Analysing the role of GIGANTEA in flowering-time regulation and light signalling of Arabidopsis

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Zusammenfassung

Die Induktion der Blütenbildung bei Pflanzen wird durch Umwelteinflüsse gesteuert. Ein sehr bedeutender Faktor für die Blühinduktion ist die Tageslänge, die sich in Abhängigkeit von der Jahreszeit ändert. Die Pflanze misst die Tageslänge durch einen internen Zeitgeber, besser bekannt unter Circadian Clock und bestimmt somit die optimale Jahreszeit zur Blütenbildung und Fortpflanzung. *Arabidopsis thaliana* ist die Modelpflanze für die Erforschung der molekularbiologischen Regulation der Blühinduktion. Da die Bildung der Blüte im Langtag gefördert wird, *Arabidopsis* aber auch im Kurztag blüht, spricht man von einer fakultativen Langtagpflanze. Pflanzen, die eine Mutation im Gen *GIGANTEA* aufweisen, haben einen Defekt in der Wahrnehmung der Tageslänge. Mutationen in *GI* verzögern die Blühinduktion im Langtag, wohingegen sie nur einen geringen Effekt unter Kurztag-Bedingungen haben. Dieser Phänotyp ist teilweise der Reduzierung der Expression des Blühzeitpunktgens *CONSTANS* (*CO*) zuzuschreiben. Zudem verursacht die Mutation im *GI* Gen einen verkürzten Rhythmus in der Expression von *CCA1* and *LHY*, zwei Genen, die in der Circadian Clock involviert sind. In *gi* Mutanten ist der Rotlichtsignalweg ebenfalls gestört, was zu verlängerten Hypokotylen führt. *GI* existiert nur einmal im Arabidopsis-Genom und codiert ein 1173 Aminosäuren langes Kernprotein. Die Sequenz des pflanzenspezifischen Proteins ist im Pflanzenreich stark konserviert. Die biochemische Funktion von GI ist bisher unbekannt. Die *GI*-Expression unterliegt zyklischen Schwankungen im circadianen Rhythmus mit einem Maximum etwa 8 bis 10 Stunden nach Sonnenaufgang. Dieser Rhythmus wird in Abhängigkeit von der Tageslänge reguliert.

Die Rolle von GI in der Regulation der Blühenindukion wurde durch drei experimentelle Ansätze untersucht. Bei Untersuchungen mit dem Zweihybrid-System der Hefe wurden 52 Proteine als mögliche Interaktionspartner von GI identifiziert. 15 Proteine wurden weiterhin auf Interaktion mit der für die Blühinduktion wichtigen C-terminalen Domäne von GI getestet. Das Protein ATA20 zeigte starke Interaktion mit GI, während 4 andere Proteine, unter anderem GIP14, schwächere Interaktion zeigten. Studien zeigten dass die Überexpression von GIP14 zu einer Verlängerung des Hypokotyls im Rotlicht führte.

Dieser Phänotyp ähnelt dem von *gi*-Pflanzen, was vermuten lässt dass GIP14 möglicherweise ein negative Regulator des GI Proteins ist.

Um das Expressionsmuster von GI zu analysieren wurde ein Fusionskonstrukt zwischen dem *GI*-Promotor und dem Reportergen *GUS* in Pflanzen transformiert. Die Expression von *GUS* wurde in jungen Blättern und den Leitgefäßen von Wurzeln, Hypokotyl, Kotyledonen und älteren Blättern nachgewiesen. Ebenfalls konnte *GUS* Expression in den Meristemen von Wurzel und Spross detektiert werden. Dies zeigt, dass *GI* in vielen Pflanzengeweben expremiert wird.

Da GI mehrere Funktionen hat, wurde durch Missexpression von *GI* untersucht, in welchen Geweben GI die Blühinduktion kontrolliert. Dies zeigte dass die Expression von *GI* in die Leitgefäße den spätblühenden Phänotyp von *gi*-Pflanzen aufheben kann.

Zusätzlich wurde ein genetischer Screen durchgeführt, um Gene zu identifizieren, deren Funktion *GI* ähnelt. Es wurde nach Mutanten gesucht, die wie *gi* den frühblühenden Phänotyp der *lhy-11cca1-1* Doppelmutante im Kurztag unterdrücken. Die *slc18* Mutation konnte mit Hilfe von 1700 F2-Hybriden auf eine Region von 126 kb auf dem unteren Arm von Chromosom 3 lokalisiert werden. Dieser Bereich enthält keine Gene, denen bisher einen Funktion im Blühinduktion zu geordnet werde kann. Bei SLC18 handelt es sich möglicherweise um einen neuen Blühzeitpunktregulator. Der Blühzeitpunkt von *slc18*-Pflanzen und die Expression der Gene *GI*, *CO* und *FT* wurden analisiert. Die Mutation soll durch Sequenzierung identifiziert werden.

Diese Studie beinhaltet eine detaillierte Expressionsanalyse von *GI*, identifiziert sowohl GI interagierende Proteine als auch Gene, deren Funktionen mit GI ähnlich sind. Dadurch werden neue Ansätze zum Verständnis der Funktion von GI gegeben.

Abstract

Many plants flower in response to environmental cues. Of particular significance in many plants species is initiation of flowering in response to seasonal changes in day length. An internal timing mechanism called the circadian clock enables measurement of daylength (a process called photoperiodism). As daylength changes throughout the year, photoperiodic control of flowering allows plants to develop flowers and reproduce in the appropriate season. *Arabidopsis thaliana* has become the species of choice in which to study the molecular-genetics of flowering-time control. This species is a facultative long-day plant that flowers much earlier under long days (LD, 16 hours light) than under short days (SD, 8 hours light); however the flowering time is intermediate if the period of light is between these extremes. A severe late flowering phenotype is caused by mutations in the *GIGANTEA* (*GI*) gene. This phenotype is at least in part caused by reducing the expression of the flowering-time gene *CONSTANS* (*CO*) and thereby delaying the time of flowering under long days. Apart from these effects, the loss-of-function *gi* mutant also shows shortened circadian rhythms in the expression of circadian controlled genes and lowers the expression of *CCA1* and *LHY*, two genes thought to be closely related to the circadian clock. Additionally, mutations in *GI* impair the transduction of the red light signal from the photoreceptor phytochrome B. *GI* is a single copy gene in *Arabidopsis* and encodes a nuclear protein of 1173 amino acids that is highly conserved in seed plants, but no homologous proteins have been found outside the plant kingdom. The biochemical function of GI is unknown, it is expressed widely throughout the plant and its transcription shows a circadian rhythm with a peak in mRNA abundance 8 till 10 hours after dawn. The timing and duration of this peak is influenced by daylength.

I addressed how GI regulates flowering time using three experimental approaches. By exploiting the yeast two hybrid system I screened for proteins interacting with GI from two libraries (total and apex from *Arabidopsis*). This identified 52 putative interacting proteins of which we selected 15 for further analysis. These 15 proteins were further tested for interaction with the C-terminal domain of GI, which is thought to be involved in flowering. One protein, ATA20, showed a strong interaction and 4 others (CSN6b, a

CHD protein-like, a member of the TCP-family and GIP14, a ZZ-finger domain family protein) weaker interactions. Recent results demonstrated that the overexpression of GIP14 caused an elongated hypocotyl phenotype under red-light, suggesting that red-light perception was impaired. This is a similar phenotype to *gi* mutant plants and suggests that GIP14 might act as a negative regulator of GI protein function. To test the detailed spatial pattern of *GI* expression, a fusion of the *GI* promoter to the GUS marker gene was constructed (*GI::GUS)* and introduced into plants. Staining of whole seedlings, stem and leaves detected *GI::GUS* expression in young leaves and in the vascular tissue of the root, hypocotyl, cotelydons and leaves. Expression was also detected in the meristem of the root and shoot. This result demonstrated that *GI* is expressed widely in plants.

To test in which tissues *GI* acts to regulate flowering, region specific promoters were used to misexpress *GI* in the gi-3 mutant. These experiments showed that expressing *GI* in the phloem companion cells rescues the late-flowering *gi-3* mutant.

Additionally, a genetic screen was performed to identify genes related to *GI* in function. *GI* increases the expression of *LHY* and *CCA1* and the proteins encoded by these genes repress *GI* expression. Mutations in *GI* also suppress the early flowering phenotype of *lhy-11 cca1-1* double mutants. An EMS mutagenesis was carried out with the *lhy11cca1-1* double mutant and several late-flowering individuals were found under SD. The late flowering *slc18* mutation was chosen for further study. Data on flowering-time and expression of *GI*, *CO* and *FT* show its significance in the flowering pathway. *slc18* was mapped by using 1700 late flowering F2 plants and located to an interval of 114 kb on the lower arm of chromosome 3. This region contains no genes with a known function in flowering-time control, suggesting that *SLC18* encodes a new floral regulator. The corresponding mutation will be finally identified by comparing the DNA sequence of genes in the region between the mutant and wild-type, and by complementation approaches.

This thesis provides new insight into GI function by determining the tissues in which *GI* acts to control flowering, proteins with which it interacts and by identifying mutations in genes with related functions to *GI*.

Abbreviations

AD:	activation domain
AGI:	Arabidopsis Genome Initiative
ABRC:	Arabidopsis Biological Resource Center
BD:	binding domain
BLAST:	Basic Local Alignment Search Tool
BSA:	bulked segregant analysis
bp:	base pair
CAPS:	Cleaved Amplified Polymorphic Sequences
CCA1:	CIRCADIAN CLOCK ASSOCIATED 1
CO:	CONSTANS
COL:	CO-like
DD:	continuous dark
DNA:	Desoxyribonucleic acid
FLC:	FLOWERING LOCUS C
FRI:	FRIGIDA
FT:	FLOWERING LOCUS T
GA:	gibberellic acid
GetCID:	Gene Transfer Clone Identification and Distribution Service
GI:	GIGANTEA
GFP:	green fluorescent protein
GUS:	β-glucuronidase
kDa:	kilo Dalton
LD:	long days
L*er*:	Landsberg *erecta*
LHY:	LATE ELONGATED HYPOCOTYL
LL:	continuous light
LUC:	luciferase
Mb:	million base pairs
NASC:	Nottingham Arabidopsis Stock Centre

PCR:	Polymerase Chain Reaction
PHY:	PHYTOCHROME
PSI-BLAST:	Position Specific Iterative BLAST
ORF:	open reading frame
QTL:	quantitative trait locus
RIL:	recombinant inbred lines
RL:	red light
RNA:	Ribonucleic acid
SAM:	shoot apical meristem
SD:	short days
SLC:	suppressor of lhy-11cca1-1
SSLP:	simple sequence length polymorphism
UTR:	untranslated region
TOC:	TIMING OF CAB
ZT:	zeitgeber time

1. Introduction

Plants are essential as the earth's primary producers. They harvest the energy of sunlight by converting light energy into chemical energy through the process known as photosynthesis. This energy is stored in carbohydrates whose breakdown produces the energy plants use to grow and reproduce. As plants are at the bottom of the food chain, this stored energy is the basis for all other life on earth. Reproduction is a central theme in the food chain. As plants reproduce, they ensure the survival and existence of the species and ensure the presence of essential energy for the organisms feeding on them and the subsequent food chain.

Flowers play an essential part in the reproduction of Angiosperms containing the organs in which meiosis, fertilization and development of the progeny occur. The various products of flowering, such as fruits and seeds are themselves an important food source, which are produced in agriculture for human consumption and consumed by many animals in nature. Flowers also produce pollen and nectar which various organisms feed upon; also resulting in the production of honey by bees. In addition flowers exist in numerous attractive variations, which are commercially grown and used as decoration. Thus flowering is an essential event in plant life, ensuring the existence of many species and providing food sources for the food chain. It contributes to important commercial fields such as the production of fruit, seeds and decorative flowers.

The timing of flowering is also important in all aspects of reproduction. At high latitudes the time that the plant flowers must be synchronised with the changing seasons so that there is sufficient time for fertilisation and seed development to occur before winter. Similarly at lower latitudes, flowering should occur when environmental conditions are most appropriate. Furthermore, to ensure the genetic diversity of many plant species it is important to have cross-pollination. Optimal cross-pollination occurs when other individuals of the same species flower at a similar time. This means that the flowering of an individual plant must be synchronised to that of the other individuals of the same species. Apart from this, plants that make use of other organisms such as insects to

disperse their pollen also need to synchronise flowering with the presence of these organisms. During evolution most species or varieties of the species have become adapted to their environment so they flower at a particular time of the year (see table 1), such that it should be possible to compose a flowering calendar for a particular location. Examples for plants grown in south-east England are shown in table 1.

Table 1. List of the average first dates of flowering of common plants and trees in south-east England (from Battey, 2000).

Hazel	January 9	Elder	May 10
Coltsfoot	January 31	Dog rose	May 23
Wood anemone	March 2	White ox-eye	May 25
Garlic hedge mustard	March 31	Greater bindweed	June 13
Horse chestnut	April 20	Harebell	June 21
Purple lilac	April 22	Madonna lily	June 21
Hawthorn	April 25	Autumn crocus	August 14
Laburnum	April 27	Ivy	September 1

In commercial applications of flowering the timing of flowering is also an important theme. Varieties of crops grown by farmers are carefully bred to optimise flowering time and yield for particular locations. Also crops are bred to widen their original geographic range ensuring optimal use of the environment such as amount of sun shine or moisture. In greenhouses the environmental signals are controlled to ensure optimal growing conditions. They use the artificial conditions to synchronise flowering of plants and to optimise production, ensure homogeneous size and ripeness of flowers and fruits.

Our research field focuses upon understanding the timing of flowering and particularly on the molecular level by using model plant species to describe the molecular mechanisms controlling flowering.

1.1. Studying flowering time

Studies on flowering time have been carried out in many plant species and systematic experimental analysis started from as early as the 1920s (Garner and Allard, 1920; Bünning *et al*., 1936). In many plants the transition from vegetative to reproductive development is controlled by environmental signals such as daylength or temperature. These signals induce floral development at the shoot apical meristem (SAM). However, these signals seem to be detected by different organs of the plant: daylength is detected in the leaves, whereas temperature is detected in the SAM (Michaels and Amasino, 2000).

The perception of daylength in the leaves was shown by grafting experiments in many species, including *Perilla crispa*. This plant is induced to flower by exposure to short days (SD), grafting a SD entrained leaf onto a long day (LD) grown plant was sufficient to trigger flowering (Zeevaart, 1985). This systemic signal must be synthesised in the leaf and then transmitted to the SAM where it induces floral development (Knott, 1934; Zeevaart, 1976). This signal, often called the floral stimulus or florigen, has proven elusive as attempts to purify it from phloem extracts failed (Corbesier *et al*., 1998) and it is believed to be a mixture of substances (Bernier *et al*, 1993).

Recently flowering-time research has focused on the annual plant *Arabidopsis thaliana* (mouse-ear cress; wall cress). *Arabidopsis thaliana* is a small dicotyledonous plant that is widely used as a model organism in plant biology. *Arabidopsis* is a member of the mustard (*Brassicaceae*) family, including cultivated species such as turnip, broccoli, radish and cabbage. Although *Arabidopsis* has no agronomic significance, it is widely used because it has many advantages for basic research in genetics and molecular studies in plants (Dean, 1993; Somerville and Koornneef, 2002).

Arabidopsis plants are small in size (around 20-30cms tall as an adult plant) and laboratory strains have a rapid 6-8 week life cycle with prolific seed production after self-fertilization. There is an extensive collection of natural accessions (>750) collected around the world that can be obtained from the two seed stock centres ABRC and NASC.

From these seed stock centres also thousands of mutants, in which the activity of almost every gene is impaired, are available to the scientific community.

The genome has a relatively simple structure of which extensive physical and genetic maps as well as the entire nucleotide sequence are available (Bevan *et al*., 1999). Whereas many crop plants have large genomes due to polyplodisation and accumulation of non-coding sequence; the relatively small *Arabidopsis* genome of 125 million base pairs (Mb) was the first plant genome to be sequenced (AGI, 2000). The complete sequence and the annotation of the predicted 25.500 genes are available from www.arabidopsis.org. Despite its small genome, comparisons with other species have shown that nearly all genes found there have a homologous gene in *Arabidopsis*. Also, the introduction of engineered DNA in *Arabidopsis* is easily obtained by infection with *Agrobacterium tumefaciens*, without the need for tissue culture procedures. All these factors make *Arabidopsis* an ideal model system for most plant processes.

1.2. Flowering of Arabidopsis

Because of these features, *Arabidopsis thaliana* has become the species of choice in which to study the molecular-genetics of flowering-time control. The various natural accessions that have been collected reflect the natural distribution (Redei, 1969). *Arabidopsis* is found over a large part of the world including various environments. The natural growth area of *Arabidopsis* covers a wide range of altitudes, latitudes, temperature and moisture. The nature of this habitat regulates the growth of the *Arabidopsis* accession; a general pattern in growth can however be seen.

In the laboratory the commonly used strains take a few days to germinate after with a vegatative growth stage follows in which a rossete of leaves is produced. After this followes the reproductive stage where a primary stem with cauline leaves and flowers is produced, followed by the production of secundary stems. Flowers self-fertilize and form

seed after which the plant dies. The lifespan of *Arabidopsis*, from germination to mature seed, takes about six weeks (Somerville and Koornneef, 2002).

The model species Arabidopsis is a facultative long-day plant, so that flowering occurs much earlier under long days of 16 hours light than under short days of 8 hours light. However, flowering time is intermediate if the period of light is between these two extremes (Reeves and Coupland, 2000). Various strains of Arabidopsis require a period of cold to induce flowering; this process is called vernalization. Without several weeks of cold (<4°C) plants stay in the vegetative stage and will only flower after a long time. Other features such as temperature and light quality also influence the time it takes to flower (Somerville and Koornneef, 2002). Flowering time is a measure for the time a plant takes to flower in a certain condition; typically this is visualised in the days to flower or in leaf number. Counting leaves is an easy way to establish flowering time as the number of (rosette) leaves is tightly linked to the time to flower (Koornneef *et al*., 1991). Many mutations that alter flowering time in Arabidopsis have been isolated and were placed in four flowering pathways that together interact in controlling flowering time (reviewed by Mouradov *et al*., 2002). These pathways are reviewed in the following sections.

1.3. Molecular-genetic analysis of flowering in Arabidopsis

As described above, flowering is controlled by environmental cues such as light and temperature, however also other cues such as nutrients and humidity influence this process. Four genetic pathways have been identified that interact to regulate flowering in response to these environmental cues. Together these pathways ensure the optimal timing of flowering; resulting in optimal production of seeds.

There are two environmental pathways, the photoperiodic and the vernalisation, that use the environmental signals daylength and temperature to promote flowering. The autonomous pathway appears to act independently of environmental signals. Finally a

hormonal pathway uses internal cues to promote flowering. An overview of the four pathways is given in the following sections.

1.3.1. The autonomous flowering pathway

The autonomous pathway controls flowering independently of environmental cues. Therefore, mutations in genes from this pathway cause later flowering under long and short days. However, these mutants are highly responsive to vermalisation and flower early if exposed to vernalization (Koornneef *et al*., 1991). Mutants in this pathway include *fca*, *fy*, *fve* and *ld*. The main function of the genes of this pathway seems to downregulate the expression of the mRNA of *FLC*, an important floral repressor gene (described below). FLC mRNA is present at much higher levels in autonomous pathway mutants than in wild-type plants. Additionally, introduction of loss-of-function alleles of *FLC* suppresses the late flowering phenotype of the autonomous pathway mutants indicating FLC activity is required for late flowering (Michaels and Amasino, 2001).

Several genes from this pathway have been isolated. *FCA*, *FLK*, *FPA* and *FY* seem to have a general role for post-transcriptional regulation, as they encode RNA processing factors. The predicted protein of FCA contains two RNP domains, believed to be RNA recognition motifs. This suggestion is supported by *in vivo* binding of RNA by FCA (Macknight *et al*., 1997). *FPA* also encodes a RNA binding protein containing RNP domains (Schomburg *et al*., 2001). *FLK*, encodes an RNA binding protein with K homology motifs (Lim *et al*., 2004) and *FY* encodes an RNA 3' end-processing factor that has been shown to interact with FCA (Simpson *et al*., 2003).

FLD and *FVE* are believed to encode factors involved in histone acetylation due to their homology to genes encoding components of the human histone deacetylase complex. These factors deacetylate the histones of *FLC*-chromatin, preventing *FLC* transcription and resulting in earlier flowering (Chou and Yang, 1998; Ausin *et al*., 2004; Kim *et al*.,

2004). *LD* encodes a transcription factor that contains a homeobox and putative nuclear localisation motifs (Lee *et al*., 1994).

Thus the central function of the autonomous pathway seems to be the downregulation of *FLC* expression by influencing mRNA stability and epigenetic control via chromatin. Indeed the autonomous pathway does not represent a single pathway but a series of protein complexes that suppress *FCA* expressionby different mechanisms.

1.3.2. The gibberellic acid pathway

The classical experiment showing that gibberellic acid (GA) promotes flowering of *Arabidopsis* was first performed in the fifties of the last century. Under short days, the exogenous application of the growth regulator GA led to a dramatic promotion of flowering (Langridge, 1957). More recently, the identification of mutants defective in GA biosynthesis or signalling confirmed that GA acts to promote flowering of Arabidopsis as well as promoting germination, shoot elongation and leaf growth (Wilson *et al*., 1992; Olszewski *et al*., 2002).

Several genes involved in GA biosynthesis have been identified and shown to influence flowering time. GA1 is a copalyl diphosphate synthase that catalyses the first step in GA biosynthesis (Sun and Kamiya, 1994). The *ga1-3* mutant has severely reduced levels of GA and flowers later than wild-type plants under LD and never flowers in SD (Wilson *et al*., 1992; Reeves and Coupland, 2001). GA20-oxidase is an enzyme in the GA-biosynthetic pathway and is regulated by environmental signals. The expression of this gene increases upon shift from SD to LD, which also induces flowering. Furthermore, overexpression of GA20-oxidase caused early-flowering in both SD and LD (Xu *et al*.,1997; Coles *et al*., 1999). This is in agreement with the effect of exogenous application of GA and suggests the GA levels are limiting on flowering time.

Mutations that affect GA signal transduction also affect flowering time. The *gibberellic acid insensitive* (*gai*) mutations, which reduces the effectiveness of GA signalling, delays flowering, particulary under SDs (Wilson *et al*., 1992; Putterill *et al*., 1995; Peng *et al*., 1997)

Promotion of flowering by the GA pathway seems to function through upregulation of *LFY* and *SOC1*, genes that play key roles in the integration of all flowering pathways. This suggestion is supported by the result that *LFY* and *SOC1* are upregulated upon overexpression and application of GA (Blazquez *et al*., 1998; Moon *et al*., 2003). Also *35S::LFY ga1-3* plants flower earlier than *ga1-3*, however there seems to be an additional role for GA as *35S::LFY* flowers earlier than the double mutant (Blazquez *et al*., 1998).

1.3.3. The vernalization pathway

Exposure of plants to low temperature for an extended period of time accelerates flowering in many plant species. This phenomenon is known as vernalization. Many naturally occurring Arabidopsis varieties flower very late without vernalization, however flower early when grown at low temperatures for several weeks (4-8 weeks) (Michaels and Amasino, 2000). Responsiveness to vernalization, however, varies between varieties of species. The requirement for vernalization is associated with varieties exhibiting the winter annual growth habit. After germination in summer they grow in the vegetative state throughout winter until the following spring when they flower. In contrast, summer annuals germinate, grow and flower in the same year; without requirement for a winter period.

Crossing summer and winter annual varieties resulted in the identification of two loci *FLC* and *FRI* required to confer the winter habit. The dominant alleles originate from the winter annuals and are required for the vernalization response (Lee and Amasino 1995; Clarke and Dean, 1994). FLC, a MADS box transcription factor functions as a repressor of flowering and is the central target for vernalization (Michaels and Amasino, 1999).

The expression of *FLC* mRNA is much higher in winter annuals that require vernalization than in summer annuals, additionally overexpression of *FLC* in early summer annuals dramatically delays flowering (Sheldon *et al*., 1999, 2000). Vernalization lowers the expression of *FLC* mRNA, and this correlates with flowering. The longer the time of cold exposure, the lower the *FLC* mRNA expression and the earlier flowering occurs (Sheldon *et al*., 2000).

The entire vernalization response however cannot simply be explained by FLC function as null alleles of *FLC* still show a response to vernalization (Michaels and Amasino, 2001). Closely related MADS box transcription factors might be redundant in function to FLC. At least one of these proteins causes late flowering when overexpressed and early flowering when inactivated. (Scortecci *et al*., 2001). However, none of the genes from this clade is as sensitive to vernalization as *FLC* is.

FRI also functions as a repressor of flowering. This gene seems to act through FLC as it increases the expression of *FLC*, whereas *flc* mutants suppress the effect of *FRI* on flowering (Michaels and Amasino, 1999). The predicted protein contains coiled-coil domains probably involved in protein-protein interaction; however the biochemical function of the protein is unknown (Johanson *et al*., 2000). Most early-flowering *Arabidopsis* varieties (such as commonly used laboratory accessions Landsberg *erecta* and Columbia) have a disrupted *FRI* gene, suggesting that these varieties originated from late-flowering varieties by inactivation of *FRI* (Johanson *et al*., 2000).

The effect of vernalization is stable throughout mitosis, meaning that *FLC* expression is also reduced in tissue formed after vernalization (Michaels and Amasino, 1999). This reduction is, however, reset upon meiosis enabling the vernalization process to occur in the offspring.

Using the vernalization requiring *fca* mutants, a new group of genes was identified that is required for vernalization. These *vernalization* (*vrn*) mutants seem to maintain downregulation of *FLC* when plants are returned to normal growth temperatures after cold treatment (Gendall *et al*., 2001). *VRN2* shows similarity to a Polycomb group

protein, which is likely to repress transcription by altering chromatin structures (Gendall *et al*., 2001). VRN1 is a putative B3-domain protein thought to interact in a non-sequence-specific manner with DNA (Levy *et al*., 2002). VRN1 is required for the methylation of lysine 9 of histone H3 (H3-K9) of *FLC* chromatin and VRN2 is thought to be involved in the H3-K27 (lysine27 of histone H3) methylation process (Bastow *et al*., 2004; Sung and Amasino, 2004).

Another vernalization insensitive mutant is *vin3*. This mutation prevents establishing of *FLC* repression in response to cold temperatures (Sung and Amasino, 2004). Upon vernalization the histone tails of *FLC* chromatin are deacetylated followed by an increase in H3-K27 and H3-K9 methylation (Bastow *et al*., 2004; Sung and Amasino, 2004). *vin3* mutants do not show any of these epigenetic marks in the *FLC* chromatin (Sung and Amasino, 2004). In addition *VIN3* mRNA levels rise during vernalization suggesting that it represents an early step in the vernalization process.

As described above, *FLC* plays an important role in the vernalization pathway in which an interplay of factors convey mitotic memory of the cold period. This mitotic memory probably occurs at the molecular level through histone modification that ensure the reduction of *FLC* expression is maintained when winterannual plants are exposed to inductive conditions in spring.

