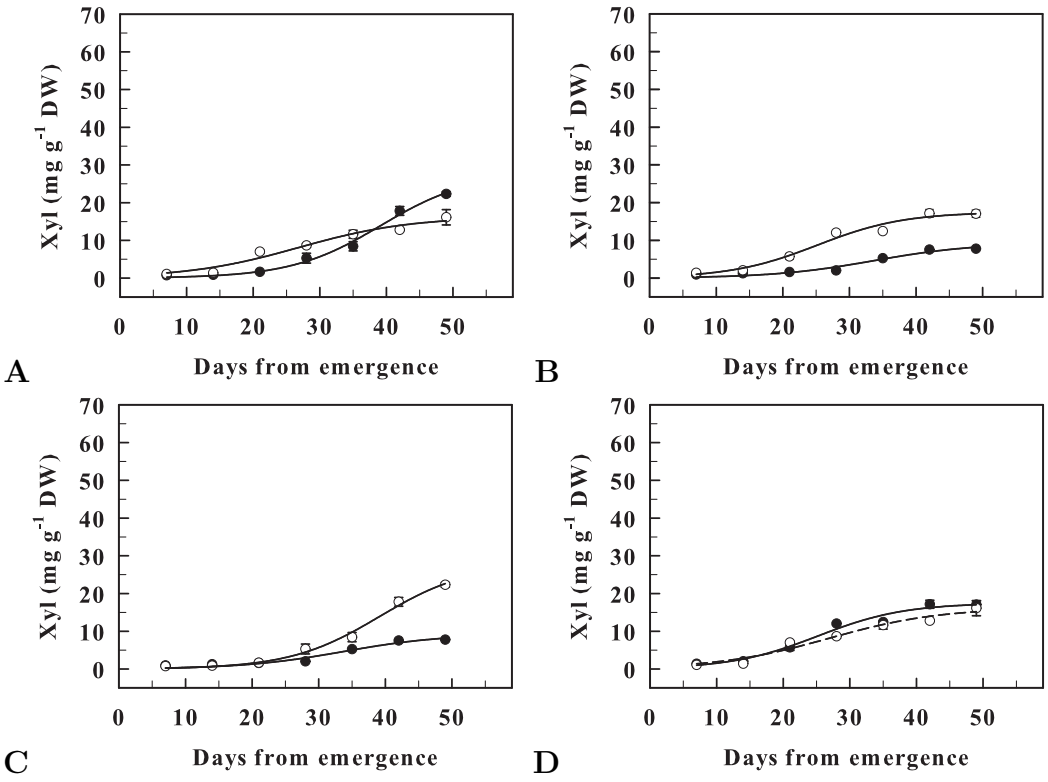

Figure A.13: **Changes in Xyl Contents in *Antirrhinum* Leaves During Development in the Summer Experiment**

Non linear regression (–) of Xyl contents in *Antirrhinum* leaves during the summer experiment. (A, B) Xyl contents in SDs (A) and LDs (B) under unshaded (○) and shaded (●) conditions. (C, D) Xyl contents in shaded (C) and unshaded (D) SD (○) and LD (●) conditions. SD shaded: r^2 0.99; SD unshaded: r^2 0.96; LD shaded: r^2 0.95; LD unshaded: r^2 0.97. Error bars indicate ± SEM.

Winter Experiment

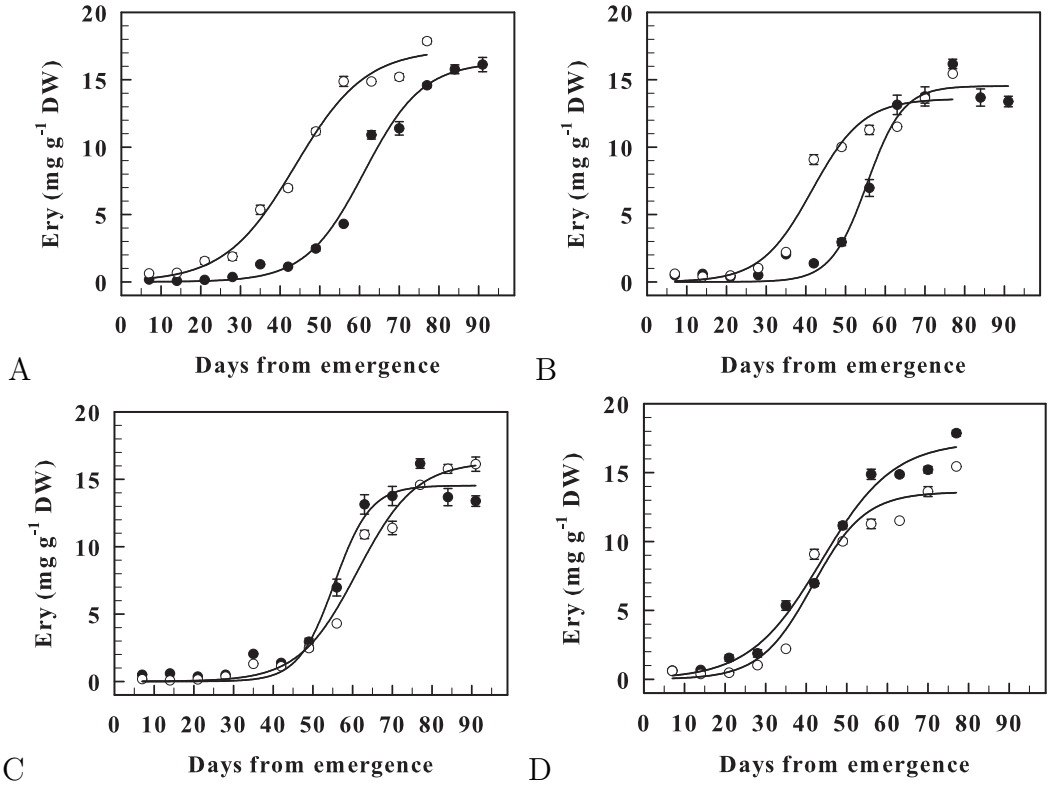

Figure A.14: **Changes in Ery Contents in *Antirrhinum* Leaves During Development in the Winter Experiment**

Non linear regression (–) of Ery contents in *Antirrhinum* leaves during the winter experiment. (A, B) Ery contents in SDs (A) and LDs (B) under unshaded (○) and shaded (●) conditions. (C, D) Ery contents in shaded (C) and unshaded (D) SD (○) and LD (●) conditions. SD shaded: r^2 0.98; SD unshaded: r^2 0.98; LD shaded: r^2 0.97; LD unshaded: r^2 0.96. Error bars indicate ± SEM.