1.3.4. The photoperiodic pathway

Seasonal changes provide important signals that promote or repress flowering of many plants. The tilted position of the earth towards the sun, causes seasonal changes in daylength that are more severe at locations further from the equator. Therefore, in winter the daylength is shorter than in summer. In nature plants react to daylength in different ways. Short-day plants flower when day length falls below a critical daylength, whereas long-day plants flower when daylength exceeds a critical daylength. As described above,

Arabidopsis is a facultative longday species, which flowers earlier in long than short-days.

Mutations that affect photoperiodism can be divided in to two classes of daylength-insensitive mutants: early-flowering mutants that flower earlier than wild-type plants in SD but are unaffected under LD, and late-flowering mutants that flower later in LD and are unaffected in SD (Reeves and Coupland, 2000). Mutations in *GIGANTEA* (*GI*), *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) cause late flowering under LDs, and these genes were placed the photoperiodic pathway based on phenotype and genetic interactions of these mutants (Koornneef *et al*., 1991). Critically, double mutants flowered no later than single mutants, demonstrating the mutations affected the same pathway.

Mutations in the *GI* gene cause a severe late flowering phenotype (Redei, 1962). This phenotype is at least in part caused by reducing the expression of the mRNA of the flowering-time gene *CO* and thereby delaying the time of flowering under long days (Suárez-López *et al*., 2001). As GI has a central role in this thesis, it will be discussed extensively below in section 1.5.

The predicted protein of CO contains two conserved regions (Putterill *et al*., 1995). The first region is located toward the amino terminus of the protein and contains two adjacent zinc finger motifs of the B-box class, similar to those found in animal B-box proteins (Putterill *et al*., 1995). The other region contains a CCT domain (Strayer *et al*., 2000; Makino *et al*., 2002; Kurup *et al*., 2000, Robson *et al*., 2001), a conserved motif in CO, CO-like (COL) and TIMING OF CAB EXPRESSION 1 (TOC1), which is located towards the carboxy terminal end of the protein.

This region similarity to the DNA binding domain of the yeast protein HAP2 (Wenkel *et al*., 2006) Fusion of CO to GFP showed that CO is a nuclear localized protein; using GFP:CO and GFP:CCT fusion proteins showed that the CCT domain is sufficient and necessary for nuclear localization (Robson *et al*., 2001). The CCT domain might have

additional roles in protein-protein interactions and DNA binding (Kurup *et al*., 2000; Wenkel *et al*.,2006).

The expression of *CO* mRNA follows a diurnal rhythm in LD conditions peaking between 16 and 24 hrs after dawn; in short days this peak is narrower and 4 hrs earlier. This rhythm is maintained when LD grown plants are transferred to LL, suggesting that the rhythmic expression of *CO* is controlled by the circadian clock (Suarez-Lopez *et al*., 2001).CO protein cannot be detected in wild-type plants due to low level expression, but can be detected in 35D::CO plants. Exposed to blue or far-red light CO protein accumulates in the nucleus of these plants, whereas in plants grown in red light or darkness the protein is absent (Valverde *et al*., 2004). The accumulation of CO protein correlates with the effects of light on flowering time, as blue or far-red light are most effective in promoting flowering. This supports the idea that flowering is promoted because *CO* mRNA coincidences with the presence of light in long-days, allowing accumulation of CO protein (Searle and Coupland, 2004).

Overexpression of the *CO* gene results in early flowering in both LD and SD conditions and also overcomes the late-flowering phenotype of mutation in *GI*. Mutations in *FT* and *SUPPRESOR OF OVEREXPRESSION OF CO 1* (*SOC1*) however suppress this early flowering of the *CO* overexpressor. As *CO* regulates the expression of *FT* and *SOC1* (Lee *et al*., 2000; Onouchi *et* al., 2000; Samach *et al*., 2000) this supports the model that CO function is positioned between GI and FT and SOC1.

FT is an early target of *CO* (Samach *et al*., 2000), and its transcript level follows the expression of CO in a circadian rhythm peaking at 20 hrs after dawn (Suarez-Lopez *et al*., 2001). *FT* expression is however absent in the *co* mutant, therefore FT is downstream of CO in the photoperiodic pathway. *FT*, which encodes a RAF-kinase inhibitor-like protein, and SOC1, which encodes a MADS-box transcription factor, are among the most potent activators of flowering as their overexpression causes extreme early flowering (Lee *et al*., 2000; Onouchi *et* al., 2000; Samach *et al*., 2000). Both are genes called floral

integrators because they are believed to integrate the various pathways that promote flowering.

1.4. The photoperiodic photoreceptors

Light plays an important role in photoperiodic flowering as it stabilises CO protein and functions as an important input signal to the circadian clock (discussed below). Therefore it is important to understand how plants sense the presence of light during the photoperiodic response.

Plants detect light through at least three families of photoreceptors. The red-light and far-red-light region of the spectrum is perceived by phytochromes. Arabidopsis plants contain 5 PHYTOCHROMES (PHY A, B, C, D and E). Analysis of the PHY mutants indicates that the light stable phyB, phyD and phyE forms mediate responses to red light and delay flowering under non-inductive conditions. The light-labile PhyA is the main photoreceptor discriminating between far-red and darkness (Quail, 2002). When grown in short days with incandescent light (rich in far-red) *phyA* mutants flower later (Johnson *et al*., 1994).

Blue light is sensed by the cryptochromes and phototropins. Phototropins 1 and 2 are important in blue-light-dependant phototropic responses, chloroplast movement and stomatal opening (Briggs and Christie, 2002). Although phototropins have a PAS domain, but no effect on photoperiodic flowering. The PAS signal-sensor domain is also present in ZTL, FKF1 and LKP2 a novel group of proteins involved in photoperiodic flowering and circadian rhythmicity (Imaizumi *et al*., 2005, Somers *et al*., 2000; Nelson *et al*., 2000; Schultz *et al*., 2001). These proteins act as ubiquitin ligases and FKF1 has been shown to mediate the degradation of cycling DOF1 (CDF1), a negative regulator of *CO* transcription (Imaizumi *et al*., 2005)

CRYPTOCHROMES (CRY) 1 and 2 show strong similarity to bacterial DNA photolyases (Cashmore *et al*., 1999) and were identified for their role in blue-light-photomorphogenesis (Ahmad and Cashmore, 1993; Lin *et al*, 1998). Mutations in *cry2* cause delayed flowering under LD (Guo *et al*., 1998).

CRY2 and PHYA seem to be the main photoreceptors promoting photoperiodic flowering in *Arabidopsis* and together they cover a large part of the light spectrum ensuring correct discrimination between day and night.

1.5. Circadian clock

Organisms experience regular changes from dark to light and light ot dark during every 24h cycle. This ongoing diurnal cycle plays an important role in the life of each organism, regulating various behavioural and physiological processes. Previously, this regulation was believed to be a direct effect of the presence or absence of light. However artificially induced changes in daily rhythms do not directly lead to a reaction of the organism; rather the rhythms seem to continue as if the organism was kept in the original environment. This suggests the existence of an internal rhythmic mechanism.

This internal rhythm, with a period of ~24 hr, is called the circadian clock, from the Latin words circa (around) and dies (day), meaning literally 'about a day' (Dunlap *et al*., 2004). It is now understood that organisms have this internal clock in order to control daily processes and anticipate changes in their environment, such as daily changes in light and temperature that occur at dawn and dusk. Anticipation to changes in the environment is important, enabling plants for instance to have their photosynthetic machinery prepared before the first sun light appears, ensuring optimal light harvest. It is also postulated that phasing light-sensitive processes, such as DNA replication, to the night would be of advantage to the organism (Pittendrigh, 1993). This anticipation can be shown by removing the external cues and keeping the organism in constant light or dark; the so-called free-running conditions. In these conditions, the rhythm continues. However in

free-running conditions, the natural rhythm is not exactly 24 hours and also dampens over time. In nature the circadian rhythm is reset every day by the environmental cues called Zeitgebers (german for 'time givers') (Zerr *et al*., 1990) that typically occur at dawn and dusk.

Circadian clocks are usually considered to comprise three parts can be discriminated: input, clock and output (McClung, 2006). The environmental cues are the inputs, which are mainly perceived by photoreceptors and by unknown temperature receptors. These receptors enable the clock to be entrained to the natural cycle of the environment. The central part is the actual clock mechanism, which coordinates downstream processes known as the output of the clock. The circadian rhythms are thus endogenously generated and self-sustaining because they continue in free-running conditions.

The current understanding of the molecular concept of the clock originates from data obtained from *Drosophila*. The protein PER was shown to play a central role in the circadian clock of *Drosophila*. The mRNA of *per* shows a circadian oscillation which is followed by the oscillation of the PER protein. The PER protein interacts with TIMELESS (TIM) to inhibit the transcriptional activation of the *per* and *tim* promotors by a heterodimer of the transcription factors CYCLE (CYC) and dCLOCK (dCLK). *clk* transcription is regulated positively by *Pdp1* and negatively by *vrille*, their transcription is also activated by the heterodimer CYC/dCLK. This combination of activation and inhibition builds the central part of the *Drosophila* circadian clock (Hardin, 2004).

The *Drosophila* circadian clock demonstrates the importance of negative feedback loops at the centre of the clock mechanism (Dunlap *et al*., 2004). Positive components promote the transcription of negative components. These negative components have two functions: blocking their own expression and promoting the expression of the positive components. These steps ensure a robust and sustainable oscillation. The principle of interlocked feedback loops on the transcriptional and translational level has been found to be the basis of all circadian clock studied. Although this principle is shared across

taxonomic groups, the proteins used to form the components of the clock are not conserved between the various model organisms (Harmer *et al*., 2001).

In plants the circadian clock seems to be formed by three interlocked feedback loops. Two Myb domain proteins CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) have a central role in this. CCA1 was originally isolated by interaction with the *LHCB1**3 promoter. Loss of CCA1 function causes a shortening of ~3 hrs of the circadian period (Green and Tobin, 1999). Overexpression of CCA1 results in arrhythmicity suggesting its role as a core component in the circadian clock (Wang and Tobin, 1998). The paralog of CCA1 is LHY which was isolated through a screen for late-flowering mutants. It turned out that this allel causes overexpression of *LHY* due to insertion of a transposon and also causes arrhythmicity (Wang and Tobin, 1998; Schaffer *et al*., 1998). Further study revealed that CCA1 and LHY cause arrhythmic late flowering plants when overexpressed and the loss of function mutants are early flowering plants with shortened rhythms. The early flowering double mutant however shows severly dampened rhythms (Mizoguchi *et al*., 2002), indicating that LHY and CCA1 are redundant factors in the centre of the circadian clock.

CCA1 and LHY form the first loop together with TIMING OF CAB EXPRESSION 1 (TOC1). *toc1-1* was the first clock mutant isolated using the firefly luciferase (LUC) reporter system. This system is widely used among the circadian research community. LUC protein catalyzes the ATP-dependant oxidative decarboxylation of luciferin. Therefore *LUC* expression can be followed as this chemical reaction releases a photon of 560nm which can be detected using luminometers or CCD-cameras (Welsh *et al*., 2005). Cloning a short fragment (*LHCB1**3) of the promoter of the rhythmic *CHLOROPHYLL A/B BINDING PROTEIN* gene (*CAB*, also called *LHCB*) upstream of *LUC* produced rhythmic light emission from single seedlings (Millar *et al*., 1992). Using this reporter abnormalities in the timing of *CAB*, resulting in the cloning of *TOC1*. TOC1 belongs to the family of pseudo response regulators and contains a CCT domain. Overexpression of *TOC1* results in arrhythmicity (Makino *et al*., 2002), suggesting its role in the circadian clock.

The exact functioning of these three genes in the clock is not fully understood. LHY and CCA1 bind to the promoter of TOC1 and overexpression of either gene inhibits *TOC1* expression; suggesting their role as negative regulators of *TOC1*. TOC1 could be the positive element in the feedback loop of the circadian clock, as LHY and CCA1 expression is greatly reduced in the *toc1-2* mutant (Alabadí *et al*., 2001, 2002; Mizoguchi *et al*., 2002). Overexpression of *TOC1* does however not lead to dramatically elevated expression of *LHY* and *CCA1* (Makino *et al*., 2002). Several other genes are required for correct *LHY* and *CCA1* expression, including *GIGANTEA* (*GI*) and *EARLY FLOWERING 4* (*ELF4*) (Mizoguchi *et al*., 2002; Doyle *et al*., 2002)

In parallel with findings in other organisms the existence of multiple interlocked feedback loops may excist in *Arabidopsis* (Locke *et al*., 2005). One of these could include the other members of the family of pseudo response regulators: PRR5, PRR7 and PRR9, as they are negative regulators of *LHY* and *CCA1* (Farré *et al*., 2005). Probably due to partial redundancy, *PRR5*, *PRR7* and *PRR9* alone have a small effect on the periodicity of the clock; the significance of these genes in the clock is however clear as the triple *prr5 prr7 prr9* mutant is arrhythmic under all conditions tested (Nakamichi *et al*., 2005). As LHY and CCA1 are positive regulators of *PRR5*, *PRR7* and *PRR9* (Farré *et al*., 2005; Mizuno and Nakamichi, 2005) this loop is closed, thereby suggesting the importance in forming an additional feedback loop.

A further loop includes the small putative Myb transcription factor LUX ARRHYTHMO (LUX) (Hazen *et al*., 2005). Overexpression and loss of function of this gene causes arrhythmicity. In the *lux* mutants expression of *LHY* and *CCA1* is repressed whereas LUX seems to be negatively regulated by LHY and CCA1 (Hazen *et al*., 2005), suggesting these proteins contribute to a third feedback loop of the *Arabidopsis* circadian clock.

Several proteins have also been proposed to function in or around the clock as they are needed for correct circadian rhythms. These proteins belong to a novel family of proteins with PAS/LOV domains, Kelch-repeats and F-boxes: ZEITLUPE (ZTL), LOV KELCH PROTEIN 2 (LKP2) and FLAVIN binding KELCH REPEAT F-BOX (FKF) (Nelson *et*

al., 2000; Somers *et al*., 2000; Schultz *et al*.,2001). These proteins seem to have a function in protein degradation in the circadian clock. Mainly ZTL is an important determinant of circadian period as it is a component of an SCF complex that recruits TOC1 for proteosomal degradation; thereby controlling the correct level of TOC1 activity.

The ability to maintain a robust ~24hr rhythm over a range of physiological temperatures is an important characteristic of the circadian clock. This is called temperature compensation, a special property of the clock that compensates for the speed of chemical reactions that varies with temperature. Recently GI was proposed to play a critical role in temperature compensation in Arabidopsis (Gould *et al*., 2006). GI seems to be essential in extending the temperature range over which robust and accurate rhythmicity can be maintained. The dynamic balance between LHY and GI seems to be needed for temperature compensation in high temperatures; whereas CCA1 seems to take over the function of LHY in lower temperatures (Gould *et al*., 2006).

1.6. Function of GIGANTEA in plants

The *gigantea* mutant was identified as a severe late flowering mutant under long day conditions. The mutant was called *gigantea* because of the larger size the plant reaches while staying in the vegetative stage for a longer time. Several *gi* mutants have been described so far; the classically obtained mutants *gi-1* to *gi-6* (Koornneef *et al.,* 1991) and the T-DNA mutants *gi-11* (Richardson *et al.,* 1998) and *gi-12* (originating from R. Amasino).

1.6.1. Flowering time of *gi* mutants

All *gi* mutations, classical alleles and T-DNA mutants, cause a similar delay in flowering. This delay is most clear under LD conditions where the corresponding wild-type flowers early. Interestingly, *gi-4* and *gi-5* show a similar delay in flowering compared to the other mutants, although no decrease was found in the transcript level of *GI* in these mutants. These mutations cause changes close to the end of the protein, indicating that the C-terminus of the GI protein is functional important for flowering (Fowler *et al*., 1999).

This observation is supported by the identification of a QTL that is involved in the temperature compensation of the circadian clock from a screen of Cvi x L*er* recombinant inbred lines (RIL). The QTL maps near *GI* and the *GI* locus show several polymorphisms between Cvi and L*er*, and two of these result in amino acid substitutions in the N-terminus of GI. This QTL does not alter flowering time behaviour, however is affected in the temperature compensation of the clock (Gould *et al*., 2006).

Additionally, the C-terminal domain of GI from *Bauhinia purpurea* (BpGI) was sufficient to rescue the late flowering phenotype of the *gi-1* mutant when expressed from the CaMV35S promoter (Chin-Fun Chen and Yang, 2003). This observation suggests that the C-terminal portion of GI is sufficient to carry out the biochemical function required for the promotion of flowering.

In SD conditions the altered flowering-time phenotype of *gi* mutants is less severe than in LD, plants flower just slightly later than the corresponding wild-type. Exceptions are the *gi-2* and *gi-12* mutants, which show also a delay in SD conditions (Fowler *et al*., 1999). Because the *gi* mutants show a severe delay in LD and not under SD the difference between LD and SD phenotype is smaller; indicating a greatly reduced daylength sensitivity compared to wild-type.

1.6.2. Isolation of the *GI* gene

Based on the robust flowering time phenotype, genetic mapping of the *gi* mutation was undertaken, showing that *GI* mapped to a region located on chromosome 1 (Koornneef *et al.,* 1991). Also the T-DNA mutant *gi-11* was shown to have a single T-DNA insertion, which was located to this region. This insertion resulted in a deletion of the 5' half of *GI* and upstream sequence. Final confirmation was obtained by identification of the mutations in the six classical *gi* mutant alleles (Fowler *et al.,* 1999).

GI was also cloned using a map-based approach and finally identified by southern blots exploiting restriction fragments of *Ler*, *gi-1* and *gi-2* probed with a BAC identified in the region. This resulted in a single band in the *gi-2* mutant whereas *Ler* and *gi-1* showed two bands, suggesting the mutation in the gi-2 mutant had caused a restriction fragment length polymorphism. End sequencing of these two fragments matched a genomic region. This region contained an open reading frame (ORF) in which deletions could be identified in both mutants (Park *et al*., 1999)

In these classical mutants the genomic region containing the gene was amplified by PCR and alterations in the coding region of the six classical alleles were identified by direct sequencing. Four of the mutations (*gi-1* to *gi-3* and *gi-6*) are predicted to introduce premature stop codons into the *GI* sequence. *gi-4* and *gi-5* are predicted to cause a frameshift and thereby alter the C-terminal part of the protein; changing the last 8 amino acids and adding 27 amino acids (figure 1; Fowler *et al.,* 1999)

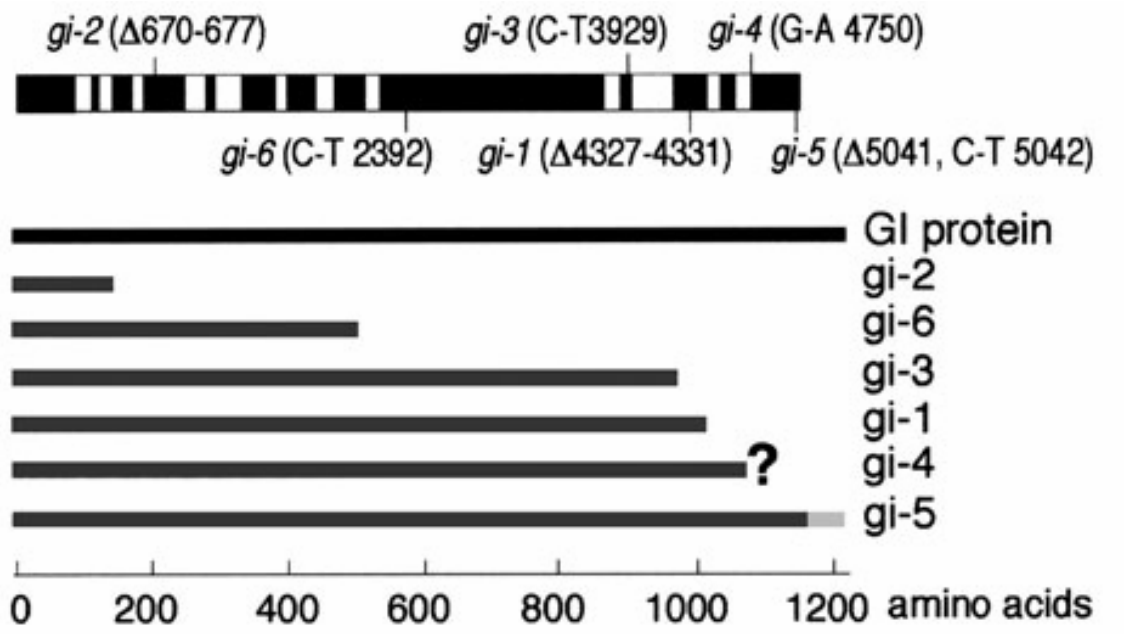

Figure 1. Putative length of GI protein in the classical *gi* mutants. Top bar presents the transcribed region of the GI gene, with introns in black and exons in white. Positions of the gi-1 to gi-6 mutations are also indicated. The putative proteins that results from these mutations are shown below. Picture from Fowler *et al*., 1999).

GI is a single copy gene in *Arabidopsis* (gene code At1G22770) and is predicted to have a coding region of 3522 bp with a 5'UTR of 318bp and a 3'UTR of 217bp. The coding region is divided over 13 exons and predicted to encode a 1173 amino acids protein of 127 kDa (Fowler *et al.,* 1999).

Database searches using BLAST indicated no significant homology of GI to other proteins but identified putative membrane spanning domains (Fowler *et al.,* 1999). However, the predicted membrane spanning regions described by Fowler *et al.* are now believed not to locate the protein to the mambrane, because GUS:GI and GFP:GI fusions were located in the nucleus (Huq *et al*., 2000; Mizoguchi *et al*., 2005). Recent use of the MEGABLAST, "search for short nearly exact matches", PSI-BLAST and domain-searches also did not reveal any significant homology (data not shown).

GI is however highly conserved in seed plants, both Angiosperms and Gymnosperms. Strong homology has been found between GI protein in several plants species, including monocotyledonous plants such as rice (*Oryza sativa*) (Hayama *et al.,* 2002) and gymnosperms such as loblolly pine (*Pinus taeda*). Homologs of GI are absent in other organisms, even in those closely related to seed plants. No homologous proteins have been found in genomes of the moss Physcomitrella (pers. comm. O. Zobell), Chlamydomonas, or of animals (Mittag *et al.,* 2005).

1.6.3. Spatial expression of GI

The expression of *GI* has been analysed by extraction of RNA from different tissues and demonstrated that *GI* is expressed in all stages of development. Samples from apices, flowers and young siliques showed highest abundance of *GI* mRNA whereas lowest levels were found in mature siliques and roots (Fowler *et al*., 1999); all samples were taken at ZT 8 when expression of *GI* is high. Although an exact study of the spatial expression of GI has previously not been done, this shows that *GI* mRNA is present in the entire plant.

The cellular expression of *GI* has been studied using GUS and GFP fusions to the N-terminus of GI. The GUS-GI fusion was targeted to the nucleus in a transient transfection assay in onion epidermal cells; this was in contrast to the GUS protein that was detected throughout the cell. On this nuclear localisation no effect of exposure to light or darkness treatment was found (Huq *et al*., 2000). Using a GFP-GI fusion, confocal microscopy showed that GI was homogenously distributed throughout the interior of the nuclear compartment. Results obtained with GUS fused to several fragments of GI suggest that the region between residues 543 and 783 is sufficient for nuclear localisation. Four separate clusters of basic amino acids in this region might function as nuclear localisation signals (Huq *et al*., 2000).

As the above localisation results were obtained in transient assays, stable transformants of *35S::GI:GFP* and *35S::GFP:GI* in the *gi-3* mutant were made to test the functionality of the fusion protein. Only *gi-3* mutants containing *35S::GI:GFP* flowered at a similar time as 35S::GI plants. In these plants GFP was only detected in the nuclei using confocal microscopy. This strongly suggests that the functionally active GI:GFP functions in the nuclei to promote flowering (Mizoguchi *et al*., 2005).

1.6.4. GI diurnal expression

GI transcript levels fluctuate within light/dark cycles of long days (LD) and short days (SD). Northern analysis of Ler samples from LD conditions showed that *GI* transcript levels peak at ZT 10 with a minimum at ZT 22 to ZT 24/0. Under SD conditions *GI* transcript also cycles, however peak levels occur earlier (at ZT 8). This peak is higher and narrower than in LD (Fowler *et al*., 1999). When LD entrained plants were prematurely transferred to darkness *GI* transcript was down regulated; indicating the reduction of GI transcript levels by dark. However the reverse experiment, SD grown plants moved to LD did not alter *GI* expression (Fowler *et al*., 1999), indicating that the presence of light does not directly up regulate *GI* expression.

The transcript levels of GI are regulated by the circadian clock. If LD entrained plants were transferred to DD or LL conditions, *GI* transcript levels continued to cycle in a similar phase as they would have done in LD, indicating regulation by the circadian clock. In DD, peak levels of *GI* expression decrease whereas trough levels increase, causing an overall reduction of amplitude (Fowler *et al*., 1999).

The regulation of *GI* transcript by the circadian clock is supported by the observation that *GI* expression is controlled by LHY and CCA1. The expression of *GI* is earlier in the single mutants *lhy12* and *cca1-1* and is dramatically early (~6 hrs) in the double mutant *lhy12cca1-1*, were the expression also rapidly dampens in LL (Mizoguchi *et al*., 2002).

1.6.5. GI feeds back into the circadian clock

GI is seen as part of the photoperiodic pathway, an output pathway of the circadian clock, because of its effect on *CO* mRNA levels. However, although *GI* expression is regulated by the circadian clock and acts in an output pathway, it also feeds back on clock function by regulating the expression of *LHY* and *CCA1*. In the loss of function mutants of *GI* the expression levels of *LHY* and *CCA1* are reduced under LD conditions (Fowler *et al*., 1999) and rapidly dampen in LL (Park *et al*., 1999; Mizoguchi *et al*., 2002). Correct high-amplitude circadian rhythms of *LHY* and *CCA1* expression in these conditions therefore depend on GI.

1.6.6. Role of GI in responses to red light

Mutations in *GI* have been shown to impair PhyB signaling during seed deetiolation in red light (Huq *et al*., 2000). This means that when *gi* mutants are grown in only red-light they have longer hypocotyls than wild-type. An elongated hypocotyls in red light is a characteristic of impaired PhyB signaling and led to the conclusion that GI plays a positiv role in PhyB signaling.

1.6.7. Characteristics of GI protein

Although little is known about the function of the GI protein, we slowly obtain some insight in the characteristics of the GI protein. Using the yeast two hybrid system, GI was identified to interact with the TRP domain of SPINDLY (SPY). Further interaction analyses confirmed interaction of SPY with GI (Tseng *et al*., 2004). SPY is a negative regulator of gibberellin signaling and contains a protein-protein interacting domain consisting of 10 tetratricopeptide repeats (TRPs) at the amino terminus. SPY is thought to be a *N*-acetylglucosamine transferase that decorates GI. *spy* mutants exhibit altered rhythms in leaf movement (Tseng *et al*., 2004), suggesting its role in correct circadian clock function by GI. Interaction of ZTL and FKF with GI have also been

reported, but their effect on the biochemical function of GI is unclear. Function of ZTL and FKF upon the circadian clock is discussed above.