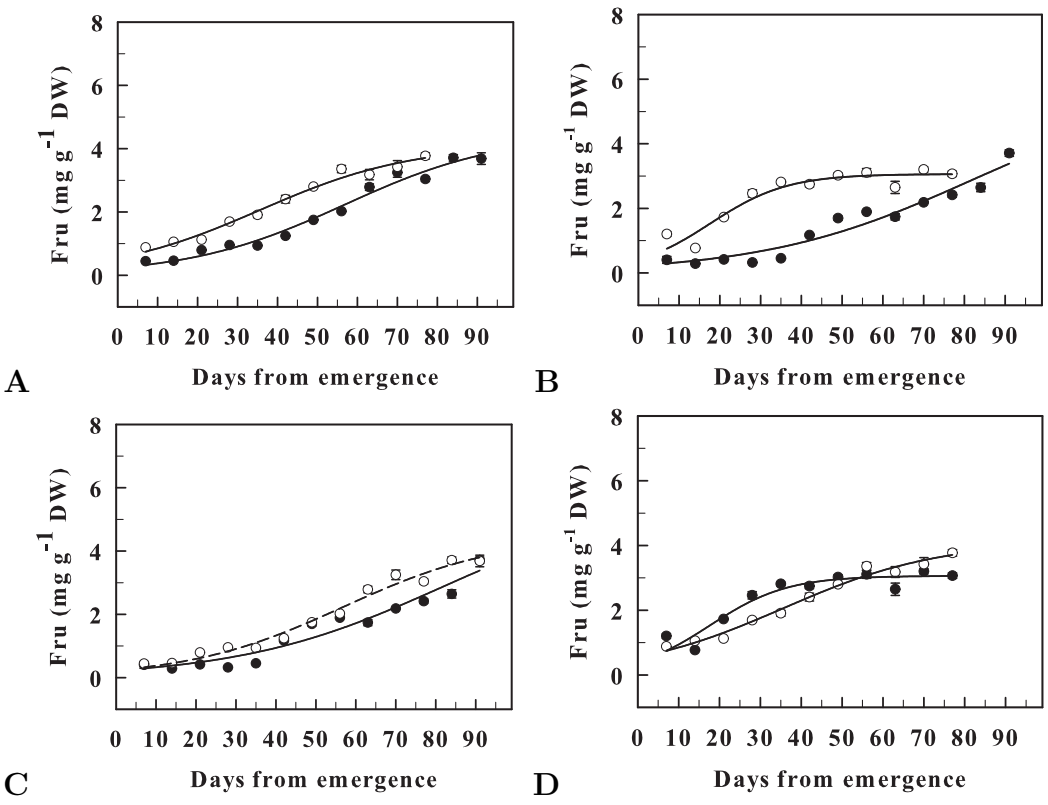

Figure A.15: **Changes in Fru Contents in *Antirrhinum* Leaves During Development in the Winter Experiment**

Non linear regression (–) of Fru contents in *Antirrhinum* leaves during the winter experiment. (A, B) Fru contents in SDs (A) and LDs (B) under unshaded (○) and shaded (●) conditions. (C, D) Fru contents in shaded (C) and unshaded (D) SD (○) and LD (●) conditions. SD shaded: r^2 0.97; SD unshaded: r^2 0.98; LD shaded: r^2 0.93; LD unshaded: r^2 0.89. Error bars indicate ± SEM.

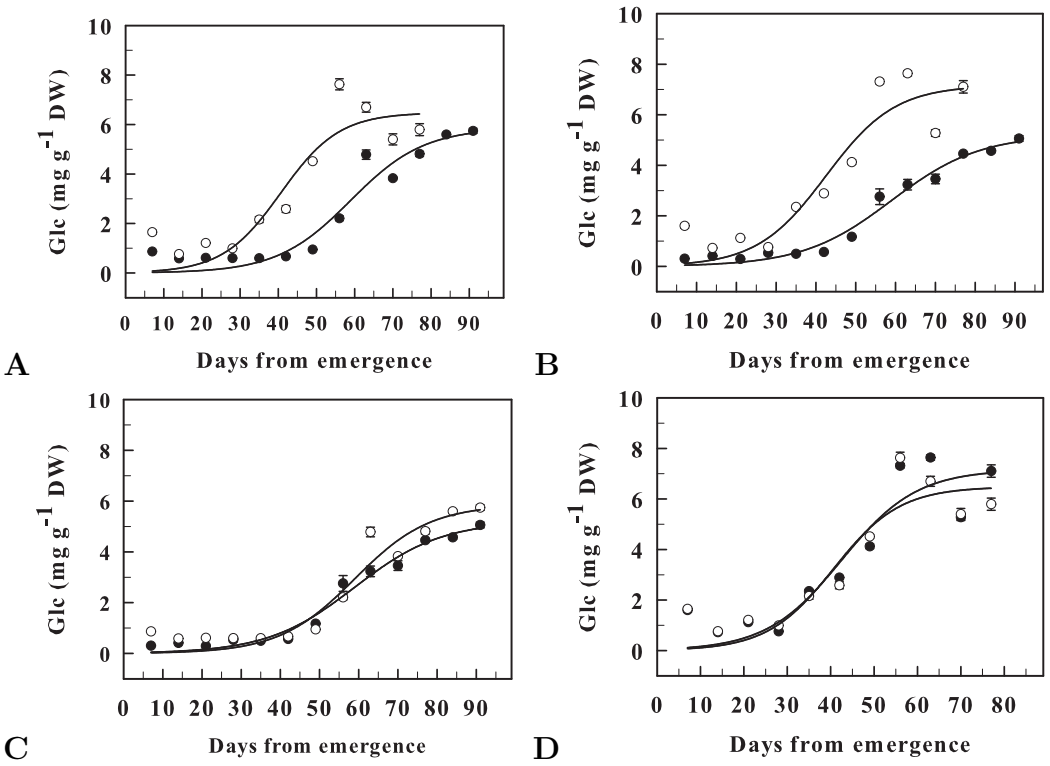

Figure A.16: **Changes in Glc Contents in *Antirrhinum* Leaves During Development in the Winter Experiment**

Non linear regression (–) of Glc contents in *Antirrhinum* leaves during the winter experiment. (A, B) Glc contents in SDs (A) and LDs (B) under unshaded (○) and shaded (●) conditions. (C, D) Glc contents in shaded (C) and unshaded (D) SD (○) and LD (●) conditions. SD shaded: r^2 0.93; SD unshaded: r^2 0.84; LD shaded: r^2 0.97; LD unshaded: r^2 0.87. Error bars indicate ± SEM.