Also recently, GI has been shown to be post-transcriptionally regulated by light and dark (David *et al*., 2006). As attempts to track GI protein with antibodies failed, tagged GI was expressed in plants by the constitutive CaMV35S and native promoters and the protein thus followed by detecting the tags to GI. They showed that using both promoters GI protein levels oscillated. This rhythmic accumulation seems to be modulated by daylength as well as phase specific factors. One mechanism by which GI protein oscillates is post-translational regulation via dark induced proteolysis by the 26S proteosome (David *et al*., 2006).

1.6.8. Additional phenotypes of the *gi* mutants

Apart from the above described phenotypes, mutations in *GI* also cause accumulation of starch (Eimert *et al*., 1995) and higher resistance to the herbicide Paraquat (Kurepa *et al*., 1998). The basis of these phenotypes is also not known. It has therefore been suggested that the resistance to Paraquat might be due to an additional mutation close by, however attempts to map or separate these phenotypes have not been successful (Wright, 2003).

1.6.9. Analysis of *GI* in other species

The *GI* orthologue of rice (*OsGI*) has been identified and its expression is regulated by the circadian clock in a similar way to *AtGI* (Hayama *et al*., 2003). Also the orthologues of *CO* (*Hd1* in rice) and *FT* (*Hd3a* in rice) have been identified and shown to be required to flower in inductive short days. Because *Hd1* represses *Hd3a* expression in long-days, the photoperiod pathway seems to be adapted to promote flowering in short days.

Promoter analyses between *Arabidopsis* and its closely related *Arabis alpina* have recently been undertaken. The analysis was done using promoter-luciferase-constructs (*AaGI::LUC* and *AtGI::LUC*) which were transformed into *Arabidopsis*. Diurnal measurement of the luminescence showed that the expression from the *AaGI*-promoter shows great similarity to endogenous *GI* expression in *Arabidopsis* (pers. comm. I. Bürstel).

In radish, constitutive overexpression of an antisense *GI* cDNA gene fragment from *Arabidopsis* delayed bolting and flowering. This provides evidence that downregulation of the *GI* gene by co-suppression could delay bolting in a cold-sensitive long-day crop.

1.7. Open questions concerning GI function and objectives of this study

The functional analysis of GI is still surrounded by many open questions. A major goal for future research is to elucidate the biochemical function of GI. One way of doing so is to identify interacting proteins that might provide indications of the processes in which GI acts. Similarly a mutant screen was performed with the objective of identifying genes related to GI in function.

Furthermore, the pattern of spatial expression of GI has not been studied in detail and knowledge about the detailed spatial expression might lead to new insights concerning the cells in which GI acts to regulate distinct processes. Therefore it would also be interesting to know where specific artificial expression of *GI* might relate to the different processes in which GI acts, such as flowering time and hypocotyl length.

2. Identification and analysis of proteins that interact with GI

2.1. Introduction

As described in chapter 1, the biochemical function of GI is unknown. Identification of proteins that interact with GI might give insight into its possible functions if the functions of the interacting proteins are known. Therefore we screened for interacting proteins with GI.

A good example in which an interacting protein can lead towards more insight in the function of a protein with unknown biochemical function, is the interaction between FT and FD. The FT protein functions downstream of CO to promote flowering. *FT* is expressed in the vascular tissue of cotyledons and leaves and encodes a small protein. *FD*, a bZIP transcription factor, is expressed in the shoot apex and required for promotion of flowering by FT. It turned out that FT and FD are interdependent partners through protein interaction. The complex of FT and FD can activate the expression of a floral identity gene *APETALA1* (*AP1*) (Wigge *et al*., 2005; Abe *et al*., 2005). Through this interaction with FD revealed that FT is a transcription factor that regulates the expression of floral identity genes, most likely to function in the shoot apex.

To identify GI interacting proteins I made use of the yeast two-hybrid system. This well-established system is used by groups working on different biological systems to search for physical interactions (such as binding) between two proteins. As this system can be used to discover protein-protein interactions, we used this system to screen for proteins interacting with GI.

The basis behind this system is the activation of a reporter gene by the GAL4 transcription factor. For the purpose of two-hybrid screening, this transcription factor is split into two separate fragments, called Binding Domain (BD) and Activating Domain (AD). The BD is the domain responsible for binding to DNA and the AD is the domain responsible for activation of transcription. Even though the transcription factor is split

into two fragments, it can still activate transcription when the two fragments are indirectly connected.

The yeast two-hybrid system that I used is based on the Gal4-activation system. The bait protein of interest is fused to the Gal4 DNA binding domain and an Arabidopsis cDNA library is fused to the Gal4 activation domain (Gal4AD). In this case, if the protein of interest interacts with a protein fused to the Gal4AD, the Gal4 activation domain is brought to DNA resulting in transcription of reporter genes. This system utilizes genetically engineered strains of yeast in which the biosynthesis of certain nutrients (usually amino acids or nucleic acids) is lacking. This yeast does therefore not grow on media lacking such nutrient. In our approach, we use Histidine and Adenine as selective markers. The activation of the reporter genes allows the yeast strain to overcome the deficit, self-produce the selective markers and thus grow on the selective media. This enables the easy selection of yeast colonies in which the two GAL4 domains are reconnected, an indication that the protein of interest interacts with a protein from the library.

2.2. Autoactivation of GI protein

In this approach, GI is our protein of interest and thus fused to the Binding Domain of GAL4. This was done by PCR amplification of the full length ORF of GI with the Gateway compatible primers GIGF and GIGR and recombined in the pDONR201. This GIGW fragment contains the full cDNA minus the STOP-codon and was recombined into the BD-vector pAS2.1 to resulting in a C-terminal fusion of GI to the binding domain of GAL4, named GI-BD.

The first step was to test whether the GI-BD protein activated transcription of the reporter genes in the absence of interacting proteins. This was done by studying the growth of yeast strains carrying the *GI-BD* plasmid plus the empty *pACT2* vector. This growth was compare to yeast strains containing the empty *pAS2.1* and *pACT2* vector or *SNF1-BD* and

SNF4-AD. The empty vectors serve as negative and the know interaction between SNF1 and SNF4 (Celenza and Carlson, 1989) serves as positive control. Because the Y2H screen will be done by mating, this was tested both in yeast strain Y187 and AH109. Suspensions of each yeast culture were spotted in 5 dilutions on medium containing an increasing concentration of the histidine inhibitor 3-Aminotriazole (3AT), ranging between 0mM and 18mM. The reporter gene *HIS3* encodes a key-enzyme in histidine synthesis, the imidazolegrycerol phosphate dehydratase and 3AT can specifically inhibit this enzyme activity in a dose-dependent way reducing auto-activation effects (Mori et al., 1995). Also the leakiness of the yeast strains are revealed, as seen by the inspecific growth of the negative control on selective medium with 0mM and 3mM 3AT. This analysis also shows the autoactivation of GI-BD, visualised by the growth of the GI-BD on selective medium. The leakiness and growth of the strain was significantly reduced using 12 mM of 3AT (figure 2). Therefore, as suitable conditions to perform the screen 12 mM of 3AT was added to the medium to prevent the appearance of false positives caused by self-activation of the reporter gene *HIS* by the bait construct and consequent leakiness of the yeast strain.

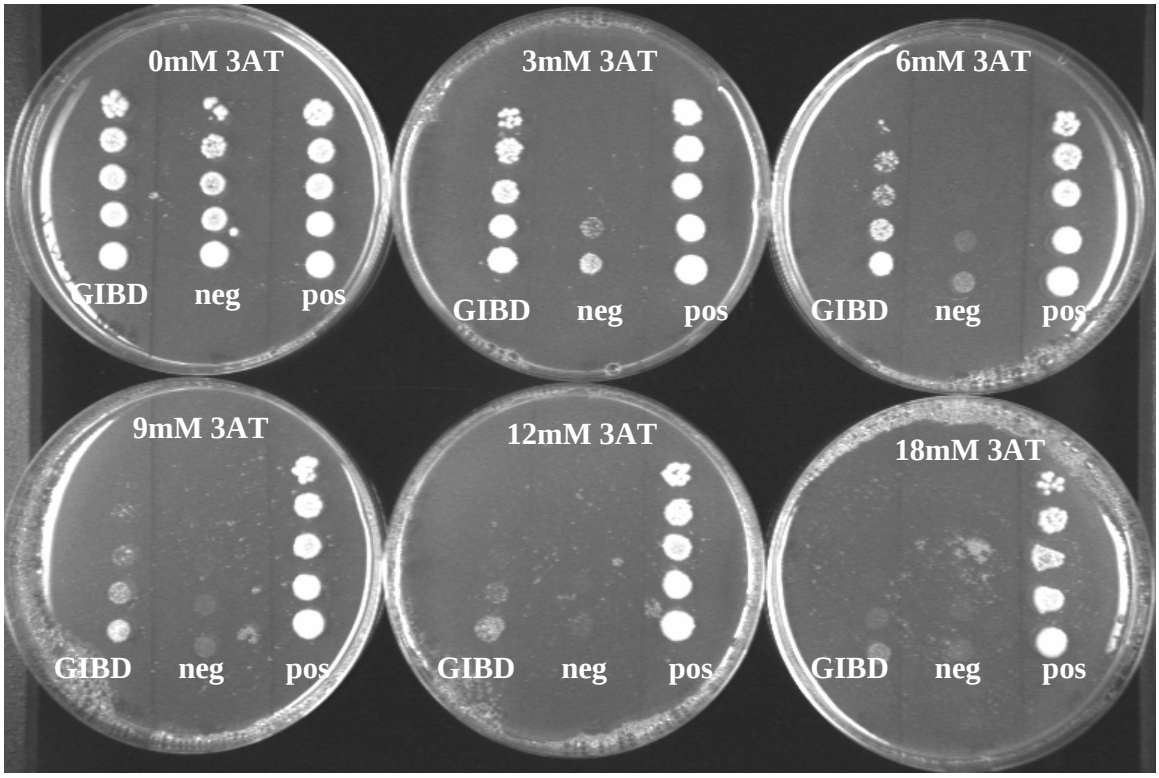

Figure 2. Analysis of self-activation of the GI-BD in yeast strain AH109. Yeast were double-transformed with *GI-BD+pAct2*, *pAS2.1 + pAct2* (negative control) and *SNF1-BD + SNF4-AD* (positive control). 5µl yeast culture, 5x, 50x, 500x and 5000x dilutions were spot onto SD-L-W-H plates with increasing 3AT concentrations.

2.3. Screening of yeast two hybrid library with GI-BD

Using these selective conditions, I screened two normalized cDNA libraries (total and apex tissue from *Arabidopsis*; kindly provided by Dr. Hans Sommer) for yeast colonies (strain AH109) able to grow on histidine selection in the presence of 12mM 3AT. Numerous colonies appeared and 192 colonies of each library were tested against an increasing concentration of 3AT (12mM→30mM). From the colonies able to grow on 30mM 3AT, 2x96 colonies were selected reducing the total number of colonies by 2.

The DNA present fused to the AD in each colony was amplified by PCR using primers from both sides of the cDNA insertion site and subsequently analysed by sequencing.

Alignment of the obtained sequences revealed 52 *GI Interacting Proteins* (*GIPs*) of which we selected 15 for further analysis (see table 1). Excluded from the selection were genes encoding non-nuclear proteins (eg *PETC*, *LOX2*, *PLC2*) and genes that are possible false positives: eukaryotic translation initiation factors (*EIFs*) and ribosomal proteins (*60S*) (pers. Comm. Dr. J. Uhrig). In addition groups of genes of unknown function and poorly assigned genes were not selected as a priority in order to keep the number of genes analyzed at a manageable level. These could be studied subsequently in another study.

Table 1 Selected putative *GI Interacting Proteins* (*GIPs*) from yeast two hybrid screen with GI.

GIP nr	Locus name	Gene ontology	homology
1	AT1G15200		pinin, nuclear protein hsSDK3 &xlSDK2
3	AT5G08330	TCP family transcription factor	PCF1 (GI:2580438) and PCF2 ((GI:2580440) Oryza sativa)
4	AT4G26430	Cop9 signalosome subunit 6 (CSN6b	
7	AT3G55980	zinc finger transcription factor (PEI1)	put. CCCH-type zinc finger protein (AT2G40140.1)
10	AT5G28770	bZIP transcr factor AtbZip63	
12	AT2G39880	MYB-transcr. factor (MYB25)	
14	AT1G56280		drought-induced protein Di19, ZZ-domain, PHD finger protein
15	AT2G37630	AS1: asymmetric leaves 1; (MYB91)	
21	AT5G64980		light-inducible protein CPRF-2, seed storage protein opaque2
22	AT5G44800	CHD protein-like	Chromodomain helicase-DNA-binding protein (CDH's)
28	AT1G73760	put. RING-zinc finger	put. Esterase-like (AT5G11910.1)
30	AT4G34990	myb-family	Myb-related protein Hv1
37	AT5G03150		TRANSPARENT TESTA 1 protein (TT1)
46	AT3G15400	ATA20	
48	AT5G67060	(bHLH088) put bHLH transcr factor	weak: Phytochrome-interacting factor 3 (bHLH8)

The next stage of the analysis was to retest the 15 selected putative interactors. Therefore, the plasmids were isolated from the positive yeast colonies, AD-plasmids amplified in *E.coli* and their sequence confirmed. Subsequently these were directly transformed into yeast strain AH109 containing either the empty *BD* or the *GI-BD* fusion to confirm the interaction. Apart from confirming the interactors, this analysis would exclude

abnormalities such as the presence of various proteins fused with the AD, giving growth of the yeast in the initial screen.

In addition, 5 of the identified putative interactors were already present in the laboratory as full-length cDNA clones and therefore these were recombined into the AD-vector. These were also transformed in yeast strain AH109 together with either empty *BD* or the *GI-BD* fusion. These 20 combinations, 15 GIPs + 5 full-length GIPs, were analysed for their interaction with GI-BD. Growth of the yeasts containing GI-BD and the various GIP-AD fusions were compared to the growth of yeasts containing an empty BD-vector and the GIPs. This was done by spotting five individual transformants per yeast line on plates lacking histidine and containing 30mM of 3AT.

Yeasts with GIPs and either GI-BD or empty BD grow well on plates containing histidine although the yeasts with the empty BD show a pink colouring due to a deficit in adenine, the other reporter (left panel figure 3). Only yeast containing GI-BD and one of the 15 retested GIPs grow on histidine selection (right panel figure 3), reconfirming that all GIPs were positive in the yeast two hybrid.

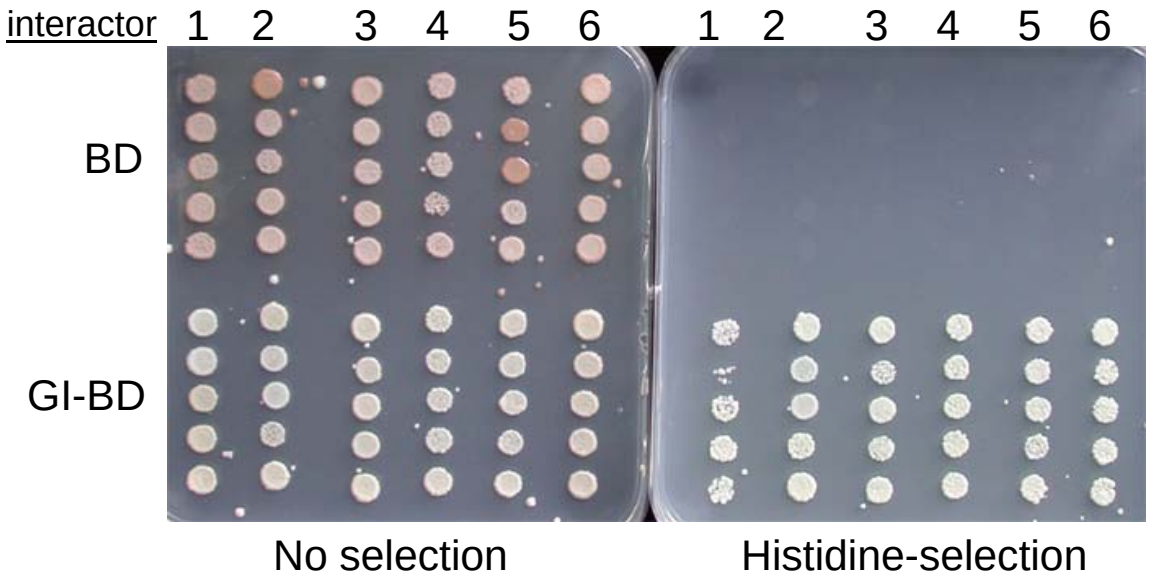

Figure 3: Confirmation of yeast two hybrid screen with GI-BD. Yeast containing either GI-BD or empty BD were transformed with the selected GIPs. Left panel shows that all combinations grow on plates without selection. Right panel shows a plate without histidine and with 30mM 3AT. This confirms the interaction of GI-BD with the GIPs. A representative part of the analysis is shown; nr1 = GIP1, 2 = GIP3, 3 = GIP4, 4= GIP7, 5 = GIP10 and 6 = GIP12

2.4. Selection of interactors with GIC, domain of GI implicated in flowering regulation

As explained above, GI seems to be involved in other processes as well as flowering. My main focus was to understand the role of GI in flowering-time, and therefore I sought a way to narrow down the number of putative interactors to those most likely to be involved in flowering-time regulation. Emily Chin-Fun Chen and Chang-Hsien Yang have shown that the C-terminal domain of GI from *Bauhinia purpurea* (BpGI) is sufficient to rescue the late flowering phenotype of the *gi-1* mutant (Chin-Fun Chen and Yang, 2003). Apart from that, screening for mutants defective in temperature compensation of the circadian clock revealed a new allele of *GI* (Gould *et al*., 2006) that implicated the C-terminal region in flowering. This allele of *GI* has a mutation in the 5' region which does not affect flowering, only circadian rhythms (pers.comm. A. Hall). These two findings suggest that the C-terminal region of GI is most likely to be involved

in flowering-time control. Further support for this idea comes from the classical alleles of GI that affect flowering, all of which contain a mutation in the 3' region of the gene.

This would suggest that testing the putative interactors against the C-terminal domain of GI would result in positive interactions with proteins most likely to be involved in flowering, therefore eliminating proteins that interact with part of GI that possibly plays no or little role in regulating flowering time.

The C-terminal domain of GI was therefore amplified. The segment of GI used was constructed to be analogous to the BpGI C-terminus used for complementation of *gi-1* (Chin-Fun Chen and Yang, 2003). The 3' half of GI was amplified using Gateway compatible primers GIC_1640 and GIGR and recombined in the pDONR207 and creating an C-terminal portion of GI starting with a startcodon at aminoacid 545 of the original GI protein. This portion of GI was recombined in pAS2.1 to create a fusion to the Gal4 DNA binding domain (GIC-BD) and transformed to yeast strain AH109. All selected putative interactors were subsequently transformed into this GIC-BD strain. To test for interaction with GIC, four individual transformants were transferred to selective plates lacking histidine and containing 30mM of 3AT. The results (figure 4) suggested that only four putative interactors bind to the C-terminus of GI; one putative interactor GIP46 (ATA20;
At3g15400) shows strong growth and 4 others, GIP3 (a TCP family transcription factor, At5g08330), GIP4 (CSN6b, At4g26430), GIP14 (ZZ-finger domain family protein, At1g56280) and GIP22 (a CDH protein-like, At5g44800), weaker growth.

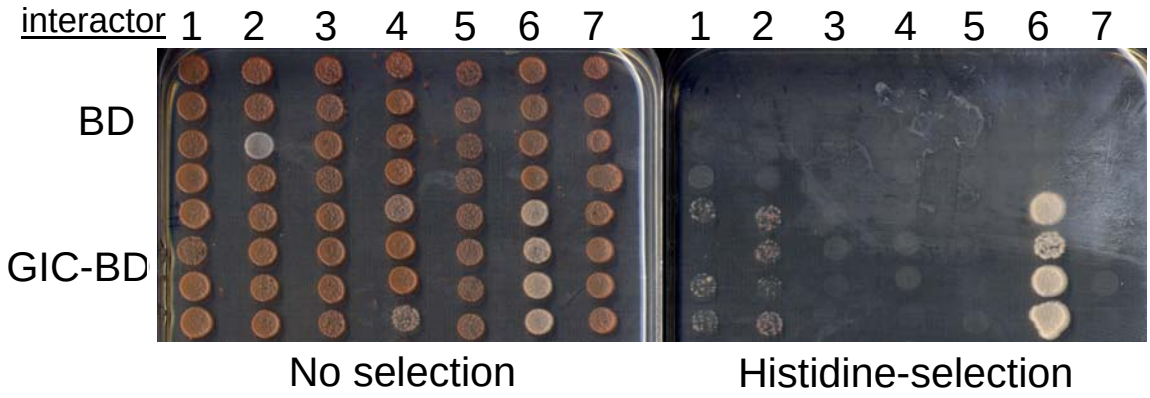

Figure 4. Testing putative interactors against GIC-BD. Yeast containing either *GI-BD* or empty BD were transformed with the 15 selected GIPs. Left panel shows that all combinations grow on plates without selection. Right panel shows a plate without histidine and with 30mM 3AT. This shows the interaction of GIC-BD with the GIP14. A representative part of the analysis is shown; nr1 = GIP28, 2 = GIP30, 3 = GIP30_FL, 4= GIP37, 5 = GIP37_FL and 6 = GIP46 and 7 = GIP48. FL stands for the full-length cDNA of the representative GIP.

Interactors not showing an interaction with the C-terminus of GI were provided to Dr. Anthony Hall and co-workers at the University of Liverpool. They will study the interaction of these proteins with the N-terminus of GI in relation to the function of GI in the temperature compensation of the circadian clock.

Discriminating between the interactors based on their interaction with the C-terminus of GI however depends on the functionality of the C-terminal region used. Therefore the *GIC-BD* construct used contain an analogous segment of *Arabidopsis* GI to the BpGI C-terminus used to complement *gi-1* (Chin-Fun Chen and Yang, 2003). Additionally the GI C-terminus fragment was cloned in pAlligatorII and pLeela by Gateway recombination. Both plasmids contain the *35S* promoter to drive expression; additionally, *GIC* expressed from the pAlligatorII construct is tagged with 3x HA. Both these constructs were transformed into the *gi-3* mutant to test for the ability to rescue the late-flowering phenotype. Both Flowering time of the T1 and T2 plants were studied however no complementation observed (no data shown). This result suggests that either the

Arabidopsis protein behaves differently to the BpGI protein, or that the different results are due to the different *gi* mutant alleles used for the complementation..

2.5. GIP14 interacts both with CO and GI in plants and causes hypocotyl elongation in red light conditions

2.5.1. GIP14 interacts both with CO and GI in plants

As interactions found in the yeast two hybrid system were identified in a hterelogous system, real interactions also need to be confirmed as positive in plants. Therefore the next steps in the identification of a protein-protein interaction involves testing these putative interactions using techniques such as FRET (Föster/Fluorescence Resonance Energy Transfer) in plants.

GIP14 was also identified in our laboratory by Dr. Stephan Wenkel in a yeast two hybrid screen using CO as bait. In order to analyse this coincidence further, and test either the small GIP14 as well as GI and CO form a higher order protein complex, interaction was studied using FRET analysis. In this experiment CO was fused to the CFP and GI was fused to YFP. After bombardment, together with unfused GIP14 these proteins were transiently expressed in *Arabidopsis* leaves (Wenkel, 2005).

We were able to show that GIP14 interacts with both CO and GI using FRET and that no FRET signal was seen between CO and GI without this GIP14 (figure 5a). Figure 5b shows the effect of photobleaching upon the energy transfer of CO to GI which is facilitated by the presents of GIP14. This suggests that GIP14 enables an interaction between GI and CO.

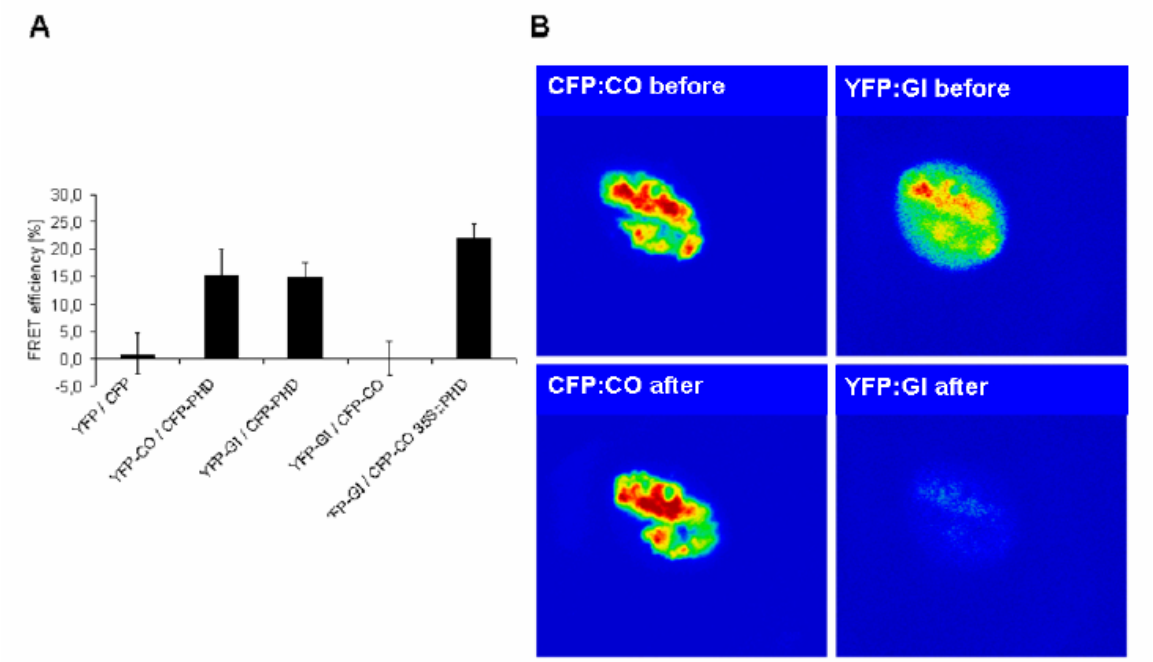

Figure 5. a. Quantification of FRET analysis of the GI-GIP14-CO interaction. b. FRET experiment of a nucleus transformed with *35S::YFP:GI*, *35S::CFP:CO* and *35S::GIP14* before and after photobleach (Wenkel, 2005).

2.5.2. *GIP14* is part of the small family of ZZ-domain proteins

Comparison of protein sequence reveals that GIP14 is part of the ZZ-finger domain protein family (figure 6). This family contains DROUGHT INDUCED 19 (*Di19*; At1g02750), which expression is strongly elevated during sustained drought conditions in both roots and leaves of *Arabidopsis*. Also HYPERSENSITIVE TO RED AND BLUE 1 (HRB1, AT5G49230) groups together with the members of the ZZ-finger domain protein family. HRB1 seems to regulate PhyB-mediation of red light signalling and Cry-mediation of blue light signalling as mutations in *HRB1* causes a short hypocotyl in blue and red light. HRB1 seems to be involved in additional light driven processes such as end-of-day far-red light response and expansion of leaf and petiole (Kang *et al*., 2005).