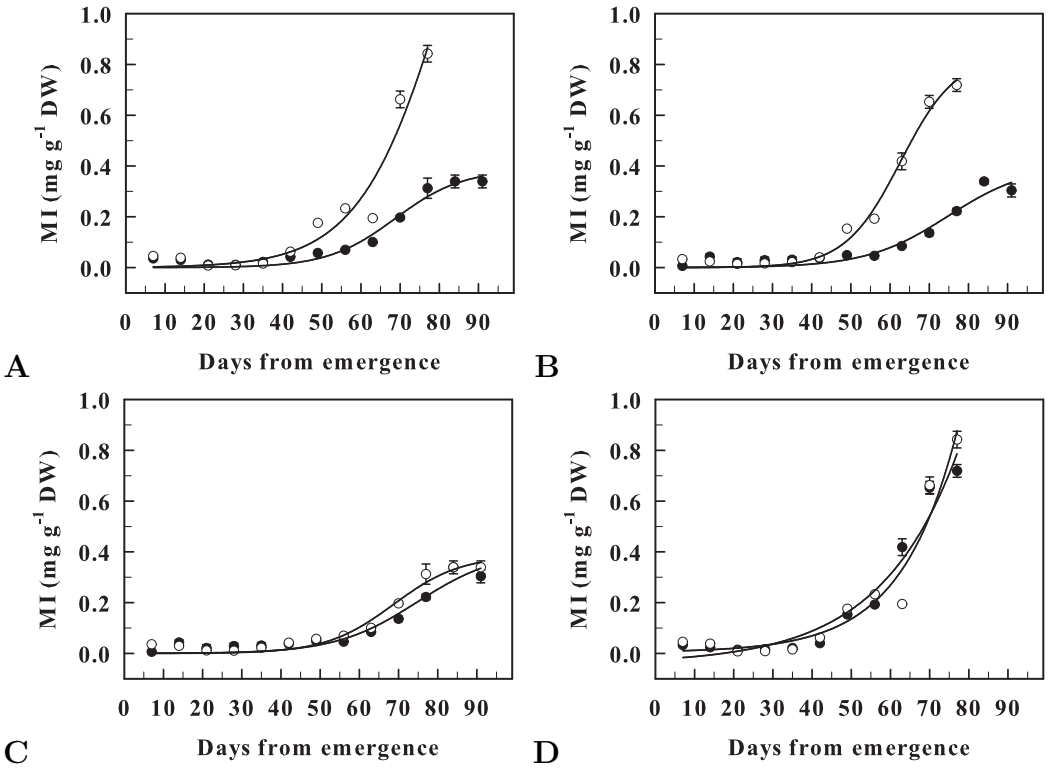

Figure A.17: **Changes in MI Contents in *Antirrhinum* Leaves During Development in the Winter Experiment**

Non linear regression (–) of MI contents in *Antirrhinum* leaves during the winter experiment. (A, B) MI contents in SDs (A) and LDs (B) under unshaded (○) and shaded (●) conditions. (C, D) MI contents in shaded (C) and unshaded (D) SD (○) and LD (●) conditions. SD shaded: r^2 0.94; SD unshaded: r^2 0.99; LD shaded: r^2 0.99; LD unshaded: r^2 0.97. Error bars indicate ± SEM.

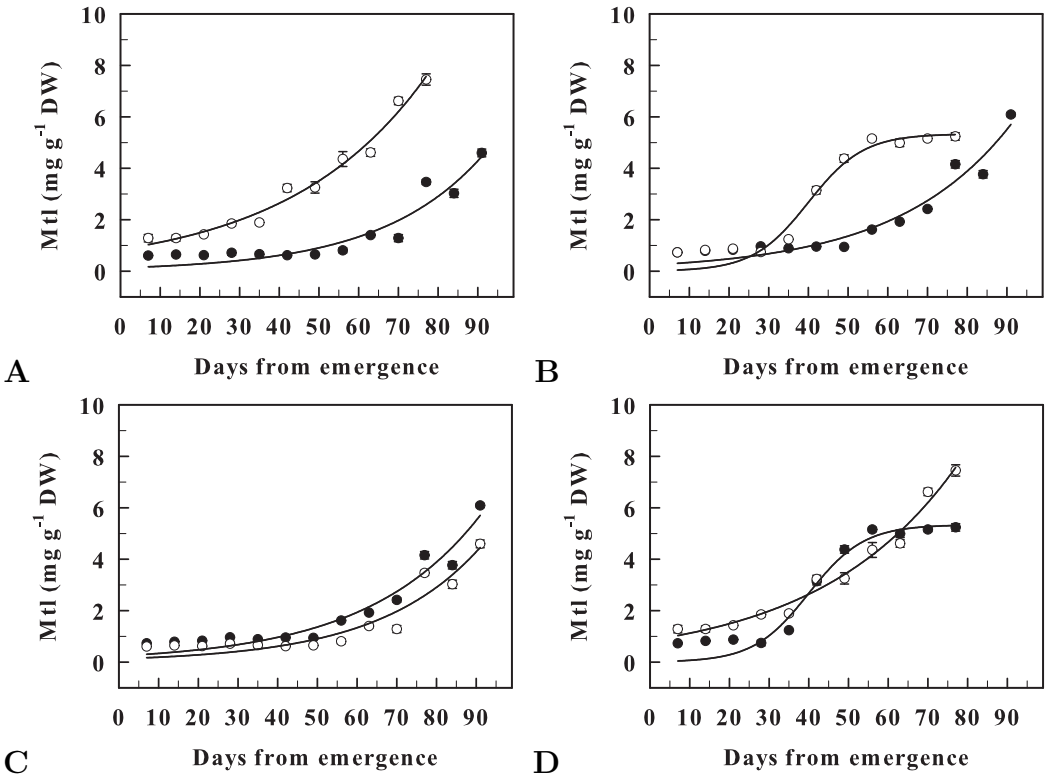

Figure A.18: **Changes in Mtl Contents in *Antirrhinum* Leaves During Development in the Winter Experiment**

Non linear regression (–) of Mtl contents in *Antirrhinum* leaves during the winter experiment. (A, B) Mtl contents in SDs (A) and LDs (B) under unshaded (○) and shaded (●) conditions. (C, D) Mtl contents in shaded (C) and unshaded (D) SD (○) and LD (●) conditions. SD shaded: r^2 0.89; SD unshaded: r^2 0.98; LD shaded: r^2 0.94; LD unshaded: r^2 0.96. Error bars indicate ± SEM.

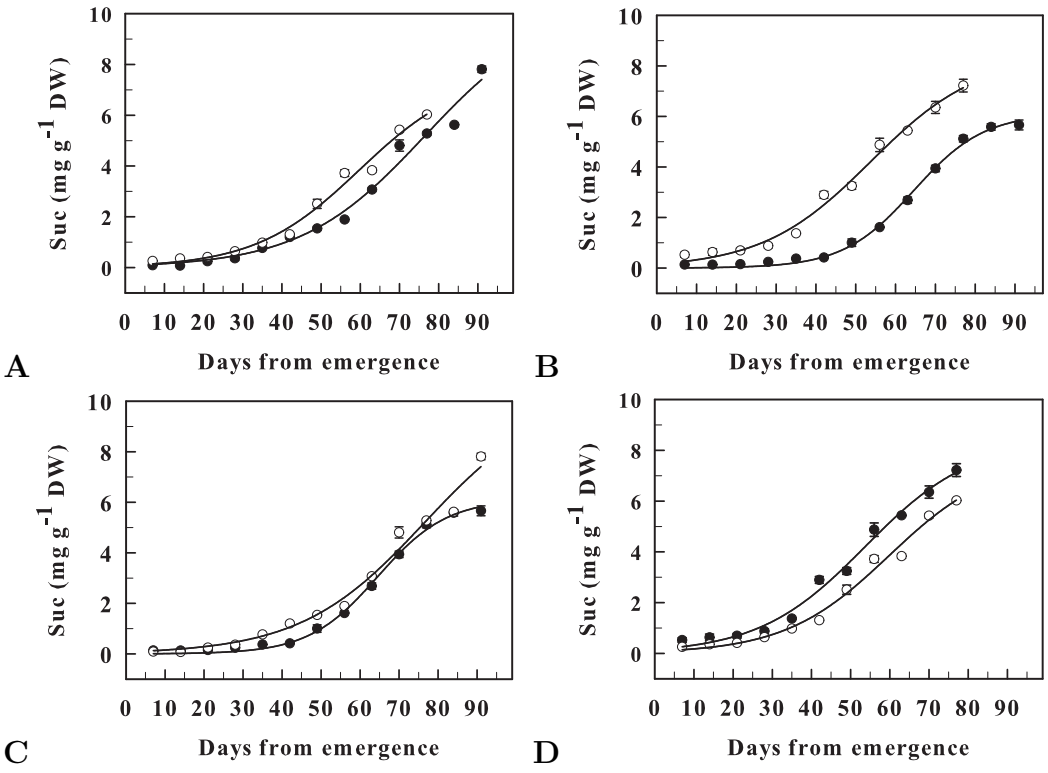

Figure A.19: **Changes in Suc Contents in *Antirrhinum* Leaves During Development in the Winter Experiment**

Non linear regression (–) of Suc contents in *Antirrhinum* leaves during the winter experiment. (A, B) Suc contents in SDs (A) and LDs (B) under unshaded (○) and shaded (●) conditions. (C, D) Suc contents in shaded (C) and unshaded (D) SD (○) and LD (●) conditions. SD shaded: r^2 0.99; SD unshaded: r^2 0.98; LD shaded: r^2 0.99; LD unshaded: r^2 0.99. Error bars indicate ± SEM.

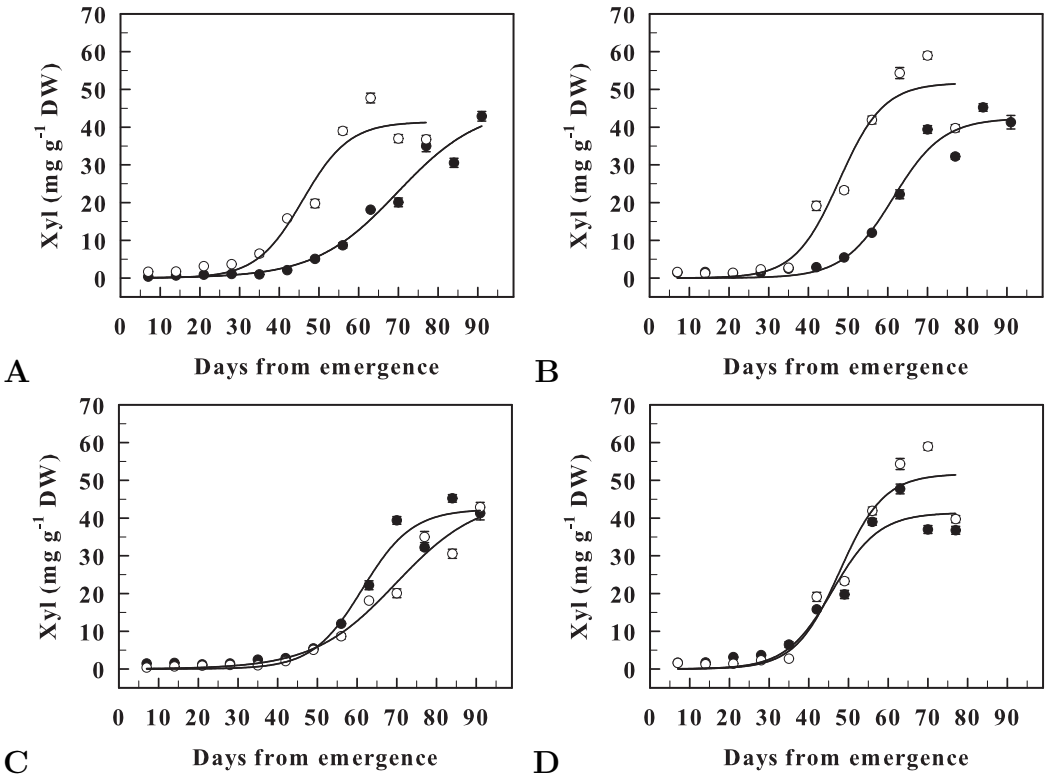

Figure A.20: **Changes in Xyl Contents in *Antirrhinum* Leaves During Development in the Winter Experiment**

Non linear regression (–) of Xyl contents in *Antirrhinum* leaves during the winter experiment. (A, B) Xyl contents in SDs (A) and LDs (B) under unshaded (○) and shaded (●) conditions. (C, D) Xyl contents in shaded (C) and unshaded (D) SD (○) and LD (●) conditions. SD shaded: r^2 0.97; SD unshaded: r^2 0.94; LD shaded: r^2 0.97; LD unshaded: r^2 0.94. Error bars indicate ± SEM.