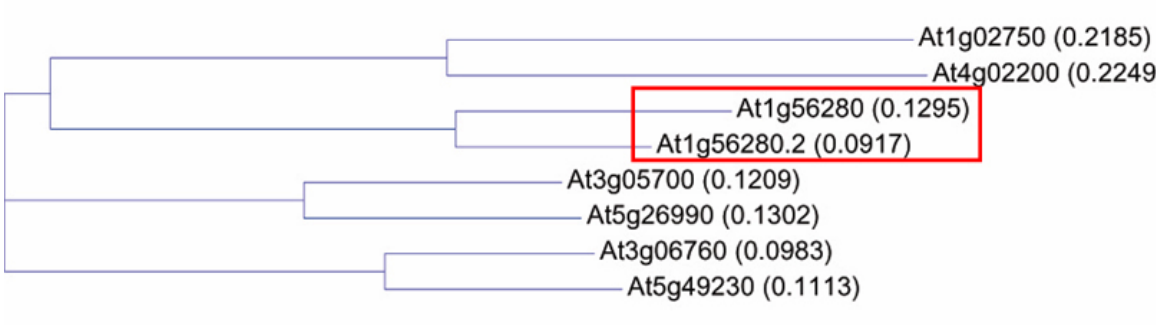

Figure 6. GIP14 belongs to a small family of ZZ-domain proteins. BLAST search revealed that the GIP14 exists in two splice variants (Wenkel, 2005)

The proteins of the ZZ-finger domain protein family group together because they all contain a ZZ-finger domain (figure 7). This ZZ-finger domain shows homology to proteins identified from mouse and human; this domain is believed to be involved in protein-protein interactions (Yu *et al*., 1997; Tanaka *et al*., 2000, Kang *et al*., 2005).

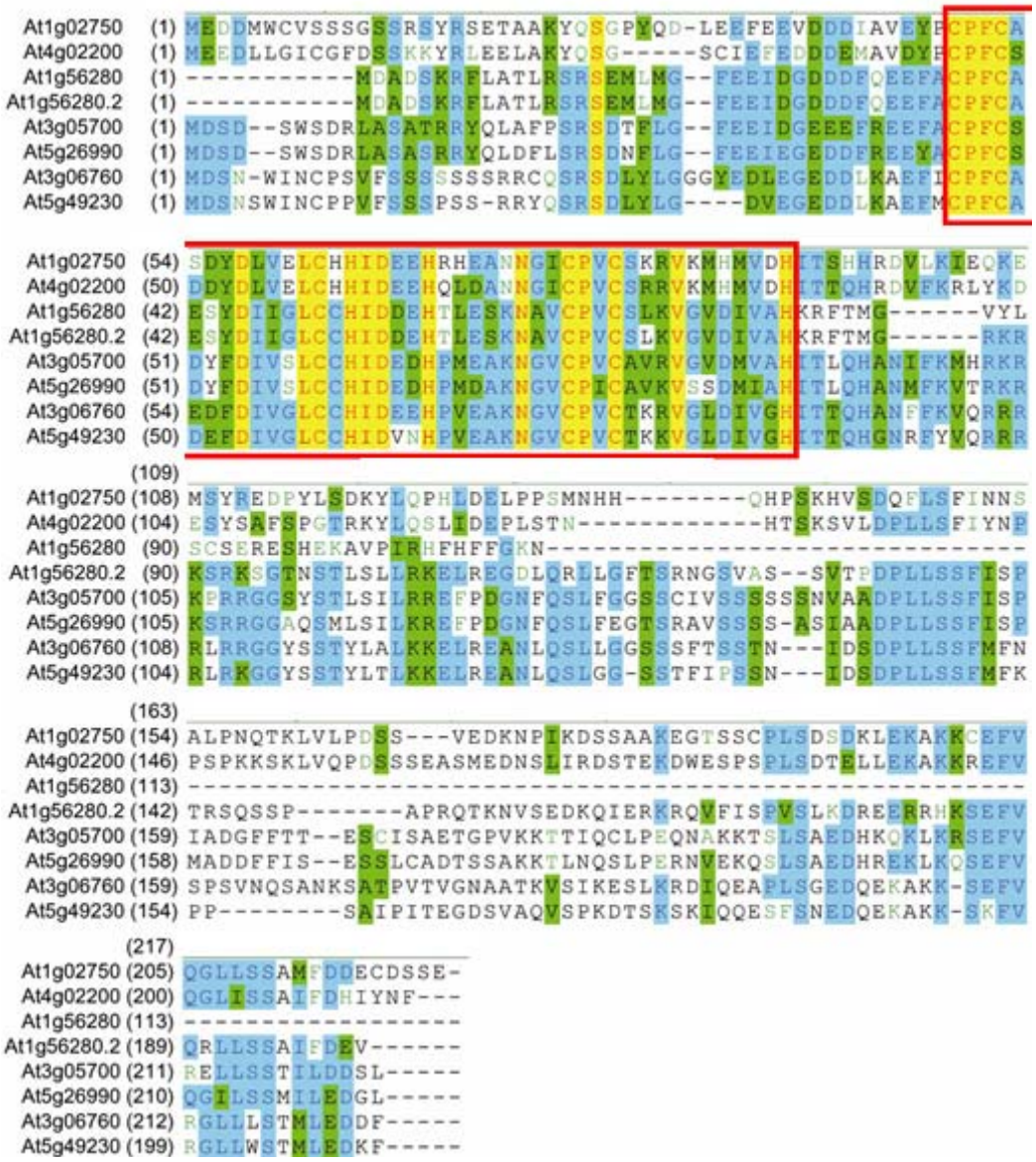

Figure 7. Alignment of all ZZ-domain proteins encoded in the Arabidopsis genome shows that GIP14 contains the ZZ-domain (red box). The various ZZ-domain proteins are less conserved in their carboxy terminus. Conserved residues in yellow; identical residues in blue and similar residues in are shown in green (Wenkel, 2005).

2.5.3. Overexpression of GIP14 causes hypocotyl-elongation in red light conditions

Attempts were undertaken to test for a phenotypic relationship between GI and GIP14. However, both overexpressors and loss of function alleles did not cause a flowering time phenotype (Wenkel, 2005). Therefore in collaboration with Stephan Wenkel we checked the hypocotyl elongation phenotype under red light. As GI has been shown to be involved in red light signalling this could provide a link with the interaction between GI and GIP14. This experiment demonstrated that when the *GIP14* is overexpressed this results in an elongated hypocotyl under red light conditions. The putative knock-out allel of the *GIP14* did not effect hypocotyl length (figure 8). This experiment clearly shows a potential relationship in function between GI and GIP14, in which GIP14 has the opposite effect to GI and may therefore interact with GI to repress its activity.

Figure 8. Analysis of hypocotyl length of gip14 knock-out and overexpression lines 13,14 and 21 compared to wild-type Columbia, gi-11 mutant and 35S::GI overexpressor (Wenkel, 2005).

2.5. Analysis of other GIC-interactors

The 4 other GIPs (GIP3, GIP4, GIP22 and GIP48) that also interacted with the C-terminus part of GI were also tested further. Full-length cDNAs were obtained and cloned in Gateway destination-vectors ready to be used for further characterisation of the possible interaction in FRET or Co-IP analysis.

These full-length cDNAs have also been expressed behind the SUCROSE-H+ SYMPORTER (SUC2; Stadler and Sauer, 1996) promoter in the Arabidopsis Columbia accession (collaboration with Dr. L. Gissot). This promoter drives expression specifically in the phloem companion cells where GI and CO acts to regulate flowering (see chapter 3). T1 data suggest that expressing these genes in the phloem does not affect the timing of flowering. T2 were tested and confirm this observation (data not shown).
SALK-insertion lines carrying T-DNA insertions in these genes have been ordered and analysis under LD conditions does not reveal any defect in flowering time of any of the 18 plants of SALK line (data not shown). None of these plants have however been tested for correct insertion in the corresponding gene nor for homozygosity or the insertion.

2.6. Discussion

Taken together this screen for interacting proteins to GI has so far not identified any partner of GI that shows a connection to the flowering time pathway. Although the screen yielded many potential interacting proteins only 5 of the selected 15 interactors were identified to interact with the C-terminus of GI in yeast. I was however not able to confirm the importance of this domain in flowering control in our conditions. Full-length cDNAs of all 4 clones have been isolated and are ready to confirm the interaction with GI in other systems such as FRET and Co-IP.

A clear connection to GI has been found with the GIP14, a ZZ-finger domain protein, which was picked up in the Y2H experiment and confirmed to bind to GI and the C-

terminus in this system. The functional connection is illustrated by the finding that overexpression of *GIP14* results in elongated hypocotyls in red light which is the opposite effect to mutations in *GI*. This suggest the possible role of the GIP14 in the regulation of red light signalling possibly in facilitating the role GI plays in this process. The interaction of GI and GIP14 has also been confirmed in FRET analysis suggesting that GIP14 might play a role in organising a complex in which both GI and CO might be present. Evidence that this protein might also have a function in the control of flowering time has however not been found.

Interestingly, HRB1, a member of the ZZ-domain family to which GIP14 belongs is known to function in red- and blue-light signaling (Kang *et al*., 2005). Since GI seems to have a function in red light (Huq *et al*., 2000) and its known that CO protein is degraded in red light (Valverde et al., 2004) it was analyzed what effect this putative interactor has in various light conditions. The experiment demonstrated that overexpressing the *GIP14* results in an elongated hypocotyl under red light conditions. This suggests the relationship in function between GI and GIP14. In regulating hypocotyl elongation under red light, GIP14 has the opposite effect to GI, together with their fysical interaction, this suggests that GIP14 interacts with GI to repress its activity. In blue, far-red and white light conditions no effect was observed, indicating the specific functioning in red light signaling of GI and GIP14.

Interesting point of discussion with every screen is the saturation, as this tells us about the possible coverage of the screen. Especially for the Y2H-screen, as this is a random search for proteins that interact with the bait. The library used was normalized to enable similar chances for a low and high expressed gene to be picked up. To get an idea of the coverage, the mating efficiency was analysed and showed a normal efficiency of 0,6% for the total and 0,3 for the apex library. This represented a total of mated cells plated of 566.000 for the total library and 395.000 for the apex library. As the library was tested to be covered once in every 100.000 mated cells; the total library was covered 6 times and the apex was covered 3 times.

As mentioned in the introduction, GI was already identified to interact with SPY (Tseng *et al*., 2004) and FKF and ZTL (pers. comm. Dr. Fabio Fornara). If the coverage of the screen was theoretical 100% these proteins should have been picked up using GI as a bait. These proteins were however not identified, suggesting that the screen was not absolute. As every screen is subjected to incomplete coverage this is not unusual, however important to realise and to note here.

3. Analysis of the spatial control of GI function

The development of flowers occurs at the shoot apical meristem (SAM) of *Arabidopsis*. Initially, in the vegetative state the SAM produces leaf primordial, but upon transition to the reproductive state flowers develop from the primordia of the SAM. An important environmental cue controlling this transition is the perception of daylength. Traditional grafting experiments showed that photoperiod is perceived in the leaves and flowering depends upon the transmission of a signal from the leaves to the apex that can cross graft junctions (Zeevaart, 1985). The wide spatial expression pattern of GI (Fowler *et al*., 1999) does not give insight as to whether it acts in the leaves or meristem to regulate flowering. Therefore I was interested to examine in which part of the plant GI is required to promote flowering. I first experimentally tested the spatial expression of *GI* using a *GI::GUS* fusion. Promoter::GUS fusions have been very useful in defining the spatial expression of *CO* and *FT*, in which *CO* expression was localized to the vascular tissue (An *et al*., 2004) and *FT* expression was found in the phloem tissue of leaves and cotyledons (Takada and Goto, 2003). Additionally, the spatial requirement for GI both in flowering time and red light signalling was studied.

3.1. The spatial expression pattern of *GI* was revealed using *GI::GUS* transgenic plants

In order to determine the detailed spatial pattern of *GI* expression, a GUS-assay was performed on plants transformed with *GI::GUS*. Stable single insertion lines were kindly provided by Dr. Hailong An. Whole seedlings, stems and leaves of 3 independent *GI::GUS* lines were stained. Blue GUS staining was detected in young leaves and in the vascular tissue of the root, hypocotyl, cotelydons and leaves. Expression was also detected at the apex of the shoot and end of the root (see figure 8).

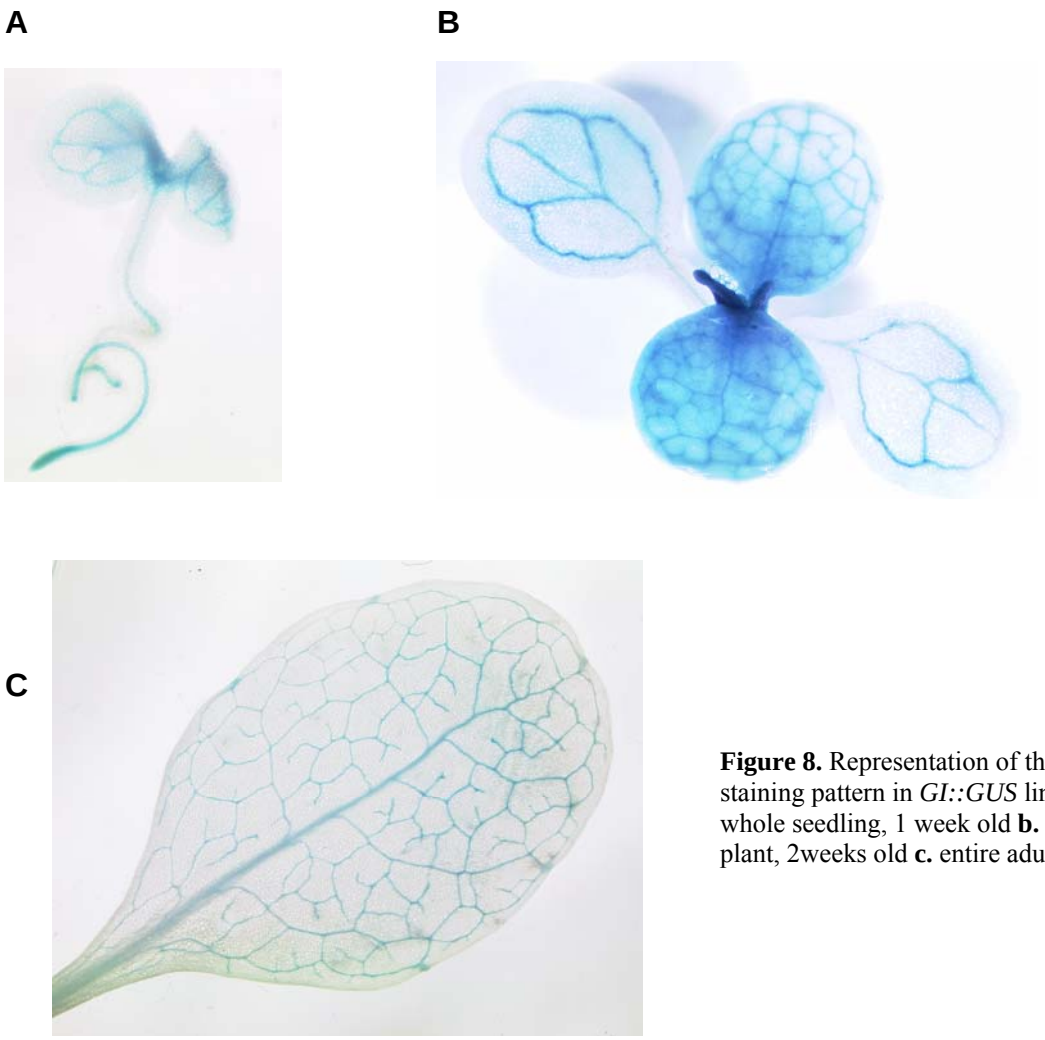

Figure 8. Representation of the GUS staining pattern in *GI::GUS* lines. **a.** whole seedling, 1 week old **b.** young plant, 2weeks old **c.** entire adult leaf.

As described in the introduction, GI is believed to be part of to the circadian clock mechanism and the control of flowering, therefore it was surprising that the expression pattern of *GI::GUS* was predominantly in the vascular tissue, since every cell is expected to contain a circadian clock. One possibility is that *GI* is expressed more widely but at lower levels, not visible compared to the heavy staining in the vasculature. Alternatively, there may be different clocks and GI may not be part of all of them.

3.2. Fusion to GI of promoters driving specific patterns of expression

In order to analyse where GI is required for the promotion of flowering and the other functions of GI, the gene was expressed in specific regions of the plant. For this directed spatial misexpression of *GI*, the *GI* cDNA was amplified using primers GIMF and GIMR

and after restriction with KPN1 and APA1 this was cloned in the binary plant transformation vector pAN2. This resulted in a construct which contained a Gateway entry site adjacent to the GI cDNA (figure 9). The various region specific promoters (see table 2) have gateway recombination sites at both ends and were recombined into the Gateway-site of this construct, forming the promoter::GI constructs. Subsequently these promoter::GI vectors were transformed, by use of *Agrobacterium*, into *gi-3* plants. The promoter::GI constructs convey resistance to the herbicide BASTA. The T1 seed was sown out and by spraying the seedlings with BASTA transformants were selected. These region specific promoters were originally cloned and provided by Dr. Hailong An (An *et al*., 2004). They have a broad coverage of the entire plant ranging from roots to shoots and meristem to vascular tissue. The constitutive promoter 35S and the native *GI* promoter were included in order to have positive controls, so that the functionality of the construct can be tested as the results expected from these promoters is known. Both rescue the late flowering of *gi* mutants, causing early flowering (Fowler *et al*., 1999; Huq *et al*., 2000).

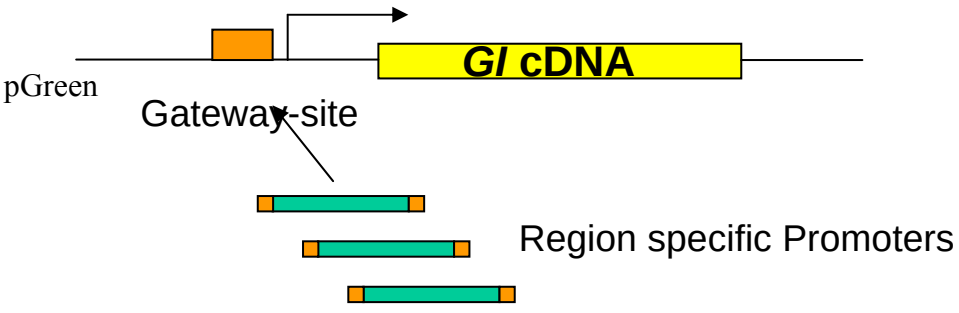

Figure 9. Schematic representation of the GATEWAY™ cloning of the various region specific promoters upstream of *GI* cDNA in the pGreen207 binary vector.

Table 2 Promoter GI fusions transformed into *gi-3*

Promoters in GATEWAY	Pattern of expression during development
35S::GI	Constitutive
GI::GI	Native
4CL1::GI	Xylem
4CL2::GI	Xylem
SUC2::GI	Phloem
RolC::GI	Phloem
TobRB7::GI	Root meristem
KNAT1::GI	Sam, hypocotyls, cortex of stem, pedistel
AG-like::GI	C specific in ABC-model (stamen/carpel)
UFO::GI	Shoot Apical Meristem (SAM)
STM::GI	Shoot Apical Meristem (SAM)
AS1-int::GI	Leaf/lateral primordia
AS1-code::GI	Leaf/lateral primordial (trunc. AS1-code)
ML1::GI	Meristem layer 1 specific

3.3. Isolation of independent homozygous single insertion lines for each promoter::GI fusion

After transformation of all *promoter::GI* fusions, the T1 generation was selected on soil by applying the herbicide BASTA (see table 3). T1 transformants were randomly picked and their progeny, the T2-generation, were studied for segregation of BASTA resistance. In order to standardise the analysis of the T2 segregation, around 120 seedlings were analysed per line, so creating an easy determination of the ratio with clear statistical significance. The expected ratio for a single insertion is 90 resistant seedlings versus 30 sensitive seedlings; using a significance of 95% the observed number of survivors should be between 80.5 and 99 (see appendix for mathematical procedure).

Those T2-lines showing a clear 3:1 segregation, an indication of a single insertions event, have been collected. T3 seeds of at least 5 single insertion lines were tested on GM-ppt plates (ppt is the equivalent of BASTA and is used in agar) in order to identify independent homozygous single insertion lines of each *promoter::GI* (see table 4). T2 plants were selected at random and 2/3th of them are expected to be homozygous for a single insertion of the constructs. The objective was to obtain 5 independent homozygous single insertion lines of each *promoter::GI*.

Table 3. Phenotypes of *promoter::GI* insertion lines in T1 generation

promoter::GI **fusions**	**Number of T1-plants**	**Flowering time**
35S::GI	32	**Early**
GI::GI	10	**Early**
4CL1::GI	>80	Late
4CL2::GI	32	Late
SUC2::GI	39	**Early**
RolC::GI	22	**Early**
TobRB7::GI	8	Late
KNAT1::GI	20	Late
AG-like::GI	6	Late
UFO::GI	5	Late
STM::GI	3	Late
AS1-int::GI	>80	Late
AS1-code::GI	14	Late
ML1::GI	10	Late

Table 4a. Selection of homozygous *35S::GI* lines

***promoter::GI* fusions**	Line	Seedlings	Survivors	3:1	Homozygous T3-lines
35S::GI	1	62	55	-	
	2	67	63	-	
	3	62	62	-	
	4	67	48	yes	4.2
	5	49	40	yes	5.11
	6	55	39	yes	6.2 6.22
	7	120	109	-	
	8	120	72	-	
	9	120	90	yes	9.1 9.8
	10	120	90	yes	10.19
	11	120	97	yes	11.2 11.9
	12	120	88	yes	
	13	120	97	-	
	14	120	90	yes	13.2 13.4 13.8 13.11
	15	120	90	yes	15.4

Table 4b. Selection of homozygous *GI::GI* lines

***promoter::GI* fusions**	Line	Seedlings	Survivors	3:1	Homozygous T3-lines
GI::GI	1	120	99	yes	1.2 1.5 1.6
	2	120	62	-	
	3	120	105	-	
	4	120	89	yes	4.1
	5	120	112	-	
	6	120	99	yes	6.1 6.4 6.5 6.6
	7	120	97	yes	7.5 7.6
	8	120	88	yes	8.3 8.4
	9	120	80	yes	9.2 9.5
	10	120	?		

Table 4c. Selection of homozygous *SUC2::GI* lines

***promoter::GI* fusions**	Line	Seedlings	Survivors	3:1	Homozygous T3-lines
SUC2::GI	1	120	89	yes	1.10 1.11
	2	120	34	-	
	3	120	118	-	
	4	120	86	yes	4.1 4.4
	5	120	40	-	
	6	120	50	-	
	7	120	65	-	
	8	120	94	yes	8.1 8.4
	9	120	87	yes	-
	10	120	81	yes	10.19

Table 4d. Selection of homozygous *RolC::GI* lines

***promoter::GI* fusions**	Line	Seedlings	Survivors	3:1	Homozygous T3-lines
RolC::GI	1	73	54	yes	1.3
	2	55	41	yes	2.13
	3	88	67	yes	3.1 3.3
	4	86	67	yes	4.2
	5	66	60	-	
	6	88	80	-	
	7	75	62	-	
	8	58	48	-	
	9	76	56	yes	9.13
	10	91	61	-	

Table 4e. Selection of homozygous *AGlike::GI* lines

***promoter::GI* fusions**	Line	Seedlings	Survivors	3:1	Homozygous T3-lines
AGlike::GI	1	120	116	These lines have no trichomes and do not segregate.	
	2	120	115		
	3	120	116		
	4	86	29	-	
	5	120	91	yes	5.1
	6	120	118	no trichomes and no segregation	

Table 4f. Selection of homozygous *ASint::GI* lines

***promoter::GI* fusions**	Line	Seedlings	Survivors	3:1	Homozygous T3-lines
ASint::GI	1	120	93	yes	1.3 1.4 1.5
	2	120	94	yes	
	3	120	86	yes	
	4	120	90	yes	
	5	120	90	yes	5.1 5.3 5.4 5.6
	6	120	111	-	
	7	120	94	yes	7.1 7.3 7.6
	8	120	88	yes	8.1 8.3 8.4 8.6
	9	120	89	yes	9.1 9.4 9.5
	10	120	89	yes	10.1 10.5 10.6

Table 4g. Selection of homozygous *AScode::GI* lines

***promoter::GI* fusions**	Line	Seedlings	Survivors	3:1	Homozygous T3-lines
AScode::GI	1	120	83	yes	1.1 1.3
	2	90	55	-	
	3	120	95	yes	3.5
	4	120	82	yes	4.1 4.2 4.4
	5	120	96	yes	5.1 5.4 5.5
	6	120	97	yes	
	7	120	93	yes	7.1 7.5
	8	120	103	-	
	9	120	118	-	
	10	120	47	-	

	11	120	99	yes	-
	12	120	100	-	

Table 4h. Selection of homozygous *4CL1::GI* lines

***promoter::GI* fusions**	Line	Seedlings	Survivors	3:1	Homozygous T3-lines
4CL1::GI	1	120	91	yes	1.2 1.5 1.6
	2	18	17	-	
	3	95	90	-	
	4	120	91	yes	4.1 4.5
	5	120	99	yes	5.2 5.3 5.6
	6	120	94	yes	6.2 6.3 6.4
	7	120	86	yes	7.4 7.5 7.6
	8	120	97	yes	
	9	120	83	yes	9.2 9.3 9.5
	10	120	94	yes	
	11	120	87	yes	
	12	120	99	yes	

Table 4i. Selection of homozygous *4CL2::GI* lines

***promoter::GI* fusions**	Line	Seedlings	Survivors	3:1	Homozygous T3-lines
4CL2::GI	1	120	100	-	
	2	120	93	yes	2.1 2.3 2.4 2.6
	3	120	87	yes	3.2 3.2
	4	120	87	yes	4.1 4.4
	5	108	84	yes	5.1 5.2 5.3

	6	120	85	yes	
	7	120	87	yes	7.6
	8	120	101	-	
	9	120	88	yes	9.1 9.2 9.3
	10	111	81	yes	
	11	120	94	yes	
	12	120	91	yes	

Table 4j. Selection of homozygous *UFO::GI* lines

***promoter::GI* fusions**	Line	Seedlings	Survivors	3:1	Homozygous T3-lines
UFO::GI	1	120	83	yes	1.6
	2	120	90	yes	2.1 2.2 2.4 2.6
	3	120	90	yes	3.1 3.2 3.6
	4	120	92	yes	4.1 4.5
	5	120	92	yes	5.3 5.5

Table 4k. Selection of homozygous *KNAT::GI* lines

***promoter::GI* fusions**	Line	Seedlings	Survivors	3:1	Homozygous T3-lines
KNAT::GI	1	120	88	yes	-
	2	111	84	yes	2.3 2.5
	3	120	84	yes	3.3
	4	120	86	yes	4.1 4.5
	5	120	90	yes	5.6
	6	120	105	-	
	7	120	86	yes	7.1 7.2 7.4 7.5 7.6
	8	108	85	yes	8.2 8.4 8.6

	9	120	90	yes	
	10	120	88	yes	

Table 4l. Selection of homozygous *TobRB::GI* lines

***promoter::GI* fusions**	Line	Seedlings	Survivors	3:1	Homozygous T3-lines
TobRB::GI	1	120	96	yes	1.1 1.6
	2	120	91	yes	2.7 2.8
	3	84	42	-	
	4	120	104	~yes	4.1 4.3
	5	0	-		
	6	0	-		
	7	120	111	-	
	8	120	94	yes	8.1 8.4 8.5

Table 4m. Selection of homozygous *STM::GI* lines

***promoter::GI* fusions**	Line	Seedlings	Survivors	3:1	Homozygous T3-lines
STM::GI	1	120	104	~yes	1.1

Table 4n. Selection of homozygous *ML1::GI* lines

***promoter::GI* fusions**	Line	Seedlings	Survivors	3:1	Homozygous T3-lines
ML1::GI	5	25	16	~yes	5.1 5.4

Identification of these homozygous lines will allow study of the spatial requirement for GI in the various processes it is involved in. In case a certain promoter fusion complements the phenotype caused by the loss of function mutant *gi-3*, this will indicate that GI functions in the tissue in which the promoter is expressed is sufficient to promote the process studied. Restoring the phenotype of *gi-3* to the wild-type phenotype or that of *GI::GI* will be scored as GI acting in those tissues. In case the promoter in question overlaps with the spatial expression pattern, as seen in the *GI::GUS* lines, this clearly suggests that GI acts in this region to control this process.