A.3.1.2 Effect of [CO_2] on Soluble Sugar Accumulation

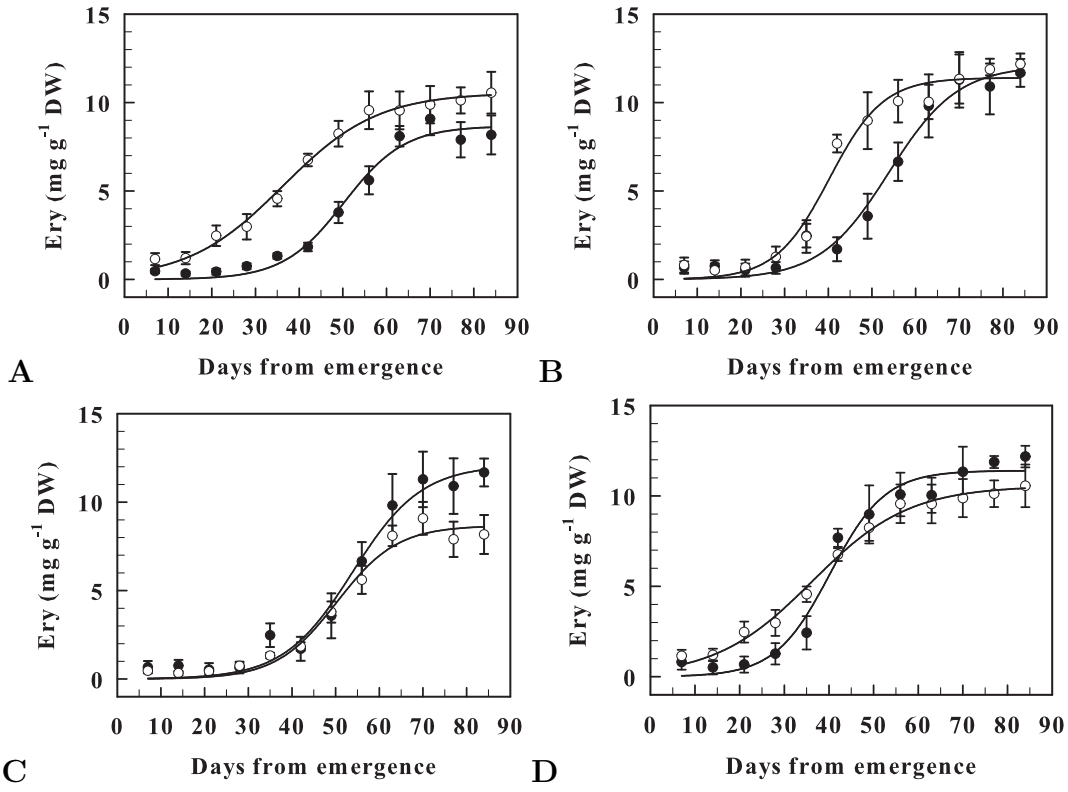

Figure A.21: **Changes in Ery Contents in *Antirrhinum* Leaves During the CO_2 Experiment**

Non linear regression (–) of Ery contents in *Antirrhinum* leaves during the CO_2 experiment. (A, B) Ery contents in SDs (A) and LDs (B) under elevated (○) and ambient (●) CO_2 conditions. (C, D) Ery contents in ambient (C) and elevated (D) SD (○) and LD (●) CO_2 conditions. SD ambient CO_2: r^2 0.98; SD elevated CO_2 : r^2 0.99; LD ambient CO_2: r^2 0.97; LD elevated CO_2: r^2 0.97. Error bars indicate ± SEM.