3.4. Expressing *GI* in the phloem is sufficient to promote flowering

The spatial involvement of GI in flowering time control was first analysed. During selection of the transformants a rough indication of the effect of the various constructs on flowering was observed. These preliminary data show that expressing *GI* from the *GI* promoter and the phloem promoters *SUC2* and *RolC* cause early flowering compared to the level *35S::GI* does. The other promoters coupled to *GI* showed no enhancement in flowering time (figure 10).

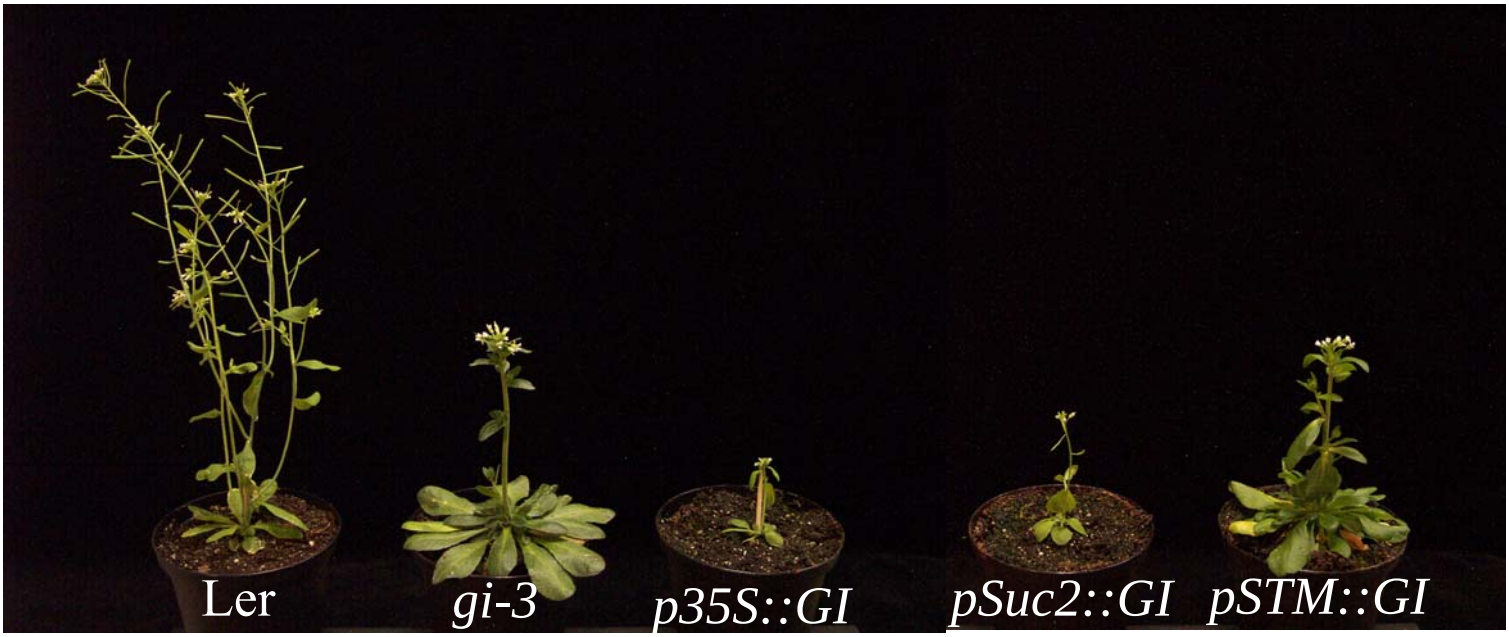

Figure 10. Representation of the flowering-time phenotypes of the misexpression lines, seen in T1 and T2.

The observation that only the promoters active in the phloem companion cells are sufficient to promote flowering was confirmed in the homozygous single insertion T3 lines (figure 11). Data from LD grown plants clearly showed that *SUC2::GI* and *RolC::GI* flowered earlier than the *gi-3* mutant and even earlier than the wild-type. The number of leaves is comparable to that of *GI::GI* and the overexpression line *35S::GI*. Illustrating the importance of *GI* expression in the phloem.

The other transgenic lines flowered with a similar number of leaves as the progenitor *gi-3* mutants. These data suggest that *GI* expression in xylem, root, SAM and leaf primordia does not lead to acceleration of flowering.

Similar results were obtained in a preliminary experiment under SD conditions (no data shown), however detailed counting of leaf number was not carried out.

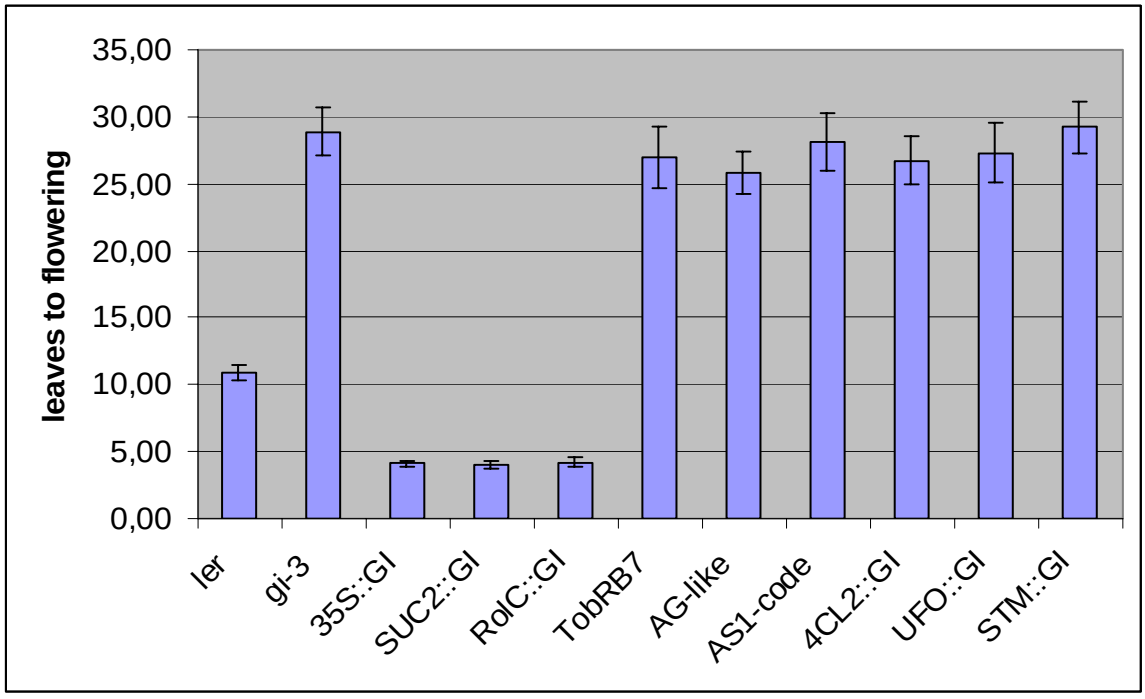

Figure 11. Longday flowering time analysis of the GI misexpression lines. Average from 2 independent lines of each *promoter::GI*. Each line: n=15

3.5. Expression of *GI* from the *AS1* promoter causes a short hypocotyl phenotype in red light

After demonstrating that GI expression in the phloem is sufficient to promote flowering, we were also interested to test how the spatial misexpression of *GI* in the *gi-3* mutant affects hypocotyl elongation. Loss of GI function causes an elongated hypocotyl under red light and therefore suggest that *GI* is implicated in PhyB signalling during seedling deetiolation. To test the spatial requirement of GI in this process, the homozygous single insertion lines were analysed for the length of their hypocotyl under red light.

Test experiments with wild-type, *35S::GI* and *gi-3* mutant plants indicated that 5 day old seedlings grown in LED chambers at 25% intensity of red light (20 μMm^{-2} s^{-1}) show the phenotypic effect of GI on hypocotyl length (data not shown).Using these conditions the hypocotyls of all misexpression lines and those of wild-type plants and *gi-3* mutants were measured. As expected the hypocotyls of the *35S::GI* plants were shorter than wild-type (Huq *et al*., 2000). Strikingly however, *AS1::GI* plants showed a similar short hypocotyls under red light as *35S::GI* (figure 12). Unfortunately the discrimination between the wild-type and the longer hypocotyl of *gi-3* was not very clear in this experiment. This result also makes it hard to distinguish whether the other promoters have an effect upon hypocotyl elongation under red light (figure 12) but they do not have as dramatic effect as *AS1code::GI*.

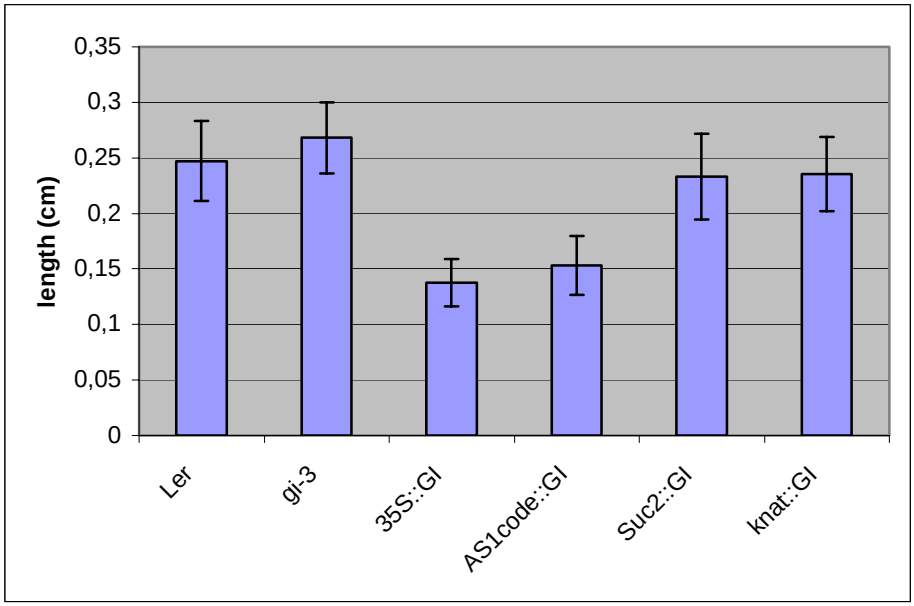

Figure 12. Analysis of the hypocotyl length of various misexpression lines of GI performed under red light conditions. Average from 2 independent lines of each *promoter::GI*. N=25

3.6. Discussion

In this experiment we tried to localize the tissue in which GI controls the various processes that it is involved in. We managed to spatially separate the role of GI in the promotion of flowering from its role in the red light signalling. Enhanced flowering was observed in the lines expressing *GI* in the phloem, whereas shortened hypocotyls under red light were the result of *GI* expression from the *AS1* promoter. From the *GI::GUS* analysis both these regions seem to overlap regions in which *GI* is expressed. Therefore there is a correlation between the misexpression experiments and the pattern of expression of GI. In order to promote flowering it is sufficient to express *GI* in the phloem and expressing *GI* from the *AS1* promoter is sufficient to enhance red light sensing. Therefore it seems possible that *GI* promotes these processes from these specific areas in wild-type plants.

The *AtSUC2* promoter is specifically expressed in the companion cells of the phloem and not in young leaf primordia or in the meristem (Imlau *et al.*, 1999; Stadler and Sauer,

1996). The expression of the *RolC* promoter is also specific to the phloem companion cells (Booker *et al*., 2003).

The *AS1* promoter confers expression in the leaf primordia and is clearly distinct from the expression of the other promoters tested, which are also expressed in early developmental stages in the SAM (see figure 13; Brand *et al*., 2001).

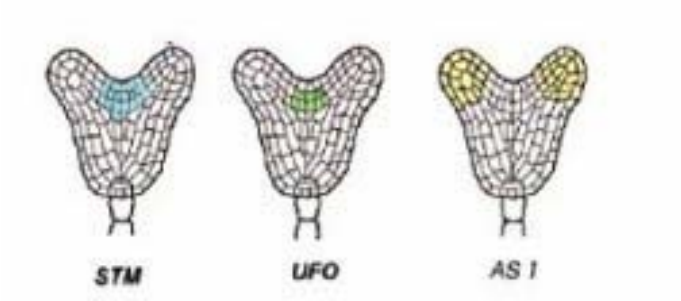

Figure 13. Expression of STM, UFO and AS1 in early development of *Arabidopsis* (figure from Brand *et al*., 2001)

As described in the introduction, promotion of flowering by GI is mainly through regulation of the expression of *CO*, a gene downstream of GI in the photoperiodic pathway (Suárez-López *et al*., 2001; Mizoguchi *et al*., 2005).

This flowering time result corresponds with the data obtained from misexpression of *CO*, revealing that expression of *CO* in the phloem is sufficient to promote flowering (An *et al*, 2004). An *et al* showed that the expression of CO is found in the phloem and shoot apical meristem (SAM); however only expressing CO from the phloem is sufficient to promote (early) flowering.

In the current working-model of flowering-time regulation GI activates *CO* expression, which in turn promotes flowering through activation of FT. Therefore it will be interesting to analyze whether and where *CO* expression is upregulated in the *GI* misexpression lines. Using standard RT-PCR no upregulation was detected in SD grown *SUC2::GI* plants compared to the *gi-3* progenitor (no data shown). Apart from the possibility that *CO* is not upregulated in the misexpression lines, the absence of the

elevated *CO* expression could have some other reasons. Especially in the fusion lines of *SUC2::GI* and *RolC::GI*, production of the *CO* transcript is expected to be elevated to a higher level, as shown for *35S::GI* (Mizoguchi *et al*., 2005). *GI* expression might results in expression of *CO* in the phloem which is a relatively small number of cells preventing easy detection.

Therefore the sensitivity might not be enough in this experiment, suggesting the use of more sensitive methods such as real-time RT-PCR. Also it might be interesting to cross these lines to a CO-reporter line in order to visualize the *CO* expression using for instance GFP or GUS markers. Apart from testing the *CO* expression levels, testing another factor in the flowering pathway such as *FT* might give insight into the way GI promotes flowering from the phloem. This could be useful if GI also promotes *FT* expression independently of CO, as was suggested by the earlier flowering *35S::GI co-2* plants compared to *co-2*. Also an RT-PCR on *CO* might be performed on the main vein of leaves by cutting this out of adult leaves. Such an experiment would increase the proportion of phloem cells in the sample and thereby possibly make it easier to detect *CO* expression in these samples.

Rescue of the elongated hypocotyl phenotype of *gi-3* by expression of *GI* from the *AS1* promoter indicates the spatial requirement for *GI* expression in the primordia of leaves. This may be explained by light being perceived in leaves and cotelydons and controlling hypocotyl length indirectly. As *AS1::GI* probably causes the expression of *GI* in these cotelydons and because *GI::GUS* analysis shows the expression of *GI* in cotyledons and young leaves this suggests that GI controls hypocotyl length from cotyledons in wild-type plants. One could imagine that in wildtype seedlings GI protein enables red light signalling in the cotelydons and thereby facilitates normal hypocotyl development.

The promoters used in this analysis were collected by Dr. Hailong An to broadly cover most parts of the plants (An *et al*., 2004). Of each area studied two promoters were included in order to have internal controls and to cover the possibility that one promoter may also be correctly expressed. Expression studies revealed that the *4CL2* promoter

might not be fully functional, however did not result in irregularities as expressing *GI* from the *4CL1* promoter does not seem to rescue the phenotypes of *gi-3* tested.

An interesting region that has not been included in this study is the region between the upper and lower epidermis of the leaves. A specific promoter for mesophyll cells might be interesting as these cells specifically act in the harvesting of light for photosynthesis. One could imagine that perception of daylength occurs mainly in these cells. Maybe GI could act in these cells to activate *CO* expression in the phloem companion cells. This region has however not been included because no clear promoter was available that was specific to these cells. The analysis of such a promoter might still be of interest as in early leaf development *GI* seems to be expressed throughout these leaves.

The *STM::GI*, *UFO::GI* and *ML1::GI* fusions should be transformed again into *gi-3*; as too few T1 plants were obtained for these constructs, respectively 5, 1 and 1. This is in contrast to the numerous transformants for the other constructs transformed with the same procedures. Unfortunately this might have resulted in an incomplete insight into the effects of misexpression of *GI*. Independent transformations have been performed which resulted in none or few T1 plants. The small number of transformants might be the result of the developmental state the *gi-3* plants, particulary in the first round of transformation; however, in the 2 succeeding attempts the plants used looked healthy and transformation was performed under several developmental stages. Somehow expression of *GI* from these promoters might prevent T1 plants be obtained. One could imagine that high expression of *GI* might negatively influence early developmental processes. In the selection process of T1 plants, densely sown seedlings were sprayed with BASTA at the point of full expansion of the cotyledons. So no notice was given to early development of these seedlings. The small number of insertion lines from these constructs seems illusive as *35S::GI* does yield transformants although the expression is constitutive.

Unfortunately the hypocotyl assay did not show clear difference in length between *gi-3* and wildtype. Although clearly *AS1:GI* results in shortened hypocotyls just like *35S::GI* does, discrimination between wildtype and the gi-mutant would enable identification of

other promoters that might restore hypocotyl length to wild-type level. This is in this assay impossible as significant length differences have not been seen between wildtype, *gi-3* and the other misexpression lines. For more detailed study of this process an alternative *gi* mutant might be better as, for instance, *gi-1* shows a better discrimination between wild-type Columbia and mutant (chapter 2).

To improve the hypocotyl experiment the hypocotyl elongation might be documented throughout the day. Hypocotyl elongation is one of the developmental processes under the control of the circadian clock and therefore the growth rate of the hypocotyl varies throughout the day. As GI possibly influences this clock output, the various lines tested might show variable times of growth. One could imagine that inhibition of hypocotyl elongation in the *gi-3* mutant is delayed and therefore interferes with the measurements.

4. Identification of mutations that suppress the early-flowering phenotype of *lhy-11cca1-1* mutants

A central aim of this thesis is to further elucidate the function of GI in plant development and particularly in flowering-time regulation. Here I have taken a classical genetic approach to isolate genes related in function to *GI*. The connective role GI plays between the circadian clock and the output pathway of flowering is not understood. One way to approach this is to identify additional proteins that play a role in this process. If the functions of these proteins are characterized then they might give some insight into the possible function of GI in flowering-time control.

As an appropriate starting point we chose the double mutant *lhy-11cca1-1*. Both *LHY* and *CCA1* repress *GI* expression and *GI* promotes the expression of these genes (Mizoguchi *et al*., 2002; Mizoguchi *et al*.,2005). Additionally this double mutant shows disrupted circadian rhythms, including the expression of *GI* which is shifted towards an earlier phase. Indicative of the role GI plays between the clock and the long-day pathway, is the observation that *lhy-11cca1-1gi-3* plants flower at a similar time to *gi-3*. This result suggests that *lhy-11cca1-1* plants flower early due to misregulation of GI, and identifying other suppressors *of lhy-11cca1-1* early flowering could identify genes needed for GI function.

4.1. Analysis of *CCR2* expression in the *lhy-11cca1-1gi-3* triple mutant

To further illustrate the role GI plays in the double mutant *lhy-11cca1-1*, the effect of GI on the output of the circadian clock has been studied. In the early flowering mutant *lhy11cca1-1*, the peak of expression of circadian controlled genes is shifted approximately 6 hours earlier (Mizoguchi *et al*., 2002). The triple mutant *lhy11cca1-1 gi-3* is late flowering; therefore we tested whether, apart from flowering-time, the expression rhythm of circadian clock controlled genes is also affected by this mutation.

Every 4 hours for 24 hours wild-type, *lhy11cca1-1* and *lhy11cca1-1 gi-3* seedlings were harvested under SDs. Total mRNA from these samples was extracted, blotted and subsequently hybridised with a specific probe to the circadian controlled gene *CCR2*. The results obtained suggest that the peak of expression of *CCR2* in the triple mutant occurs 4 hours earlier than in wild-type seedlings, and seems to be approximately 2 hours later than in the *lhy11cca1-1* double mutant (see figure 14). Therefore, addition of the *gi-3* mutation slightly delays the peak of expression of *CCR2*, but the expression rhythm is not reverted to wild-type. To confirm this observation, the analysis needs to be performed at a higher time resolution and is being continued by Dr. F. Cremer using *CCR2::LUC* to analyse the expression of this circadian controlled gene in the double and triple background and under different conditions.

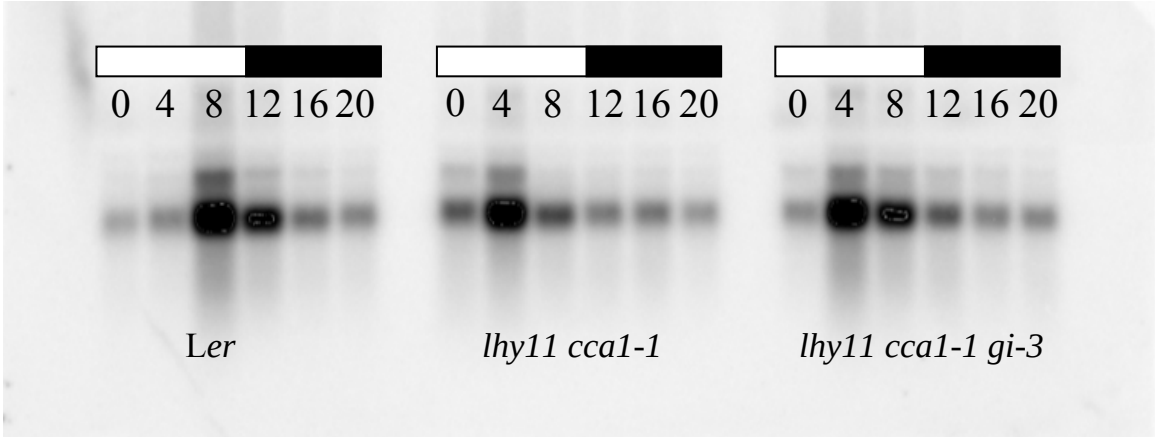

Figure 14. Expression of *CCR2* mRNA in La-er, *lhy11cca1-1* and *lhy11cca1-1 gi-3* lines. Ten day old seedlings were harvested every 4 hours starting at dawn (ZT=0). Total mRNA was extracted, blotted and hybridised with a *CCR2* specific probe

4.2. Characterizing the *suppressor of lhy11cca1-1* (*slc*) lines

In order to isolate genes that are related to *GI* in function, late-flowering mutants were screened for, in the background of *lhy-11cca1-1*. An EMS mutagenised collection of

seeds derived from the *lhy11cca1-1* double mutant were screened. This mutagenesis was started by Dr. T. Mizoguchi, who subsequently screened the collection for individuals that flower later than the double mutant. In order to obtain the highest discrimination this screen was performed under SD conditions. The individuals that flowered later than the *lhy-11cca1-1* progenitor were called *suppressor of lhy11cca1-1* (*SLC*) and are predicted to carry mutations in genes related to *GI* function or other genes of the photoperiodic pathway. Those mutants with a flowering time later than the progenitor will be referred to as late flowering, although they may not be later flowering than wild-type plants.

After the first round of selection for late flowering M2 plants performed by Dr. T. Mizoguchi, my project started by characterising the M3 generation of the SLC lines that derived from this screen. Important first step was to determine whether contaminant plant material was present. This was tested by confirming the homozygousity of the *lhy11* and *cca1-1* mutations. All mutant lines were confirmed to be homozygous for hygromycin resistance (carried by the *Ds* element in the *lhy11* allele) and kanamycin resistance (carried by the T-DNA in the *cca1-1* allele). Some lines however were shown not to be viable or to have a severely reduced viability, bringing down the number of lines to 20 (data not shown).

Additionally *FLC* expression tested in the mutants. High *FLC* mRNA levels cause late flowering, through for example mutations of the autonomous pathway. I wished to exclude these and to concentrate on the photoperiod pathway. Therefore the M3 progeny was analysed for normal *FLC*-expression to exclude mutations in the autonomous pathway. The *FLC* expression was tested by northern analysis of mRNA isolated from 10 day old seedlings. This experiment revealed that SLC lines 13, 16, 17 and 24 show clear elevated expression of *FLC* compared to the parental line *lhy11cca1-1* (figure 15); therefore these lines were excluded. *lhy11cca1-1fca* was also used as a control for the effect of an autonomous pathway mutant upon *lhy11cca1-1*. The *lhy11cca1-1fca* triple mutant was late flowering and showed elevated *FLC* expression. Note that the *gi-3* mutation did not have an effect upon the *FLC* expression, as expected and suggesting that mutations with related effects would also not affect FLC mmRNA levels.