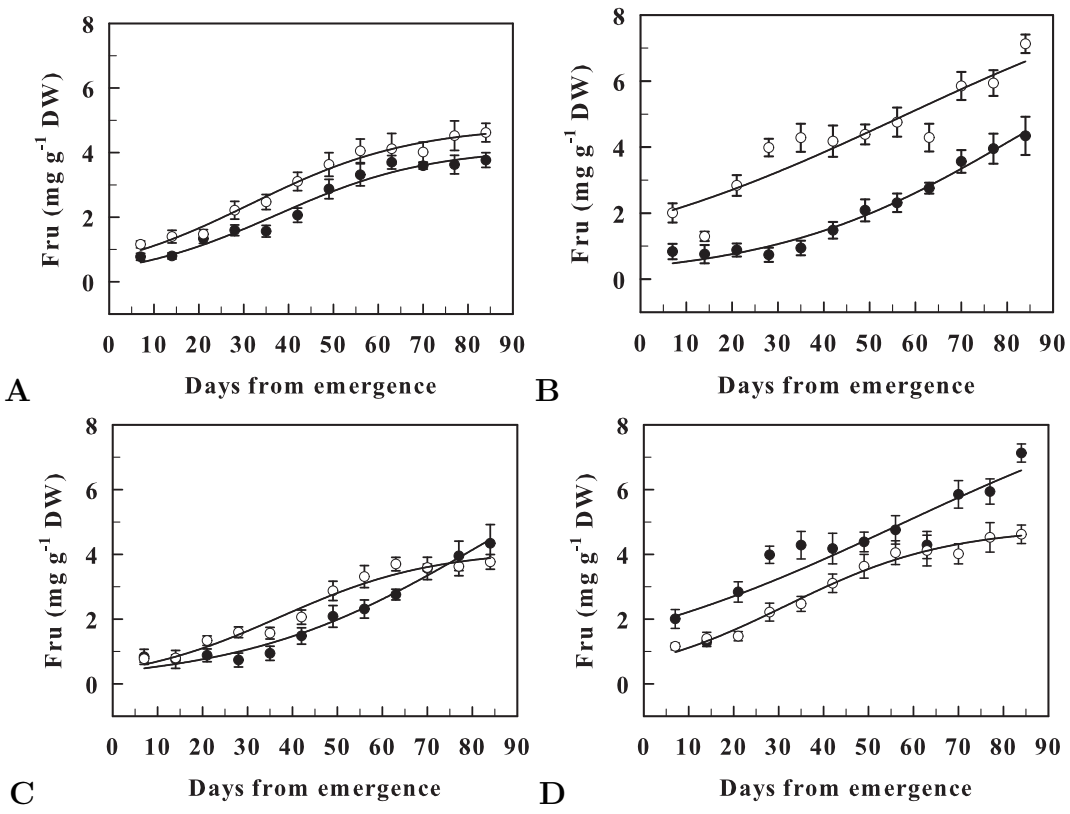

Figure A.22: **Changes in Fru Contents in *Antirrhinum* Leaves During the CO_2 Experiment**

Non linear regression (–) of Fru contents in *Antirrhinum* leaves during the CO_2 experiment. (A, B) Fru contents in SDs (A) and LDs (B) under elevated (○) and ambient (●) CO_2 conditions. (C, D) Fru contents in ambient (C) and elevated (D) SD (○) and LD (●) CO_2 conditions. SD ambient CO_2: r^2 0.96; SD elevated CO_2: r^2 0.98; LD ambient CO_2: r^2 0.97; LD elevated CO_2: r^2 0.86. Error bars indicate ± SEM.

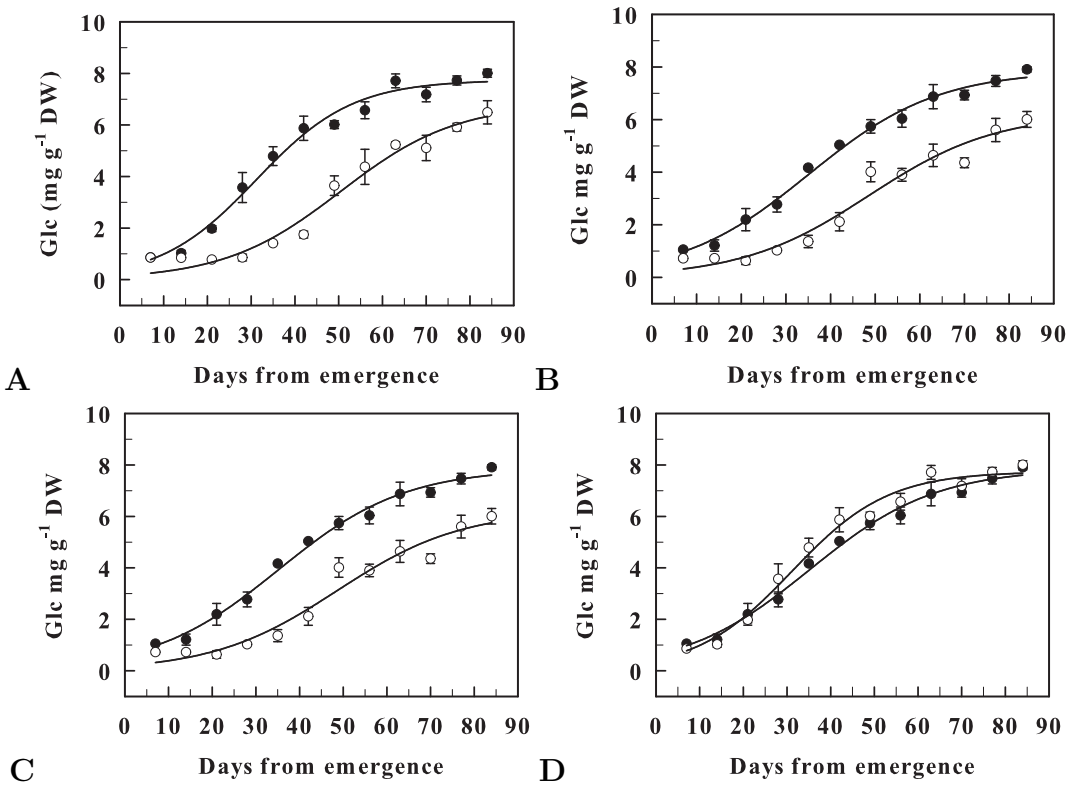

Figure A.23: **Changes in Glc Contents in *Antirrhinum* Leaves During the CO_2 Experiment**

Non linear regression (–) of Glc contents in *Antirrhinum* leaves during the CO_2 experiment. (A, B) Glc contents in SDs (A) and LDs (B) under elevated (○) and ambient (●) CO_2 conditions. (C, D) Glc contents in ambient (C) and elevated (D) SD (○) and LD (●) CO_2 conditions. SD ambient CO_2: r^2 0.97; SD elevated CO_2: r^2 0.98; LD ambient CO_2: r^2 0.95; LD elevated CO_2: r^2 0.99. Error bars indicate ± SEM.