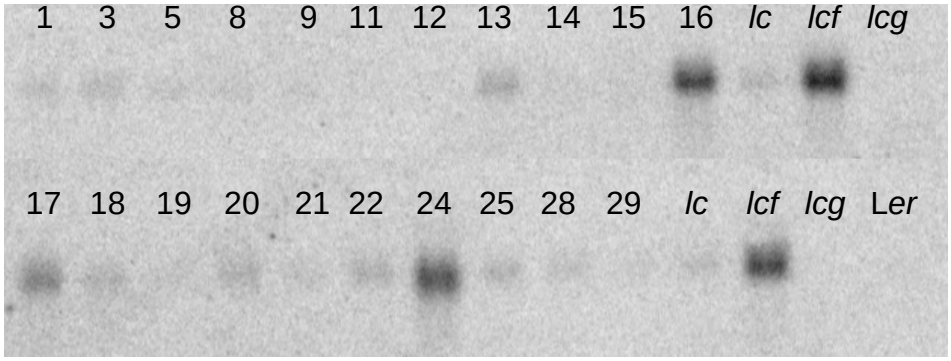

Figure 15. *FLC* expression in 10 day old seedlings of the *slc* line. Numbers indicate the *slc*-line number. *lc= lhy-1cca1-1*, *lcf= lhy-1cca1-1fca*, *lcg= lhy-1cca1-1gi-3*, L*er*=Landsberg *erecta*

In order to measure the flowering time of these lines precisely, they were scored under 10 hr SDs. This experimental demonstrated that certain lines (*slc1*, *2*, *8*, *9*, *11* and *21*) could not be clearly confirmed as late flowering when compared to the parental line *lhy11cca1-1* (figure 16a). This may be due to the use of slightly different light conditions used here, compared to previous experiments. Especially the lines carrying the lowest isolation numbers were not later flowering than *lhy-11cca1-1*, suggesting that these were the first lines to be picked in the screen. The lines with the highest isolation numbers were confirmed to flower later than the *lhy-11cca1-1* progenitor (figure 16b.). Additionally, *slc25* was dwarfed and excluded from further analysis. The few lines (*slc12*, *14*, *15* and *20*) that segregated for flowering time (data not shown) were also excluded, because the M2 was expected to be homozygous for recessive mutations and thus should yield homozygous M3 lines. The late-flowering individuals from the 3 segregating lines were taken to the M4 to analyse flowering time again however no late flowering under SD could be observed and these lines were not continued with.

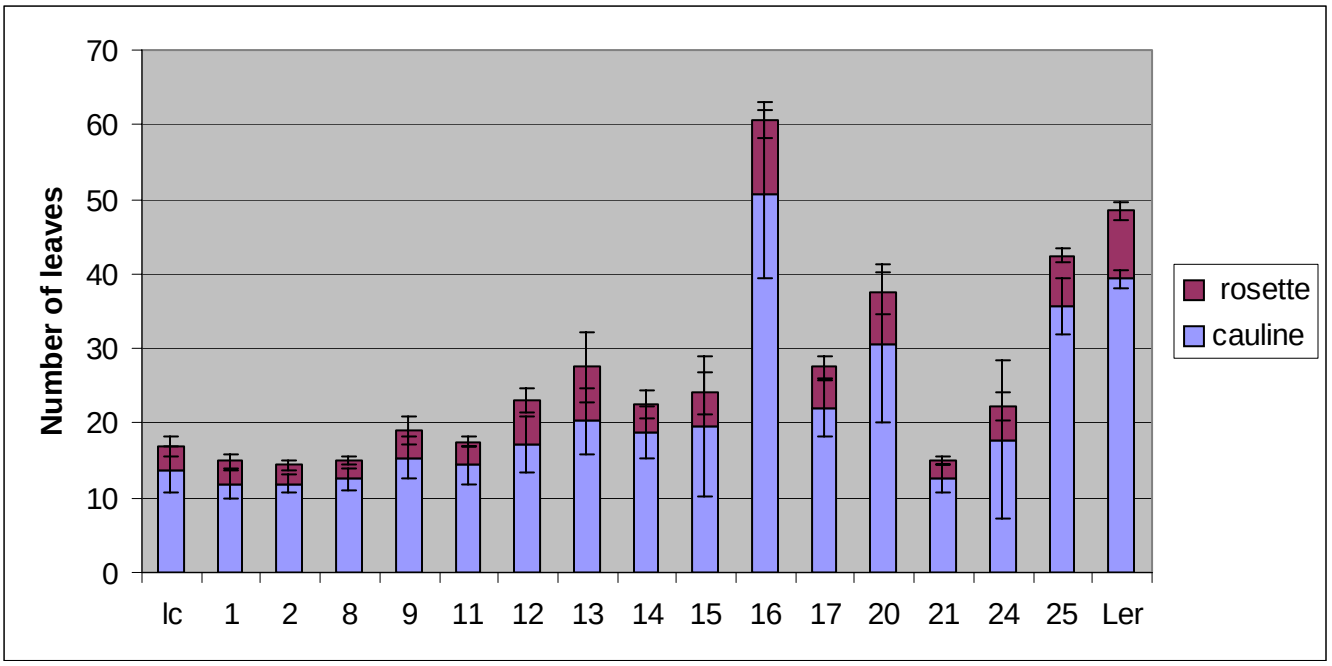

Figure 16a. Flowering-time of excluded *slc* lines in SD conditions. lc = lhy-11cca1-1, numbers correspond to the number of the slc mutant. These lines were excluded because of high levels of FLC mRNA, a weak flowering-time phenotype or segregation of the phenotype.

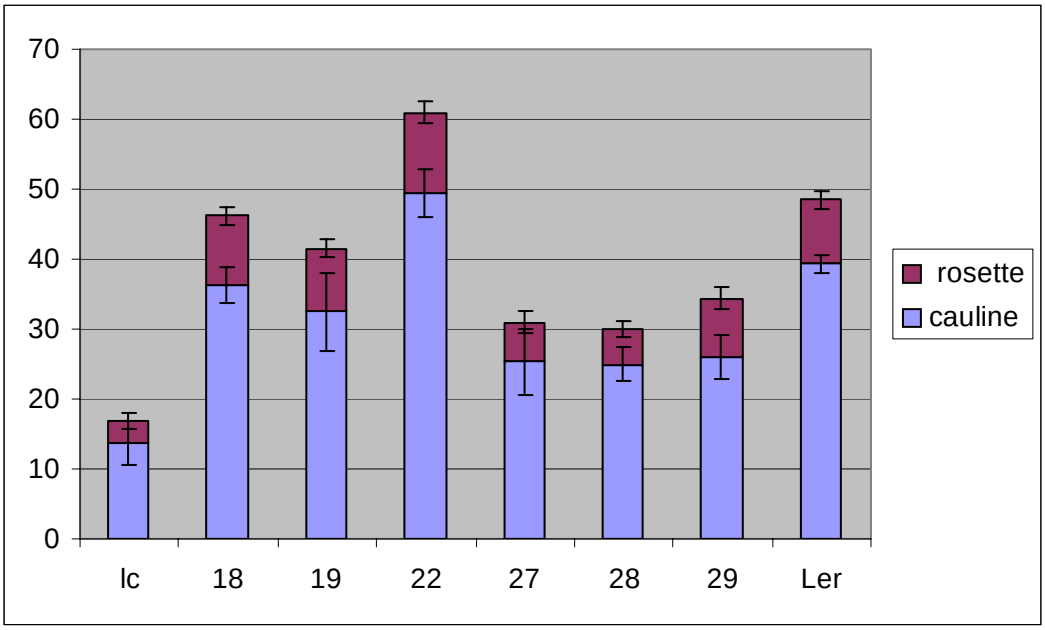

Figure 16b. Flowering-time of *slc* lines in SD conditions. lc = lhy-11cca1-1, numbers correspond to the number of the slc mutant.

4.3. Bulked Segregant Analysis (BSA) of the *slc* lines

The number of SLC-lines was reduced to 6 by the initial characterization of excluding lines that show high *FLC* expression, show no confirmed late flowering time phenotype, exhibit strange segregation or are unviable. The remaining lines were were studied further to attempt to locate the mutations. The mapping strategy that was deployed started with rough mapping using Bulked Segregant Analysis (BSA) (Michelmore *et al*, 1991). BSA involves creating a pool of a small number of mutant phenotype individuals from an F2 hybrid population and compairing these with a pool pf wild-type phenotype plants. Using such a small bulk of individuals can reveal a rough mapping position deploying a small number of markers: about 5 to 6 markers per chromosome. For more exact positioning, I used 35 markers in total to provide a more precise location of the mutations but still keep the total number of PCR reactions relatively low (see table 5). These markers were kindly provided by Dr. I. Searle.

Table 5. Markers per chromosome. Sequences of the primers appear in the materials and methods.

Chromosome I	Chromosome II	Chromosome III	Chromosome IV	Chromosome V
Nga63	F18P14	Nga172	Ciw5	MOJB
F7K19	Ciw3	Nga162	T26M18	Nga151a
F12K8	F26B6	Ciw11a	FCAALL	Ciw8a
Ciw1	F4P9	MZN14	F26K10	Nga76
Nga280	T2H17	T32N15	F6E21	Ciw9
NF5I14a		Ciw4	T16L1	MQB2
AthATPase		F27K19	F23E13	Nga106
		Nga6	T9A14	

In order to deploy the SSLP and CAPS markers the mutant lines were crossed to another *Arabidopsis* accession. Crossing two accessions results in a random distribution of both

genomes in the F2 generation. As the mutant originates from L*er*, plants that show the desired phenotype must carry L*er* DNA at the site of the mutation. As typically mutations are recessive, the mutation should be localized where all late-flowering F2 individuals are homozygous for L*er*. The rest of the chromosome should behave randomly when a second mutation influencing the phenotype studied is absent.

In this study the *slc* mutants were generated from the *lhy-1cca1-1* parental line with Landsberg *erecta* (Ler) accession background. Therefore another accession, Columbia (Col) was chosen for crossing. In order to avoid segregation of the *lhy-1* and *cca1-1* alleles, these alleles in the mapping population were incorporated into the Columbia background. This was done by crossing the *lhy-1cca1-1* (L*er*) parental line 5 times back to Columbia to remove most of the Landsberg background, resulting in the BC5F3 line. This line was chosen for the mapping cross because a large number of markers have been optimised for use in L*er*-Col and Col is the accession whose whole genome was most thoroughly sequenced. Resistance to both kanamycin and hygromycin of the *lhy-1cca1-1* BC5F3 line confirmed the presence of *lhy-11* and *cca1-1*.

The F1 collection was obtained by using the SLC lines as females and the BC5F3 *lhy-1cca1-1* (Col) as pollen donor. This was done to confirm that the cross was successful; because most mutations are be recessive and therefore F1 plants should be early flowering, similar to *lhy-1cca1-1* BC5F3 in SD. In case the cross failed and the seeds are due to self-fertilization, they should exhibit a phenotype similar to the female parent and be late flowering under SD conditions. Unfortunately, it was impossible to cross *slc19* even in the reciprocal cross. In order to determine the effect of the hybrid upon flowering time, a control cross of the parental L*er* to Columbia was also made. All F1 plants, from both mutant and control crosses, were analysed and as expected showed early flowering and a Columbia-type phenotype. Seeds of all F1 plants were harvested independently.

The F2 generation from an individual F1 plant from each cross was sown out to find late individuals that could be used for the BSA analysis. The size of each population included 150 individuals that were all scored for flowering time. The plants were grouped based

on their number of rosette leaves and compared to the control *lhy-11cca1-1* cross of the BC5F3 Col x Ler. Firstly, this revealed a typical bell shaped curve for the control cross, which is due to the combinations of allelic variation between the two accessions. The latest individuals in this control population flower with 13 leaves (figure 17). Secondly, this experiment revealed that due to the large variation in flowering time, it was not possible to discriminate late-flowering individuals for the mutants *slc19*, *slc22* and *slc29*. This was possibly due to the fact that the flowering time of the F2 populations shows a continuum and therefore no mutant individuals could be definitely identified. It is therefore impossible to discriminate between the late-flowering individuals that were created due to the combination of alleles causing late-flowering from the accessions and the effect of the mutation. In contrast, for *slc22* and *slc29* a small number of late-flowering individuals were found that flowered later that all plants in the cross between accessions, however the number of plants was too small to be used for BSA analysis (figure 17) and therefore mapping was not continued.

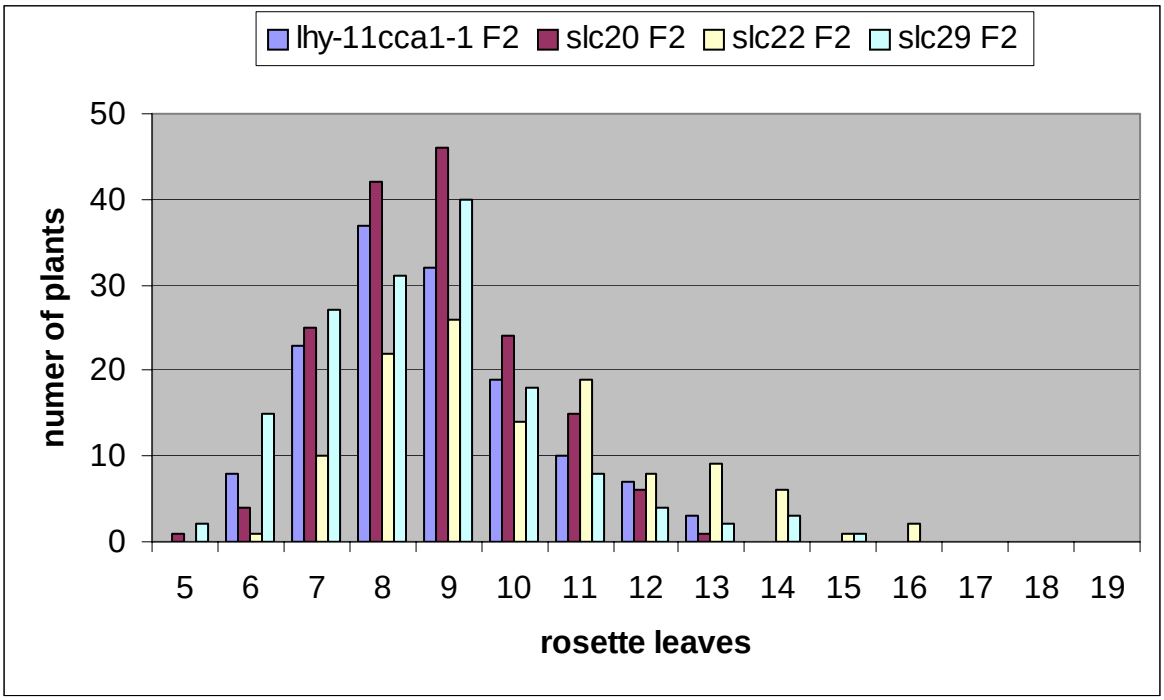

Figure 17. Graphical representation of flowering time in the F2 population of *slc20*, *slc22*, *slc29* and the control *lhy-11cca1-1* crosses.

For three F2 populations, those of *slc18*, *slc27* and *slc28*, a number of plants could clearly be scored as mutant individuals (figure 18) and be used for the BSA analysis. As plants in the control cross flower with up to 13 leaves, those individuals flowering with 15 leaves and more were assumed to be homozygous for the mutation.

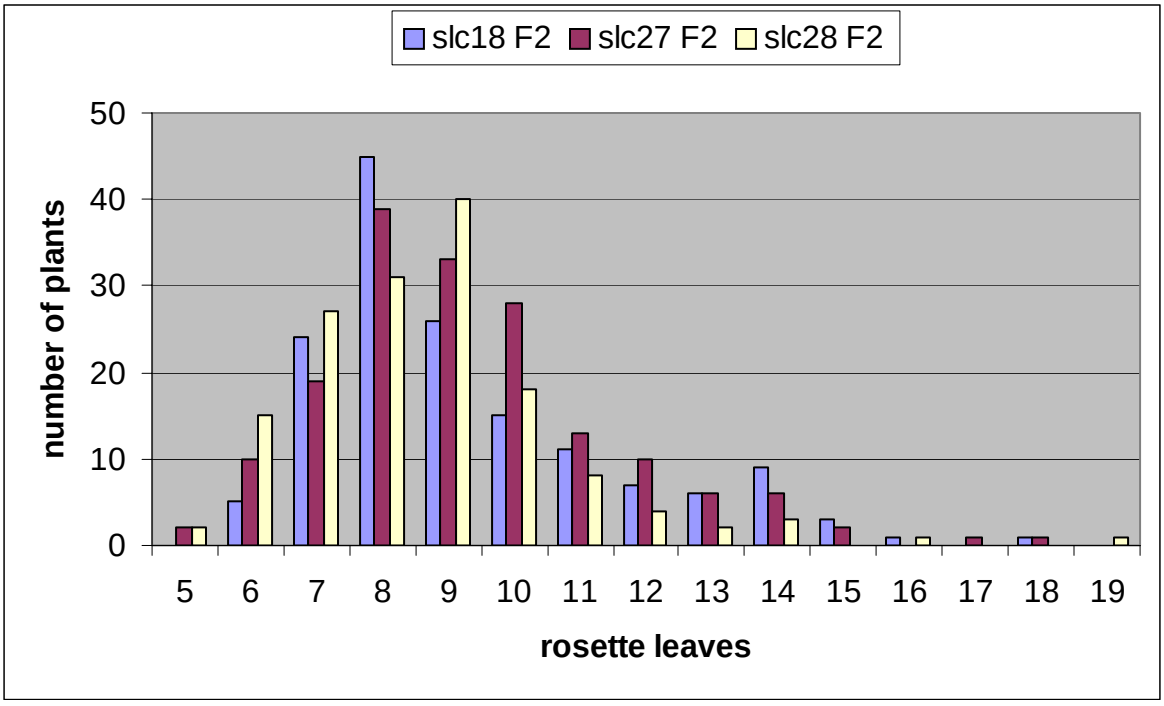

Figure 18. Graphical representation of flowering time in the F2 population of *slc18*, *slc27* and *slc28*.

The late-flowering individuals were used to roughly map the region of the mutation. This was done using the BSA strategy exploiting the polymorphisms between L*er* and Col for 35 co-dominant markers covering the genome. The DNA of six individual late-flowering plants was combined and by PCR analysis the genotype was compared to the genotype of a bulk of 6 individuals with an intermediate flowering time, 8 rosette leaves. This revealed that *slc18* was homozygous L*er* for the markers T32N15, ciw4, F27K19 and nga6. These markers correspond to positions on the lower arm of chromosome 3, below 19Mb from the top of the chromosome. For *slc27* the markers FCAALL and F26K10 were homozygous L*er*, both localized to a region on chromosome 4 between 8 Mb and 14 Mb from the top. For the bulk of *slc28* there were two regions found that gave

homozygous L*er*; the marker F12K8 on chromosome 1 and F26B3 and T2H7 on chromosome 2.

Using the BSA analysis a region was found to be homozygous for L*er*. In the F2 plants derived from each mutant, and these regions are likely to define the intervals in which the mutations causing the phenotypes are located. Importantly, in the bulk plants showing an intermediate flowering time those regions are heterozygous.

Therefore using 6 individuals per bulk, the BSA analysis resulted in the following possible positions of the mutations (see figure 19):

-*slc18* on the lower arm of Chromosome 3

-*slc27* on the middle of Chromosome 4

-*slc28* on the lower arm of Chromosome 1 or 2

These positions were confirmed by using a total of 12 late individuals from the corresponding populations. The individual DNA samples of these 12 plants were all homozygous for these markers.

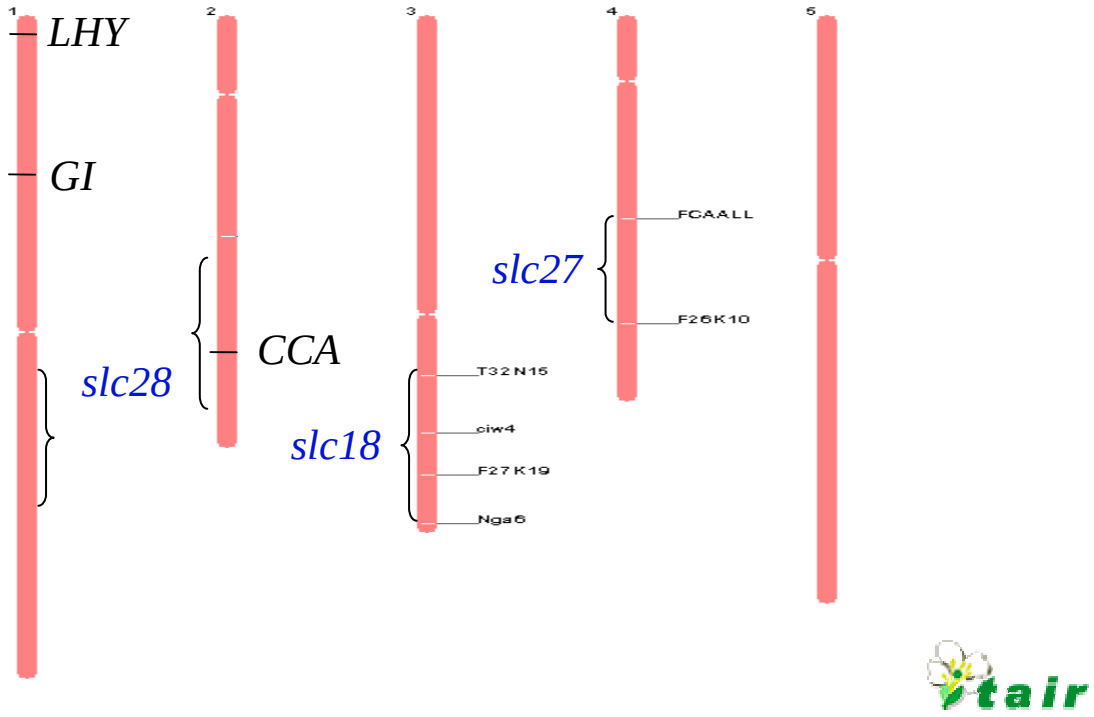

Figure 19. Putative chromosomal positions of slc18, slc27 and slc28 after BSA analysis.

Analysis of the variation in flowering time in the F2 populations also gave insight into the ecotypic variation in flowering time. Unfortunately a large number of mutant individuals could not de recovered from the F2 hybrid population. Therefore the F2 populations made with the *slc18*, *slc27*, *slc28* mutants and the control cross of *lhy-11cca1-1* were sown in SD10, SD8 and SD6 (the numbers here indicate the daily amount of hours of light) to analyse whether shorter daylengths would improve the severity of the late-flowering phenotype caused by the mutations. This experiment revealed that SD8 is the best condition to screen for late-flowering individuals as it gave good separation of early vs. late-flowering plants (data not shown). Although SD6 gave a slightly better separation, this also delayed flowering dramatically and therefore was not practical to work with.

Under SD8 conditions, the segregation of early versus late flowering plants was also analysed and *slc18* showed a more clearly penetrant phenotype than the other 3 mutations. *slc18* F2 population segregated 4:1, which is close to the Mendalian segregation of 3:1 in case of a recessive mutation. The *slc27* and *slc28* populations gave a segregation of respectively 6:1 and 8:1, indicating that a larger population would be needed to identify enough late flowering individuals to map the mutation at high resolution.

4.4. Genetic analysis of the *slc18* mutant

The *slc18* mutants flower later than the parental line *lhy-11cca1-1* (L*er*) and for the BSA mapping the mutation was assumed to be recessive. Back crossing the mutants to the progenitor line would demonstrate the heritability of the mutation in the F1 and F2 generations. In all crossings undertaken here, the mutant was used as female. In the F1, all plants were as early flowering as the *lhy-11cca1-1* control, already indicating the recessive nature of the *slc* mutations. This corresponds with the expectation, as most EMS generated mutations are recessive and thus would phenocopy *lhy-11cca1-1* that is

homozygous for the wild-type *SLC18* allele. Segregation data were obtained for the F2 generation and showed a 1:3 segregation for mutant to wild-type phenotype.
Late-flowering plants were harvested to obtain an *slc18lhy-11cca1-1* line background mutations that might also be generated by the EMS mutagenesis were removed.

Simultaneous to this crossing, the mutants were backcrossed to the L*er* wild-type to segregate the *slc* mutations away from the *lhy-11* and *cca1-1* mutations. This was done by pollinating the *slc* mutants with L*er* pollen and selecting the late-flowering F2 plants under long days. From the 300 F2 plants, about $1/6^{th}$ of these (47) were later flowering than the *L*er wild-type. The offspring of these plants was tested for hygromycin and kanamycin resistance. Resistance to these antibiotics is linked to the *lhy-11* and *cca1-1* mutations, therefore lines were selected in which all seedlings die in the presence of either antibiotic. These 5 lines, 3.1, 3.4, 3.18, 3.20 and 3.25, are likely to be the single *slc18* mutants. These single *slc18* mutant plants give insight into the flowering phenotype caused by the mutation in the presence of functioning LHY and CCA1 proteins.

The crosses of the *slc18* mutant to the *lhy-11cca1-1* line and L*er* were analysed for flowering time under SD and LD conditions. The crosses to the parental line *lhy-11cca1-1* showed a confirmation of the initial phenotype, the mutants were delayed in flowering compared to the parental line *lhy-11cca1-1* under SD conditions. Under LD, the mutants are not later than the lhy-11cca1-1 double mutant (figure 20). The flowering time of plants carrying the single mutation, crossed to the L*er* wild-type and without *lhy-11* and *cca1-1*, was also analysed. This showed that in SD conditions the mutant flowers later than the L*er* wild-type, with approximately 4 rosette leaves (figure 20). In LDs the single mutant flowers similar to that of Ler, or maybe even slightly later (~1 leaf). Therefore, the mutated gene seems to function exclusively in SD conditions, as both single and triple mutants are later flowering than their corresponding controls. Under LD conditions the *slc18* mutation does not seem to affect flowering time.

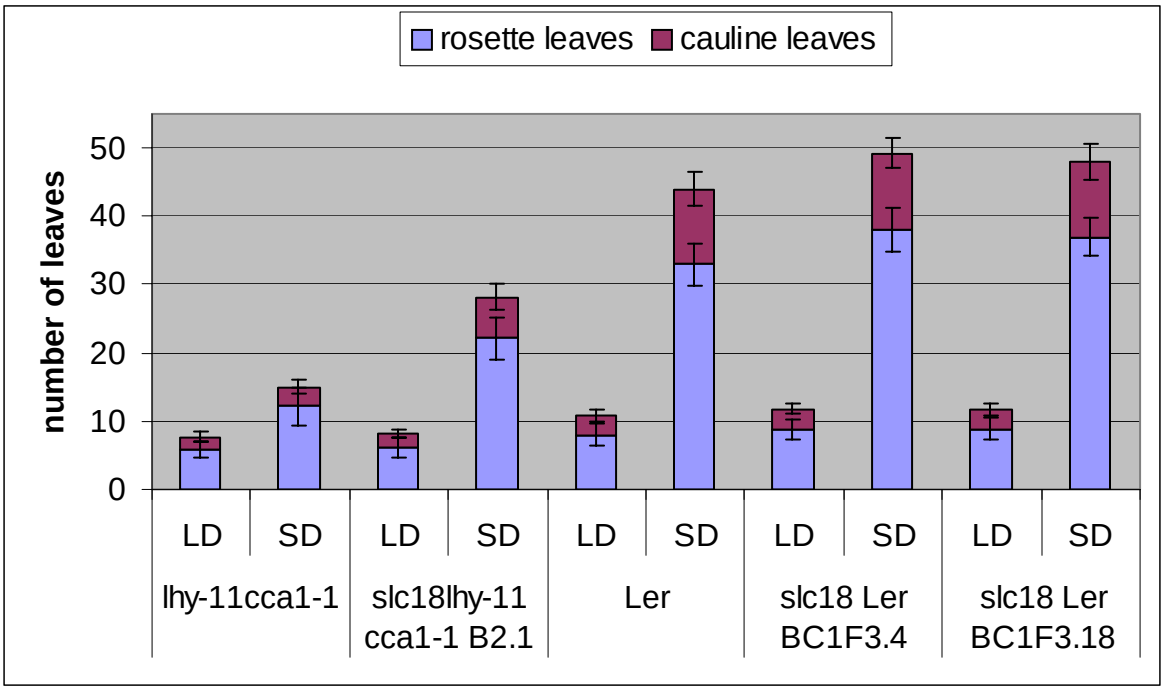

Figure 20. Flowering time of L*er*, the single mutant *slc18*, *lhy-11cca1-1* and the triple mutant *slc18lhy-11cca1-1* in LD and SD conditions of the growbanks.