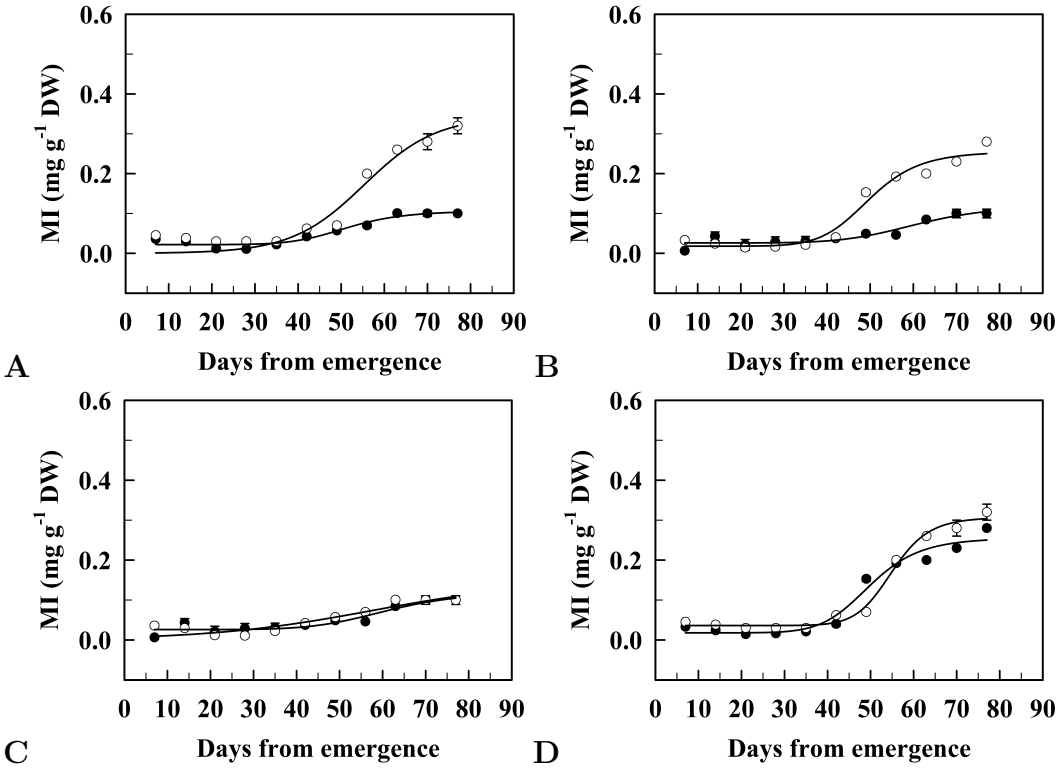

Figure A.24: **Changes in MI Contents in *Antirrhinum* Leaves During the CO_2 Experiment**

Non linear regression (–) of MI contents in *Antirrhinum* leaves during the CO_2 experiment. (A, B) MI contents in SDs (A) and LDs (B) under elevated (○) and ambient (●) CO_2 conditions. (C, D) MI contents in ambient (C) and elevated (D) SD (○) and LD (●) CO_2 conditions. SD ambient CO_2: r^2 0.94; SD elevated CO_2: r^2 0.95; LD ambient CO_2: r^2 0.89; LD elevated CO_2: r^2 0.96. Error bars indicate ± SEM.

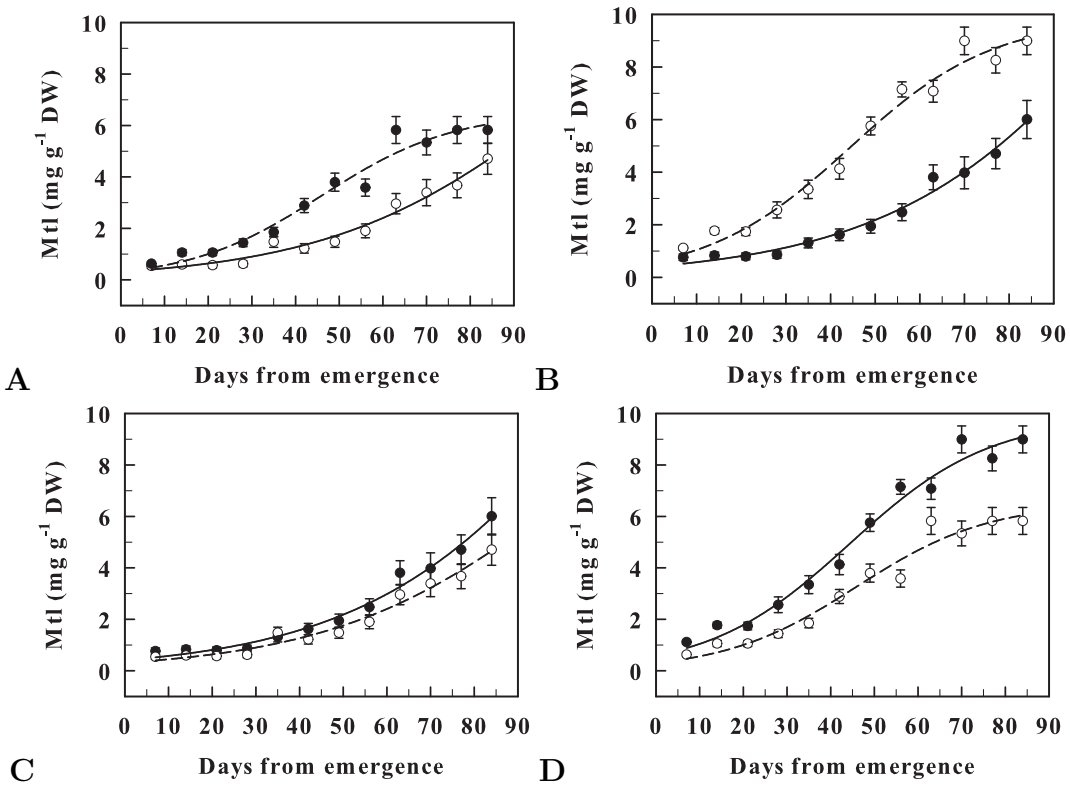

Figure A.25: **Changes in Mtl Contents in *Antirrhinum* Leaves During the CO_2 Experiment**

Non linear regression (–) of Mtl contents in *Antirrhinum* leaves during the CO_2 experiment. (A, B) Mtl contents in SDs (A) and LDs (B) under elevated (○) and ambient (●) CO_2 conditions. (C, D) Mtl contents in ambient (C) and elevated (D) SD (○) and LD (●) CO_2 conditions. SD ambient CO_2: r^2 0.97; SD elevated CO_2: r^2 0.98; LD ambient CO_2: r^2 0.95; LD elevated CO_2: r^2 0.99. Error bars indicate ± SEM.