4.5. *SLC18* mutation was fine mapped to 126kb on lower arm of chromosome 3

For further mapping *slc18* mutation was chosen, because late flowering individuals were relatively easy to identify in the *slc18* F2 hybrid population and also no known genes affecting flowering time are present in this interval.

Two populations were screened for late flowering individuals under SD8. This yielded a total of 1700 late flowering F2 plants. Firstly, a small population of 250 late flowering individuals was used to check for heterozygousity with the markers defining the interval on the lower arm of chromosome 3, T32N15, ciw4, F27K19 and nga6. Individuals that are heterozygous for one marker and homozygous L*er* for the other, are useful to narrow down the region containing the mutation. As the mutation locates to the region that is L*er* in all plants that have the mutation, heterozygous individuals have the potential to define

a smaller region containing the mutation. Analysis this medium size for heterozygous individuals resulted in the identification of 15 plants that were heterozygous for the more inward marker F27K19 (~21 Mb), however no heterozygous individuals were identified with the lower marker nga6 (~23 Mb). With the use of new marker sets, the region was divided into segments of about 1 Mb. Subsequent PCR analysis showed that 4 individuals were found breakpoints were found for the marker Sorb10 which localises to 21.91 Mb on chromosome 3.

These Sorb markers (kindly donated by Dr. K. Göllner) were used to screen the large population of *slc18* F2 hybrids. This yielded new individuals that are heterozygous for these markers. Subsequently the heterozygous individuals were used to narrow down the region containing the mutation. This was done by using several CAPS and SSLP markers placed inwards (see Materials and Methods) taking smaller steps as the region and the number of heterozygous individuals declined. Markers were developed by making use of the polymorphism releases from Cereon (Methods) in which the polymorphisms between the L*er* and Col ecotypes are listed. This list is free to use for non-commercial institutions and was used to develop markers in the region of interest. This procedure resulted in identification of an interval of 126 kb between 22,203 and 22,329 MB on chromosome 3 (figure 21). This region contains one breakpoint between marker T2O9Nde1 and T2O9Cla1 from the individual plant EY311. On the other side the breakpoint lies between the markers T8B10Smi1 and F27H5Alu1 from the individual plant EC30. No other markers and plants were found to be heterozygous in this region. Therefore the crossovers in these plants define the boundaries of the region in which the mutation lies. The late-flowering phenotype of the individuals listed in figure 19 has been confirmed in the F3 generation; in which all F3 plants were late-flowering in SDs. The region between markers T2O9Nde1 and T8B10Smi1 contains 30 annotated genes according to the sequence information from www.arabidopsis.com (table 5). Additional markers cannot be developed, as no polymorphisms exist between the outer and inner markers.

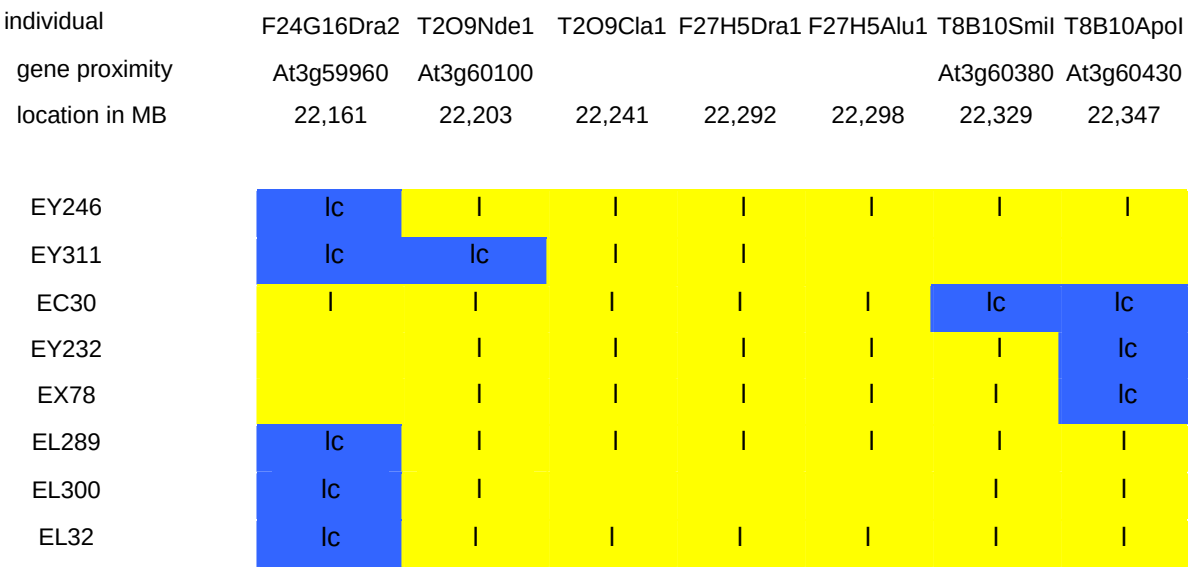

Figure 21. Individual *slc18* F2 hybrid plants were genotyped for markers on chromosome 3. The blue boxes indicate that the marker is scored as heterozygous (L*er*/Col = lc), yellow boxes indicate that the marker is scored as homozygous L*er* (l).

Table 5. Overview of annotations between marker T2O9Nde1 and T8B10Smi1

Chromosome 3: 22203000 to 22328998 nt.
Includes annotation units T2O9 F27H5 T8B10
Total genes in region: 30 (loci in this region: 35)

Location (Mb)	Locus	Gene Model: Description
22,204	AT3G60100	citrate synthase
22,208	AT3G60110	DNA-binding bromodomain-containing protein
22,217	AT3G60120	glycosyl hydrolase family 1 protein
22,221	AT3G60130	glycosyl hydrolase family 1 protein / beta-glucosidase, putative (YLS1),

22,227	AT3G60140	DIN2:in similar to beta-glucosidase and member of glycoside hydrolase family 1.
22,233	AT3G60150	similar to expressed protein [Arabidopsis thaliana] (TAIR:At2g44525.1)
22,234	AT3G60160	ATMRP9: MRP subfamily; ABC transporter family protein
22,243	AT3G60170	copia-like retrotransposon family
22,253	AT3G60180	uridylate kinase, putative uridine monophosphate kinase, putative UMP kinase
22,255	AT3G60190	ADL4:dynamin-like protein 4 (ADL4)
22,26	AT3G60200	expressed protein, hypothetical proteins
22,262	AT3G60210	chloroplast chaperonin 10, putative
22,265	AT3G60220	ATL4:RING-H2 zinc finger protein ATL4 (C3HC4-type RING finger) family protein
22,272	AT3G60230	expressed protein
22,274	AT3G60240	EIF4G:protein synthesis initiation factor 4G (EIF4G)
22,279	AT3G60245	60S ribosomal protein L37a (RPL37aC)
22,281	AT3G60250	CKB3:Regulatory (beta) subunit of the protein kinase CK2
22,284	AT3G60260	phagocytosis and cell motility protein ELMO1-related
22,289	AT3G60270	uclacyanin, putative, similar to uclacyanin 3 GI:3395770 from (Arabidopsis thaliana)
22,29	AT3G60280	UCC3 (uclacyanin 3):blue copper-binding protein III
22,293	AT3G60290	similar to oxidoreductase, 2OG-Fe(II) oxygenase family protein
22,296	AT3G60300	RWD domain-containing protein, contains weak similarity to RING finger protein 25

22,298 AT3G60310 expressed protein

22,302 AT3G60320 expressed protein

22,309 AT3G60330 ATPase, putative proton pump, similar to P-type H(+)-transporting ATPase

22,315 AT3G60340 palmitoyl protein thioesterase family protein

22,317 AT3G60350 armadillo/beta-catenin repeat family protein / F-box family protein

22,323 AT3G60360 expressed protein

22,325 AT3G60370 immunophilin / FKBP-type peptidyl-prolyl cis-trans isomerase family protein

22,327 AT3G60380 expressed protein

4.6. Alternative approach to define position of *slc18* mutation

During the process of positional cloning of *slc18*, the mutation was defined in a region of 0.25 MB and we chose to deploy a complementation approach to help identify the gene. Introducing a transgene containing the correct version of the mutated sequence would be expected to rescue the mutant phenotype and thereby define the position of the mutation. I carried this out by transforming the mutant with Bacterial Artificial Chromosomes (BACs) that overlap each other and together span this region. A combination of several transformable JAtY BACs available from Gene Transfer Clone Identification and Distribution Service (GeTCID) cover the region (figure 22). In case one or several overlapping BACs rescue the phenotype, this will confirm the mutation is present in this region and define the region to the position of the BAC or a smaller region contained in overlapping region of the BACs. As the BACs are about 60-80kb in length and overlap in several places; this would narrow down the region containing the mutation to about 20kb.

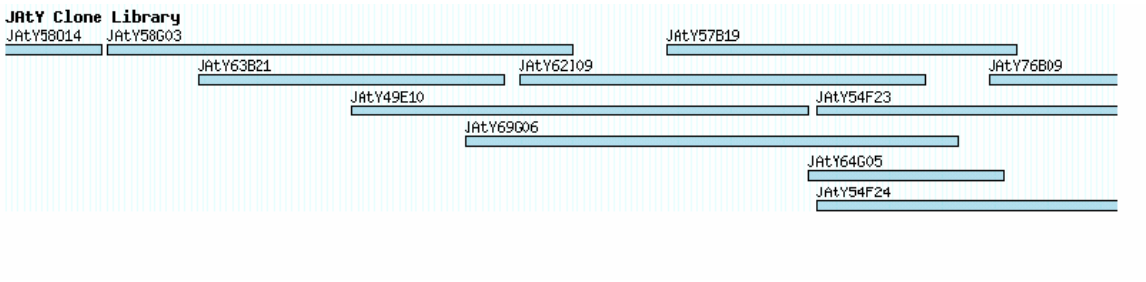

Figure 22. Distribution of JAtY BACs between 22,161 and 22,347Mb on the lower arm of Chromosome 3

E. coli containing the desired JAtY clones were ordered from the GetCID (methods) and subsequently plated on LB-kan agar. By testing the colonies with six PCR markers in the region, the *Arabidopsis* DNA in each JatY was confirmed (see table 6). DNA was extracted, cleaned and transformed in *Agrobacterium* strain C58. For transformation, the *slc18* mutant was dipped with *Agrobacterium* containing the 10 selected JAtY clones (see table 6) that together span the region of interest. The double mutant *lhy-11cca1-1* and wild-type L*er* were also dipped as control. Selection of T1 plants by BASTA was done under SD conditions and simultaneously tested for flowering time. Unfortunately no transformants were isolated for *slc18* and *lhy-11cca1-1*; and only a few transformants were isolated from the control L*er*.

Table 6. Confirmation of position of the JAtY BACs. PCR amplification was done with the positional markers distributed over the region. The X indicates the presence of a PCR product.

JAtY		F24G16Dra2	T2O9Nde1	T2O9Cla1	F27H5Dra1	F27H5Alu1	T8B10Apo1
Position		22,161Mb	22,203Mb	22,241Mb	22,292Mb	22,298Mb	22,347Mb
1	58O14	X					
2	58G03		X	X			
3	63B21		X	X			
4	49E10			X	X		
5	69G06			X	X	X	
6	58F01			X	X	X	
7	67B14				X	X	
8	77C08						
9	62I09				X	X	
10	62J16				X	X	
11	57B19				X	X	
12	64G05					X	
13	54F23					X	X
14	54F24					X	X
15	76B09						X

Transformation of *Arabidopsis* with BACs can occur at low frequency, and therefore I used a high number of plants in this approach. A total of 20 pots were dipped with Agrobacterium containing each construct, each pot contained 6 plants and therefore a total number of 1200 *slc18* plants did not yield a single transformant.

In addition to this approach we chose to cross the *slc18* single mutant in the wild-type background with two Near Isogenic Lines (NILs) obtained from Prof. Dr. M. Koornneef. This collection of NILs consists of several lines with L*er* as genetic background apart from a small portion that is Cvi. As the genome is largely L*er*, crossing this to a mutant in

the L*er* background would exclude variations in the phenotype of the cross due to allelic variation other than the mutations. One could use these lines to confirm or further narrow down the region, as the phenotype can be more precisely scored. We used these NIL lines, which contain Cvi on the lower arm of chromosome 3, to confirm the location of the mutation. NIL176 (176-46-86-11-1, descent of CvL 176) is scored for Cvi at 20,7Mb and 22,9Mb and NIL8 (8-73-25, descent of CvL 8) is Cvi around 22,9 Mb. The *slc18* single mutant was crossed with these NILs and F1 plants were wild-type flowering as expected. In case of correct localisation of the mutation to the lower arm of chromosome 3, all late flowering individuals in the F2 generation under SD should be homozygous for L*er* and the other individuals should be heterozygous L*er*/Cvi or homozygous for Cvi. This material is available for testing with the markers used for the mapping.

4.7. Sequence comparison between *slc18* and *lhy-11cca1-1*

As can be seen in table 5, there are 30 candidate genes for *slc18* in the interval. One approach to identify the gene is to amplify each of the genes. In the region and compare the amplified sequence from *slc18* with the control sequence amplified from *lhy-11cca1-1*. During the process of positioning the mutation, the sequence of two genes was analysed. The first sequence analysed was the ORF of *CASEIN KINASE II BETA –3 CHAIN* (*CKB3*) because this protein is reported to interact with CCA1 (Daniel *et al*., 2004) and the gene is located near the middle of the defined region. Additionally the ORF of *SQUAMOSA-PROMOTER-BINDING-PROTEIN-LIKE12 (SPL12*) was sequenced as SPL12 is a homologue of SPL8 which functions as a local regulator of GA-dependant developmental processes (Zhang *et al*., 2006). In both cases the ORF was amplified from genomic DNA from *slc18* and *lhy-11cca1-1*, after which the sequences were compared. This analysis did not reveal any mutation, thereby excluding these genes as *SLC18*. More precise mapping also excluded *SPL12*.

Additional sequence comparison is thus needed to identify the mutated gene that causes the phenotype. Due to the fact that there are still 30 genes are located in 126kb (see table

5); sequence comparison will start with the ORF of the most likely candidates in the middle of the region.

4.8. Analysis of *GI, CO* and *FT* expression in the *slc18* mutant

Although the responsible mutation has not been identified yet, analysing the expression of genes from the photoperiodic pathway might give insight into the function of the gene that is affected. Therefore the expression patterns of the mRNAs of *GI*, *CO* and *FT* were studied over a short day. This was done by growing the backcrossed triple *slc18lhy-11cca1-1* mutant in a SD cabinet together with L*er* and *lhy-11cca1-1*. During the day 10 after sowing, seedlings were collected every 4 hours. mRNA was isolated from these samples and using RT-PCR the expression of *GI*, *CO* and *FT* mRNA was studied. As reported by Mizoguchi *et al*, 2005, the expression of *GI*, *CO* and *FT* is altered in *lhy-11cca1-1* compared to wild-type (figure 23). It is suggested that the alteration of FT expression, due to early expression of *GI* and *CO*, causes the early flowering of the *lhy-11cca1-1* double mutant. As shown in this chapter the delay in flowering of the *lhy-11cca1-1gi-3* triple mutant could be the result of the delay in *GI* expression compared to the lhy-11cca1-1 double. Therefore one could imagine that in the *slc18* mutant, which is also delayed in flowering, a similar change could also have taken place. The analysis of the mRNA of *GI*, *CO* and *FT* however does not show a change in *FT* expression (figure 23).

Both a decrease or delay in expression of *GI*, *CO* and *FT* could lead to a delay in flowering, however not seen in the *slc18* mutant. This result suggests that *SLC18* acts downstream of *FT* transcription.

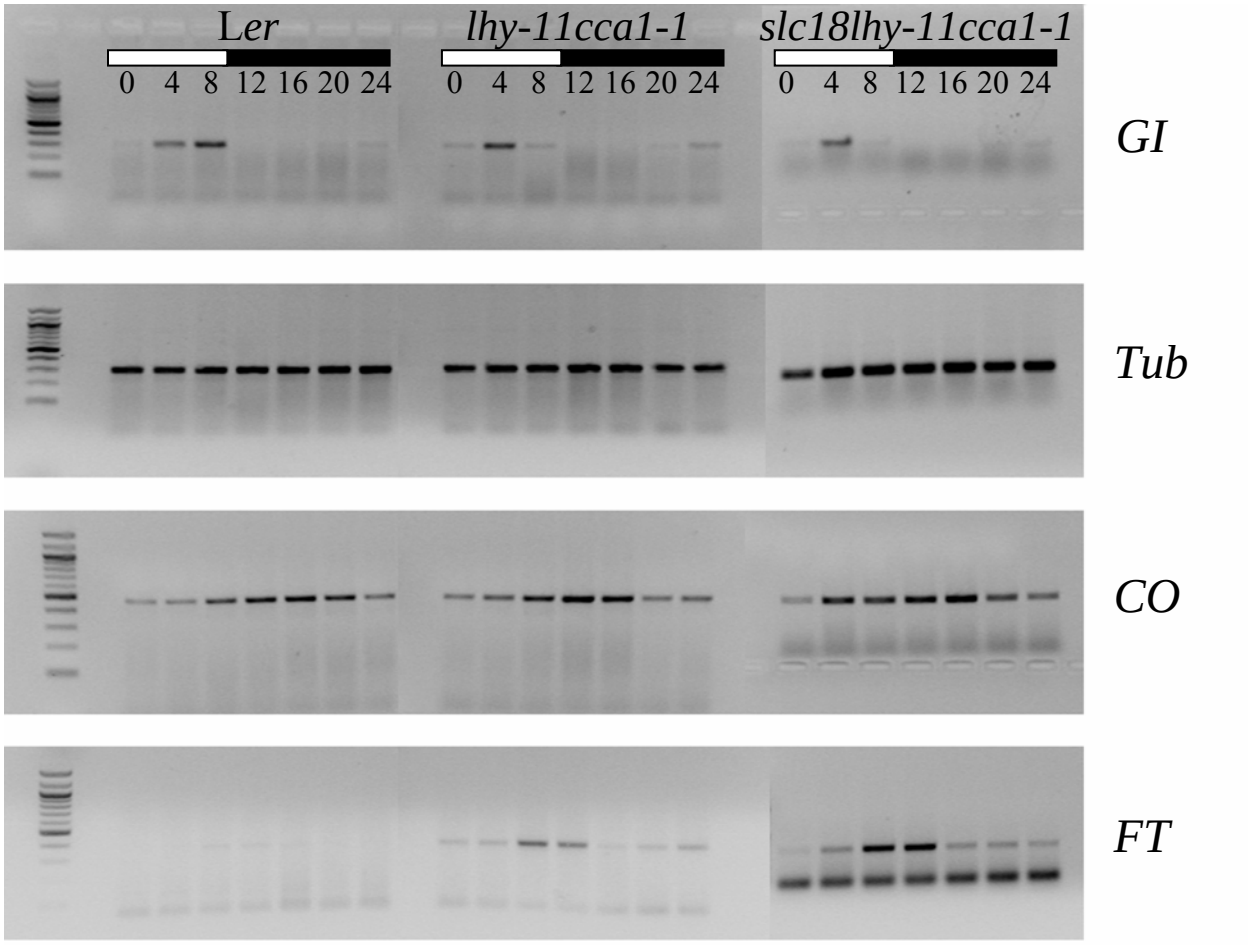

Figure 23. Analysis of *GI*, *CO* and *FT* mRNA in the L*er* wild-type, *lhy-11cca1-1* and *slc18lhy-11cca1-1*. The expression of *GI*, *CO* and *FT* seems not to be affected by the *slc18* mutation.

4.9. Discussion

To further elucidate the function of GI in flowering-time regulation of Arabidopsis, I used a genetic approach to screen for mutations with an effect related to the function to GI. An EMS mutagenised collection of seeds derived from the *lhy11cca1-1* double mutant was used. After excluding a large number of the *suppressors of lhy11cca1-1* based on molecular and phenotypic criteria, *slc18*, *slc28* and *slc29* were continued for mapping. F2 hybrid populations were created and a preliminary map position was identified. *slc18* was the most promising due to its clear and penetrant mutant phenotype and because of the absence of genes known to regulate flowering-time in the interval to which it was mapped. Using a population of 1700 F2 plants, *slc18* was localized to a smaller region on chromosome 3 between markers T2O9Nde1 and T8B10Smi1. This region contains 30 annotated genes. The DNA sequence of two genes, CKB3 and SPL12, were analysed, but did not contain a mutation in *slc18*. The putative tertiary structure of the N-terminal region of GI seems to show similarities to helices of ß-glucanases (pers. comm. F. Cremer), therefore the ß-glucosidase located in this region may be a good candidate for sequence analysis between *slc18* and the wild-type. The mutation responsible for the suppression of the early flowering of the *lhy11cca1-1* double mutant will be identified by sequence comparison. The *SLC18* gene must encode a novel protein specifically active to promote flowering in short-day conditions.

After isolation of the *SLC18* gene, obtaining an overexpressor line of *SLC18* and a loss-of-function allele will allow the effect of SLC18 on flowering-time to be tested more thoroughly. Identifying the gene will also enable us to analyse the diurnal expression of *SLC18* in SD and LD conditions. Together with the possible known features of the protein or its homologues this would she more light on the promotion of flowering in short days.

Using large populations of F2 plants, it should also be possible to map at higher resolution the *slc28* and *slc29* mutations. This would probably require analysing F2 populations of up to 10.000 plants. Possibly, population size could be reduced by

screening in conditions shorter than 8 hrs of light per day as this would allow identification of more mutant plants. Such a condition might also be used to try isolating more late-flowering individuals for the mutant lines *slc22* and *slc29*, as already some late-flowering plants could be identified in SD8. For *slc19* such an approach would not be succesful as none late-flowering individuals were found in SD8 conditions. The drawback of shorter conditions is that this would elongate the time to flower and thereby the duration of the experiment.

Compared to wild-type, *slc18* seems to flower a little bit later in LD conditions. This however could be the result of our procedure to isolate the single mutant, as the late flowering individuals from the F2 backcross were isolated and subsequently tested for their absence of the *lhy-11* and *cca1-1* mutations. Analysing the flowering-time phenotype of the *slc18* single mutant which could be obtained from the stockcentre as a T-DNA insertion after identification of the gene should confirm its role in flowering in the wild-type background.

The complementation with the JAtY BACs was unsuccessful. Although GetCID also found a low transformation rate´with these cosmids, transformation failed using a great number of plants. Also transforming the L*er* control did not yield transformants, suggesting that strain the transformation procedure was very difficult. Possibly using a more virulent *Agrobacterium* might yield transformants.

5. Materials and Methods

5.1. Materials

5.1.1. General molecular biological techniques

Standard molecular biological techniques such as working with DNA (PCR-amplification, separation on agarose gels, restriction digests, etc.), protein (SDS-PAGE, western blot etc.) and preparation of buffers and media were carried out as described by Sambrook and Russell (Sambrook and Russell, 2001).

5.1.2. Antibiotics

Ampicillin (1000 x): 100 mg/mL in H2O
Chloramphenicol (300 x): 10 mg/mL in H2O
Gentamycin (1000 x): 25 mg/mL in H2O
Kanamycin (1000 x): 50 mg/mL in H2O
Rifampicin (500 x): 25 mg/mL in ethanol
Tetracycline (2500 x): 5 mg/mL in H2O

Stock solutions stored at –20° C.

5.1.3. Bacterial strains

E. coli strains
DH5α (genotype: F- φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 tonA)
DB3.1 (genotype : F- gyrA462 endA1 (sr1-recA) mcrB mrr hsdS20(rB-, mB-) supE44 ara-14 galK2 lacY1 proA2 rpsL20(SmR) xyl-5 - leu mtl1)

Agrobacterium strains
GV3101 (pMP90RK, GmR, KmR), RifR (Koncz and Schell 1986)
C58 (pGV 2260, KmR) (provided by GetCID, Norwich)

5.1.4. Plant material

All *Arabidopsis* seed originate from seed collection of Coupland laboratory;
apart from the RIL lines that were kindly donated by Prof. Dr. M. Koornneef as well as the knock-out lines for selected GIPs which were obtained from NASC (Nottingham Arabidopsis Stock Centre).