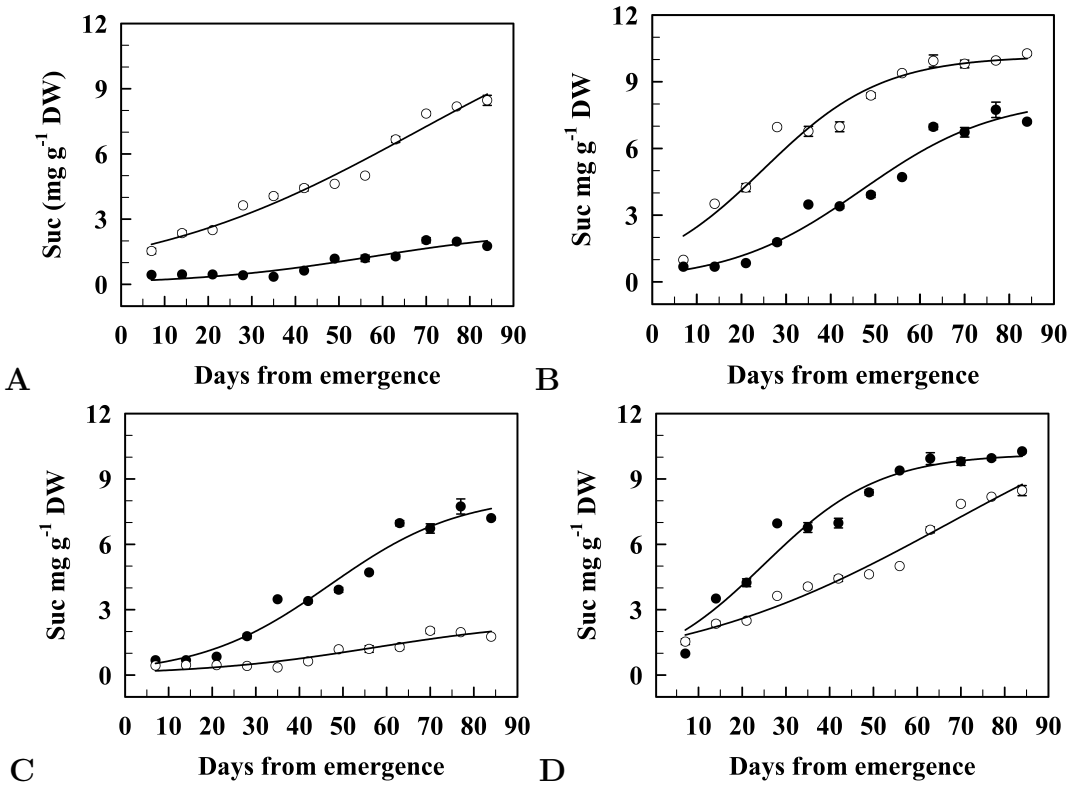

Figure A.26: **Changes in Suc Contents in *Antirrhinum* Leaves During the CO_2 Experiment**

Non linear regression (–) of Suc contents in *Antirrhinum* leaves during the CO_2 enrichment experiment. (A, B) Suc contents in SDs (A) and LDs (B) under elevated (○) and ambient (●) CO_2 conditions. (C, D) Suc contents in ambient (C) and elevated (D) SD (○) and LD (●) CO_2 conditions. SD ambient CO_2: r^2 0.89; SD elevated CO_2: r^2 0.96; LD ambient CO_2: r^2 0.89; LD elevated CO_2: r^2 0.95. Error bars indicate ± SEM.

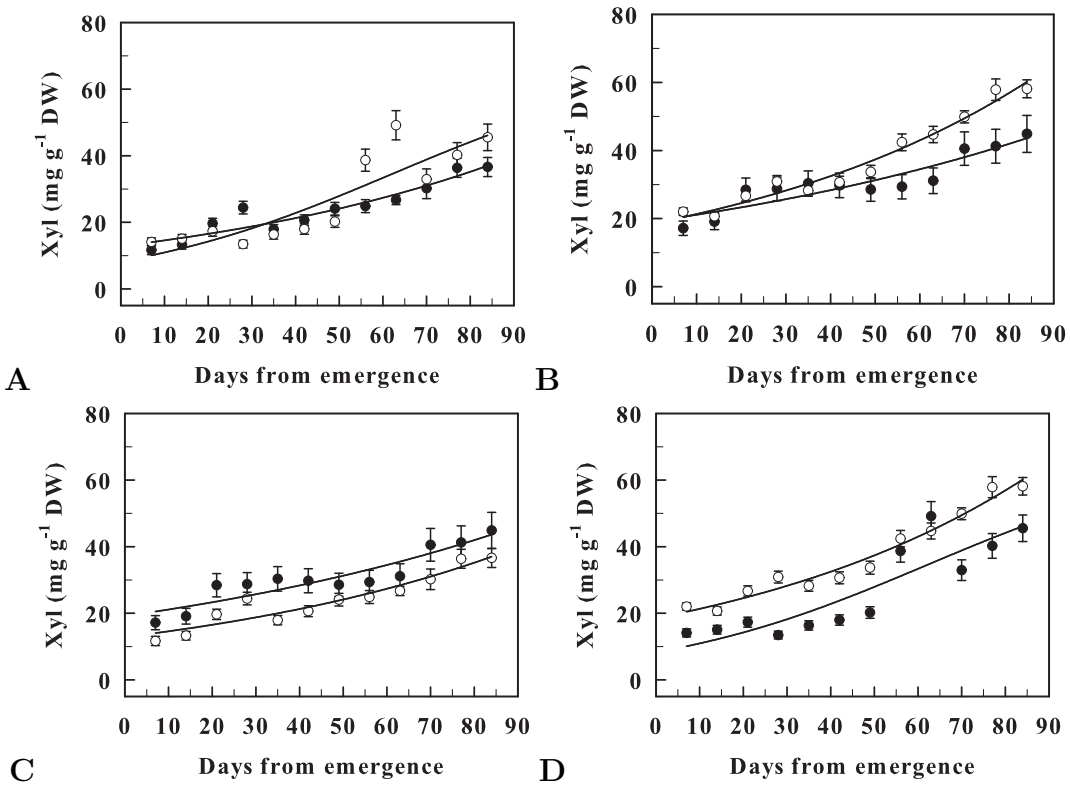

Figure A.27: **Changes in Xyl Contents in *Antirrhinum* Leaves During the CO_2 Experiment**

Non linear regression (–) of Xyl contents in *Antirrhinum* leaves during the CO_2 experiment. (A, B) Xyl contents in SDs (A) and LDs (B) under elevated (○) and ambient (●) CO_2 conditions. (C, D) Xyl contents in ambient (C) and elevated (D) SD (○) and LD (●) CO_2 conditions. SD ambient CO_2: r^2 0.89; SD elevated CO_2: r^2 0.77; LD ambient CO_2: r^2 0.84; LD elevated CO_2: r^2 0.89. Error bars indicate ± SEM.