5.1.5. Vectors

Donor vector for GI cDNA
35S::GI, DNA stock nr. 261

Gateway cloning vectors

pDONR201	entry clone (Invitrogen), kanamycine-resistant
pDONR207	entry clone (Invitrogen), gentamycine-resistant

Yeast-vectors

pAS2.1	Gateway compatible bait vector containing the BD of GAL4
pACT2	prey vector containing the GAL4-activation domain (Clontech)
pGADT7	prey vector containing the GAL4-activation domain (Clontech)
pDEST22	prey vector containing the GAL4-activation domain (Invitrogen)
pDEST32	bait vector containing the GAL4-binding domain (Invitrogen)

Vectors for plant expression

pAN2	based on pGreen0229, Gateway® attB sites
pLeela	35S-promoter, Gateway® attB sites
pAligator2	double 35S-promoter triple HA-tag, Gateway® attB sites
pSUC2	SUC2-promoter, Gateway® attB sites

Vectors for FRET
pENSG-YFPN 35S-promoter, Gateway® attB sites, YFP
pENSG-CFPN 35S-promoter, Gateway® attB sites, CFP

BACs for complementation
JAtY clones were obtained from the GetCID, Norwich

5.1.6. Oligonucleotides

Primers were designed using http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi (Rozen and Skaletsky 2000) and synthesized by SIGMA, INVITROGEN, PROMEGA or OPERON

Name	**Sequence (5'-3')**
GI cloning primers	
GIGF	GYF-GCATGGCTAGTTCATCTTCATCT
GIGR	GYF-TTATTGGGACAAGGATATAGT
GIC_1640	GYF-AAATGGCGAGAAAAGCTAGATCGAGTTTTACCAC
GIMF	GGTACCTGGATGGCTAGTTCATCTTCATCT
GIMR	GGGCCCTTATTGGGACAAGGATATAGT

BSA primers

nga63-F	GCCTAAACCAAGGCACAGAAG
nga63-R	TCATCAGTATTCGACCCAAG
F27K19_F	TGCTTTTGAAGAGATGGTTATTAGG
F27K19_R	CCCCATTTCACTTATCATTGG
F12K8_F	ACCAACACCACAACAAACGAC
F12K8_R	CTTTTTCTGTTCTTCCGCTATTC
CIW1-F	ACATTTTCTCAATCCTTACTC
CIW1-R	GAGAGCTTCTTTATTTGTGAT
nga280-F	CCTGATCTCACGGACAATAGTG
nga280-R	GGCTCCATAAAAAGTGCACC
NF5I14a-F	GTTGAGTCTTGGCATCACAGTTC
NF5I14a-R	CTGCCTGAAATTGTCGAAAC
ATPASE-F	CCTGGGAACGGTTCGATTCGAG
ATPASE-R	GTTCACAGAGAGACTCATAAACCA
F18P14-F	ATTCCCGCAATTTATTTTGTTC
F18P14-R	GTTTGATGGCAGATTTGTTTTC
Ciw3-F	GGAAACTCAATGAAATCCACTT
Ciw3-R	GTGAACTTGTTGTGAGCTTTGA
F26B6_F	CTCTATCTGCCCACGAACAAG
F26B6_R	GCCATTGCAAAAGAACATCAG
F4P9_F	TGGTCCATACCCATTTCATAAC
F4P9_R	ATGAATTTTCATTCTACTGTTTTG
F2H17_F	ATTGCATACCACGCAGTTCAC
F2H17_R	CCATTTTGCCCTTTCCTTCTAC
nga172-F	AGCTGCTTCCTTATAGCGTCC
nga172-R	CCATCCGAATGCCATTGTTC
nga162-F	CATGCAATTTGCATCTGAGG
nga162-R	CTCTGTCACTCTTTTCCTCTGG
Ciw11a-F	GTTTTTTTCTAATCCCCGAGTTGAG

Ciw11a-F	GAAGAAATTCCTAAAGCATTC
MZN14-F	CAATACACTTTATCCAGATGCTG
MZN14-R	GGGATTTGTTGATTGAAAAAGGAC
T32N15_F	ATCTGAAAATCCTTGAGTGAG
T32N15_R	TTGTGACGAATAGTGAAAGGAGAG
ciw4-F	GTTCATTAAACTTGCGTGTGT
ciw4-R	TACGGTCAGATTGAGTGATTC
F27K19_F	TGCTTTTGAAGAGATGGTTATTAGG
F27K19_R	CCCCATTTCACTTATCATTGG
Nga6-R	AGCGAATCCGAAAATAATGGAG
Nga6-F	TGGATTTCTTCCTCTCTTCAC
ciw5-F	GGTTAAAAATTAGGGTTACGA
ciw5-R	AGATTTACGTGGAAGCAAT
T26M18-F	CAATTAGCGGAGGCCACTTC
T26M18-R	GGGCAAAAGCTTCCAGTAC
FCAALL-F	CCACCGTCAACATCCCTAAC
FCAALL-R	GCTCTTATACTTCTCAGCTCTTGTC
F26K10-F	AGAGAGCACGATGCCTGATAG
F26K10-R	AATGCTTCAGCGATTGAGAAC
F6E21-F	TTCTTTGTTCAAGTTCCATGTCTC
F6E21-R	CGGTGATTGTCTCAAGTGTTTG
T16L1_F	TGGGGGCAATGTATTTTACAC
T16L1_R	AATTTTCTTCAACGCCTTGTG
F23E13-F	TGACCGTTGAAAGTGTTGTTG
F23E13-R	GCCCGAGAAGCCTGATAG
T9A14_F	AACACCGTTGATGCAAAGTTC
T9A14_R	GCACAATCTTTGGGTTCAATC
MOJB-F	GAAGATGAAAGATTTTAGGAGGAC
MOJB-R	GTTTGTAGGAGAAGGGGACAAG
nga151a-F	ATCTCATACTGACCCATATGTTCC
nga151b-R	ATTGTACAGTCTAAAAGCGAGAG

ciw8a-F	TACTAGTGAAACCTTTCTCAG
ciw8a-R	TTTTATGTTTTCTTCAATCAGTTAG
nga76-F	GGAGAAAATGTCACTCTCCAC
nga77-R	AGGCATGGGAGACATTTACG
ciw9-F	CAGACGTATCAAATGACAAATG
ciw9-R	GACTACTGCTCAAACTATTCGG
MQB2-F	CTTTGATAGTAACCTTTTTCAAACCA
MQB2-R	TGCCATTTATTTGGTCAACAC
Nga106-F	GTTATGGAGTTTCTAGGGCAC
Nga106-R	TGCCCCATTTTGTTCTTCTC

Fine mapping primers

Sorb10_F	CATTGTGTAAGTTTAGTTTTAATTCAT
Sorb10_R	CTGCACATTTCCTTGCAAA
F24G16Dra2f	AAAATATGTTGCCGGGACTG
F24G16Dra2r	GGGTATCATCCGCTACAACG
T2O9Nde1_f	GAATATCAAACCGCCCATTC
T2O9Nde1_r	AAGAGTGGAGCCATCTGACG
T2O9Cla1_f	TTGATTTCCCTGCCTTATGC
T2O9Cla1_r	GGAGTTCGAACCCTAACTGG
F27H5Dra1_f	GTGAGGAGGACGATGACGAC
F27H5Dra1_r	CCGTACTTCGAAGATTTGGTG
F27H5Alu1_f	AGCTCATGCATCAACGTCAC
F27H5Alu1_r	CCGTTCTGAAGCCTGTAAGC
T8B10Smi1_f	TGGCGACTCTTCTCCTTGAC
T8B10Smi1_r	TGATTTCGTCGGTGAGACTG
T8B10Apo1_f	TCTCAGATCCATTTTCCCATTC
T8B10Apo1_r	ACTTCAGGCTTAAAAGCGATTG

Real-time quantitative PCR

GIQF	CCCAAGTAGTGAGAATGACT
GIQR	CACCACTACACCATCGGAA

COQF	CATGGAAACTGGTGTTGTGC
COQR	TATCTCAGGACCCTGGCTTC
FTQF	ACAACTGGAACAACCTTTGGCAATG
FTQR	ACTATAGGCATCATGACCGTTCGTTACTCG
actinF	GGT GAT GGT GTG TCT
actinR	ACT GAG CAC AAT GTT AC

GYF = Gateway attB1 extension 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3'
GYR = Gateway attB2 extension 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3'

5.1.7. Enzymes

Taq-DNA Polymerase, Homemade (Standard PCR reactions)
Expand High Fidelity Taq polymerase, ROCHE (Mannheim)
Pfu DNA Polymerase, STRATAGENE (Heidelberg)
T4 DNA ligase, ROCHE (Mannheim)
Superscript II RT, INVITROGEN (Heidelberg)
GATEWAY® -Technology, INVITROGEN (Heidelberg)
BP-Clonase, INVITROGEN (Heidelberg)
LR-Clonase, INVITROGEN (Heidelberg)
Lysozyme, ROCHE (Mannheim)
DNaseI, ROCHE (Mannheim)
Restriction enzymes, New England Biolabs (Frankfurt am Main)

5.1.8. Chemicals

Laboratory grade chemicals and reagents were purchased from ROTH (Karlsruhe), SERVA (Heidelberg), BOEHRINGER (Mannheim), MERCK (Darmstadt), BECKMANN (München), GIBCO BRL (Neu Isenburg) and SIGMA (Deisenhofen) unless otherwise stated.

5.1.9. Internet resources

http://jicgenomelab.co.uk/libraries.html
http://www.arabidopsis.org
http://ftp.arabidopsis.org/browse/Cereon/index.jsp
http://www.ncbi.nlm.nih.gov/BLAST/
http://www.tigr.org/tdb/e2k1/ath1/ath1.shtml
http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi
http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
https://www.genevestigator.ethz.ch/

5.2. Methods

5.2.1. Growth conditions of Arabidopsis plants

Plants were grown on soil in controlled environment rooms under LDs (10-hours light/6-hour day extension/8-hours dark), SD10 (10-hours light/14-hours dark) or SD8 (8-hours light/16-hours dark) as described (Putterill et al., 1995), or on MS agar under true LDs (16-hours light/8-hours dark) or SD8. Large scale mapping population was grown in the SD8 greenhouse. Flowering time was measured by scoring the number of rosette and cauline leaves on the main stem of at least 20 individuals. Data are expressed as mean±s.d.

Seedlings for hypocotyl measurements were grown on MS agar in continues Red Light emitted by LEDs (25% intensity = 20 μMm-2 s-1). After 7 days plates were scanned in and hypocotyl length was measured using Image J from WCIF (http://www.uhnresearch.ca/facilities/wcif/imagej/)

5.2.2. Yeast two hybrid screen

For the GIGANTEA yeast-two-hybrid screen two constructs were used, the full-length GI cDNA and the GI C-terminus (GIC). They were amplified by PCR using the High Fidelety TAQ using respectively the primer pair GIGF and GIGR, and GIC_1640 and GIGR. Via standard BP-recombination to pDONR201 or pDONR207 they were recombined in pAS2.1 by standard gateway LR recombination.

Direct transformation of plasmids in yeast was done according the following protocol. A single colony of the yeast (Saccharomyces cerevisiae) strain was inoculated in 5ml YPAD and grown overnight. 1ml of overnight culture was then transferred to 50ml YPAD and grown for four hours at 30°C. After incubation the mixture was centrifuged at 2000g for five minutes and resuspended in 1ml of 100mM Lithium acetate pH 7.5. After transfer to an Eppendorf tube, cells were pelleted by centrifugation and resuspended in 500µl Lithium acetate pH 7.5. Aliquots of 50µl were used for transformation. These 50µl aliquots were transferred to Eppendorf tubes and cells were pelleted by centrifugation. After removing the supernatant, 240µl 50% PEG, 36µl 1M Lithium acetate pH 7.5, 25µl 2mg/ml ssDNA and 50µl water containing 0.5µg of plasmid DNA were added. Cells were resuspended by pipetting, incubated for 30 minutes at 30°C followed by incubation for 30 minutes at 42°C. Finally, cells were pelleted by centrifugation, resuspended in 100µl 1M sorbitol and spread on selective medium.

For the screenings with GI two different cDNA libraries were used. A library containing Arabidopsis thaliana cDNAs from whole plants and a library containing cDNAs from the Arabidopsis shoot apex. Both libraries (constructed by Dr. Hans Sommer, MPIZ Cologne) were cloned into the pGADT7 vector and transformed into the yeast strain Y187. The libraries were introduced into the bait containing yeast strains by mating.

This was done by growing a 50ml bait-culture (GI in AH109 strain) in synthetic drop-out medium (SD-medium) supplemented with 4% glucose and all necessary amino acids except tryptophan (Trp). Cells were counted in a hemocytometer and the volume of 3 x 108 cells was calculated. This volume was then transferred to a 50ml Falcon tube. The library containing cells were thawn at 42°C and 5ml of the library were transferred to the bait-containing falcon tube (bait/library ratio = 2.5). The mixture was centrifuged for five minutes at 4000rpm and the pellet was resuspended in 6ml YCM pH3.5. After 100 minutes incubation at 30°C with constant shaking, 5ml were transferred to 500ml sterile water and mixed well. 236ml were transferred onto a 47mm membrane filter (450nm; PALL Gelman Lab.) and incubated for five hours at 30°C on YCM pH 4.5 plates. The filter was then transferred on SD-Trp-Leu-His plates and incubated over night. The filter was overlaid in a 50ml falcon with 1M sorbitol and vortexed. The filter was removed and the tube centrifuged at 4000rpm for five minutes. The pellet was resuspended in 10ml 1M sorbitol. Cells were counted and 1µl / 0.1µl / 0.01µl / 0.001µl were plated on SD-Trp-Leu plates to calculate mating efficiency (cells on SD-Trp- Leu / all cells). Cells were spread on large SD-Trp-Leu-His plates and the amount of 3- Aminotriazole determined in the pre-screening was added. Plates were incubated for seven to ten days at 30°C. Single colonies emerging on the plates were isolated and transferred to 96-well plates containing 20µl 1M sorbitol. Cells were spread on SD-Trp-Leu-His plates using a hedgehog and transferred to 30°C. After the appearance of colonies, these plates were kept at 4°C as a masterplates. To identify interactors 2µl from the 96-well plates were used in a 25µl colony PCR reaction using the 3AD/5AD primer pair. After purification of the PCR products using the QIAGEN PCR-purification kit, PCR fragments were sequenced using T7-primer.

5.2.3. Transformation of Arabidopsis leaves by particle bombardment

For each bombardment experiment 5µg of plasmid DNA was used per construct. The final volume of DNA should not exceed 5µl. For ten bombardments 30µg of gold (size 1.0 micron) was washed with 1ml of 70% ethanol for 15 minutes while shaking. The gold-ethanol mixture was spun down for a few seconds in a microcentrifuge and washed three times with sterile water. Finally the gold particles were resuspended in 500µl of sterile 50% glycerol. To each DNA-mix of each bombardment experiment 50µl of the gold-glycerol mix was added under constant shaking, followed by the addition of 50µl of 2.5M CaCl2 and 20µl 0.1M spermidine. The mixtures were incubated for another three minutes shaking and spun down in a microcentrifuge. After two washes, first with 140µl of 70% ethanol, second with 140µl of 100% ethanol, the DNA-gold mixture was resuspended in 50µl of 100% ethanol. For each bombardment using the BIORAD biolistic system, 25µl of the DNA-gold mixture was used.

5.2.4. Confocal microscopy and in vivo analysis of protein-protein interactions using Foerster resonance energy transfer (FRET)

To generate fluorescent proteins we used pENSG-YFP:GW and pENSG-CFP:GW which are Gateway destination vectors yielding N-terminal fusions of YFP/CFP driven by the 35Spromoter (kind gift from Dr. Nieves Medina-Escobar, MPIZ Cologne). Plasmids were transformed by particle bombardment on Arabidopsis leaves. Co-localization studies and FRET analysis were performed 14-16 hours after bombardment. Colocalization of YFP, CFP and dsRED was performed using a Leica TCS SP2 AOBS confocal microscope allowing a flexible selection of emission bandwidths and imultaneous multicolor-imaging. For FRET analysis transformed Arabidopsis epidermal cells expressing equal levels of CFP and YFP were selected. Analysis of FRET was performed using a Zeiss LCS510 META confocal microscope equipped with argon ion and He-Ne lasers. FRET was quantified using the acceptor-photobleaching (APB) technology and FRET-efficiencies were determined according to Karpova and Bhat (Karpova et al., 2003; Bhat et al., 2004).

5.2.5. Agrobacterium-mediated transformation of Arabidopsis plants

Transformation of A. tumefaciens

An aliquot (50µl) of an electrocompetent agrobacteria (strains GV3101 pMP90 or pMP90RK) was thawn on ice. 1µl of plasmid DNA was added, cell and DNA were mixed by flicking the tube and than transferred to an electroporation cuvette. An electric pulse was applied (2.5kV/cm, 25µF, 400Ω for 8-12ms), cells were resuspended with 1ml LB medium and transferred to a 15ml Falcon tube. After incubation at 28°C with constant shaking, 100µl were plated on LB plates containing appropriate antibiotics.

Transformation of Arabidopsis plants

Plasmid carrying Agrobacterium strains were grown overnight in one liter LB medium and appropriate antibiotics. Cells were harvested by a 30 minute centrifugation at 4.000rpm and pellets were resuspended in one liter transformation buffer (2.2g MS salts, 50g sucrose, 0.6g MES, 300µl silvet, pH 5.7). Arabidopsis plants (nine plants per pot) were dipped in the transformation mixture for two minutes and than bagged with plastic for 24 hours. After this 24 hour incubation, plastic bags were removed. After seed set plants were bagged for seed collection.

Selection of transformed Arabidopsis plants

The bulked seeds were sown on soil and the transformed T1 seedlings were selected after 1-2 weeks by spraying with the herbicide BASTA. Only those plants equipped with the vector, which also contains a resistance gene against the herbicide, survive this treatment. Spraying was repeated after 3 days.

5.2.6. GUS assay

To visualize the spatial expression pattern GI, the promoters was amplified by PCR, recombined into the pDONR207 plasmid and finally introduced into the pGPTV-BAR plasmid in front of the β-glucuronidase gene. Transgenic GI::GUS-expressing plants were kindly provided by Dr. Hailong An. Tissue was harvested and incubated for 10 minutes in heptane to remove cuticular waxes and dried at room temperature for about 5 minutes. The tissue was then submerged in GUSsolution (for 400ml: 15.6ml 1M NaH2PO4, 24.4ml 1M Na2HPO4, 360ml H2O, 263mg K3Fe(CN)6, 200mg x-Gluc, 400µl triton-X100) overnight at 37°C. The solution was removed and 70% ethanol was added to remove the chlorophyll, followed by washes of 100% ethanol until the green color was completely removed and the blue GUS-pattern became visible. For long time storage, GUS solution was kept at -20°C, after thawing at 4°C.

5.2.7. Isolation of genomic DNA from plant tissue

DNA from plants was isolated using the method described by Edwards et al. (Edwards et al., 1991). For genotyping, a single Arabidopsis leaf was harvested per sample and frozen in liquid nitrogen. Samples were macerated using disposable grinders or an electric drill. Immediately after maceration 400µl of extraction buffer (200mM Tris pH 7.5, 250mM NaCl, 25mM EDTA, 0.5% SDS) was added and the sample was vortexed for five seconds. Samples were kept after this step at room temperature until the last sample was extracted. The extracts were centrifuged for 5 minutes and 300µl of supernatant was transferred to a new tube containing 300µl of isopropanol and vortexed. After a two minute rest, samples were centrifuged at 14,000 rpm. The supernatants were discarded and pellets dried at room temperature. After dissolving the pellets in 50µl TE, 1µl was used for PCR. DNA was stored at -20°C.

For quick screening of a large amount of plants, DNA was extracted with the 'boiling' method (Berendzen *et al*., 2005).

5.2.8. RNA isolation from plant tissue

To analyze expression levels of genes after hormone treatments or in time-course experiments, half a 1.5ml Eppendorf tube was filled with seedlings grown on plates. Total RNA was extracted using the RNeasy kit from Qiagen according to the manufacturer's instructions.

5.2.9. Reverse transcription

Equal amounts of RNA were used for reverse transcription (usually between 1 and 5µg). Reverse transcription was carried out in 20µl final volume using the SuperscriptII reverse transcriptase from Invitrogen according to the manufacturers instructions. Independent

5.2.10. PCR and RT-PCR reactions

Standard PCR reactions were performed on a Mastercycler (Eppendorf, Hamburg).
For RT-PCR, 2µl of a ten-fold dilution of the cDNA produced by reverse transcription was used. The amplification mix contained the following: 2µl PCR buffer, 1µl 10µmM of each primer, 0.5µl 10mM dNTPs, 0.25µl Taq polymerase and 13.25µl water.

5.2.11. DNA sequencing

DNA sequences were determined by the Automatische DNA-Isolierung und Sequenzierung (ADIS-Unit) at the MPIZ on Applied Biosystems (Weiterstadt, Germany) Abi Prism 377 and 3700 sequencers using Big Dye-terminator chemistry (Sanger et al. 1977). PCR products were purified with the QIAGEN Extract Kit

5.2.12. Mapping

The mapping strategy that was deployed started with rough mapping using Bulked Segregant Analysis (BSA) (Michelmore *et al*, 1991). BSA involves creating a pool of a small number of mutant phenotype individuals from an F2 hybrid population and compairing these with a pool of wild-type phenotype plants. In case of a putative association with markers on a chromosome, additional F2 plants were screened with the respective markers.

6. Final discussion

This study aims to contribute to a better understanding of the role of GI in the promotion of flowering, light signalling and functioning of the circadian clock in Arabidopsis. I used the following approaches to reach these goals: 1. identify proteins interacting with GI, 2. show where GI acts to promote flowering and 3. identify and characterize a new mutation delaying flowering time.

The screen for interacting proteins to GI was done using the Y2H system. This yielded numerous proteins that potentially interact with GI, and the most promising of these were retested for their interaction with the C-terminal half of GI, which has been implicated in flowering-time control. Five GI INTERACTING PROTEINS (GIP) scored positive in interaction with this part of the GI protein. Overexpression of one of these proteins interacting with the C-terminal, the ZZ-Type Zinc Finger protein *GIP14*, causes an elongated hypocotyl and as this is a similar phenotype to *gi* mutants, GIP14 might therefore function in red light signalling as an antagonist of GI. GI might play a role in degrading this ZZ-Type Zinc Finger Protein and thereby activate red light signalling by removing GIP14. This observation poses the question of whether the effect of GIP14 on red light signalling is regulated by GI function. Experimentally this could be tested by overexpressing *GIP14* in a *gi* mutant and comparing the effect upon hypocotyl elongation with *GIP14* overexpression in wild-type plants. If GI inhibits GIP14 activity, then overexpression of GIP14 in a *gi* mutant background would be expected to produce an even stronger elongated hypocotyl phenotype. Interestingly this protein was also found to interact with the CO protein, although this might suggest a putative bridging function of GIP14 between GI and CO, a link to the function of CO has not been found.

The functional characterisation the other GIPs was not continued because they did not have an obvious phenotype in flowering time or in other processes related to GI functioning. However, material has been generated to perform interaction studies using FRET and Co-IP in plant cells or in vitro respectively. One could test the mutant lines from these genes for an effect on hypocotyl length in red light or for a circadian

phenotype by leaf movement or altered circadian gene expression rhythms. One particularly interesting interactor with GI is CSN6b, a subunit of the COP9 signalosome. Absence of a mutant phenotype in the *CSN6b* gene could be explained because of the presence of the nearly identical gene *CSN6a*. Further exploration of this interaction might be interesting, because the COP9 signalosome regulates photomorphogenesis. The signalosome is believed to regulate photomorphogenesis by influencing the activity of the E3 ubiquitin ligase COP1 (Chen *et al*., 2006). This ubiquitin ligase both delays flowering and inhibits photomorphogenesis. GI could therefore play a role in regulating signalosome activity and thereby influence photomorphogenesis and flowering via COP1. The significance of the interaction between Gi and CSN6b needs to be tested by wider range of methods including FRET and *in vitro* co-immunoprecipitations and then the effect of GI on the stability of COP1 target proteins could be explored.

Regional misexpression of *GI* yielded several single insertion homozygous lines of each promoter::*GI* construct expressing GI at different stages throughout development. These lines uncovered interesting requirements for GI in different tissues to regulate flowering time and hypocotyl elongation. We showed that *GI* expression is needed in the phloem companion cells to promote enhancement in flowering of the *gi-3* mutant. This observation is in agreement with the results obtained in a similar experiment for CO. Specific expression of *CO* (An *et al*., 2004). Together with the expression data of *GI::GUS* and the misexpression data of *CO*, the results fully make sense for the expression of *GI* in the phloem. As this experiment located the region in which GI is needed to promote flowering, this is also important information in considering proteins that interact with GI. If these interacting proteins are involved in the regulation of GI activity in flowering then they should be expressed in the phloem.

The misexpression experiments show that GI can promote flowering from the leaf. To test the requirement for *GI* expression in the phloem, one could perform a RNA silencing experiment of *GI* using phloem specific promoters in wild-type plants. Similarly, synthetic microRNAs, also known as artificial miRNAs (amiRNAs) could be used, which have been shown to act in a cell autonomous manner (Schwab *et al*., 2006).

Additionally, the elongated hypocotyl of the *gi-3* mutant can be restored by expressing GI specifically from the *AS1* promoter, showing the need for *GI* expression in the leaf primordia. Therefore, interestingly, we could separate two functions of GI by the different spatial requirements of *GI* expression in flowering-time and hypocotyl elongation. Separation of the functions of GI in flowering-time control and circadian clock function has already been suggested (Mizoguchi *et al*., 2005), however not been shown by spatial requirements for expression of GI. Requirement for GI in the leaf primordia is consistent with *GI::GUS* expression in that region and it therefore seems plausible that light is perceived in these tissues and then a signal is directed to the hypocotyl that influences the elongation of the hypocotyl. GI would than have a role in regulating this signal.

A misexpression analysis was also done with the circadian clock genes *LHY* and *CCA1*, which revealed that these genes also influence flowering time from the phloem. If expressed from a phloem specific promoter, misexpression of these genes results in late flowering Arabidopsis plants (pers comm. L. Corbesier). This is in correspondence with the early flowering phenotype of the *lhy* and *cca1* mutants and the late flowering of *35S::LHY* or *35S::CCA1* plants. LHY and CCA1 therefore perform their function in flowering-time control from the phloem, however in an opposite manner to that of GI and CO. This delay in flowering time from expression of *LHY* or *CCA1* in the phloem has been shown to be due to downregulation of *CO* and *FT* mRNA levels (pers comm. L. Corbesier). *GI* expression is however not changed, when tested in mRNA samples extracted from whole leaves, suggesting that *GI* is expressed widely in the leaf and that downregulation in the phloem companion cells by overexpression of *CCA1* and *LHY* has little effect on total mRNA levels. If *GI* levels are down in the phloem companion cells, this would explain why *CO* and *FT* expression is low and these plants are late flowering. This could be tested by in situ hybridization. When *GI* levels are unchanged, this could suggest that LHY and CCA1 function by regulating *CO* mRNA levels directly or by regulating GI protein levels, thereby regulating *CO* expression.

To fully complete the misexpression experiment with *GI*, flowering time under short-days and the effect upon circadian rhythms has to be done. The latter could can be done by testing the rhythm of a circadian controlled gene such as CCR2 or by analysing the circadian leaf movements. To provide a clear discrimination for the hypocotyl elongation, the red light experiment might be repeated by measuring hypocotyl length throughout the day or by using a different gi mutant such as *gi-1* with a larger difference between mutant and wild-type.

The *slc18* mutation was mapped to a short interval conaining 30 genes. 'Candidate' genes can now be tested for the presence of polymorphisms that might be the cause of the phenotype in flowering time. Analysis of the flowering time of the triple *slc18lhy-11cca1-1* and the single *slc18* mutant reveal that the delay in flowering is specific to SD conditions. It seems however that this delay in flowering time is not due to changes in the expression of *GI*, *CO* or *FT*. The nature and function of this illusive mutation is still to be unravelled, but the analysis presented here indicates that SLC18 is a novel flowering-time gene acting downstream of LHY/CCA1 and independently or downstream of FT.

The analysis presented in this thesis has provided novel insights into the function of GI in light regulation and flowering-time control. Finally determining the biochemical function of this intriguing, plant-specific protein will be important in understanding its roles in different biological processes.

7. Literature

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8. Appendix

8.1 Mathematics towards determination of 3:1 segregation

Tested number of seedlings = 120
Expected number of resistant seedlings = 90
Observed resistant seedlings = b
Expected dead seedlings = 30
Observed dead seedlings = a
a = 120 - b

95% significance by 3:1 segragation: $\chi^2 \leq 3{,}8$

$3{,}8 = (| a-30 | -0{,}5)^2 / 30 + (| b-90 | -0{,}5)^2 / 90$
$3{,}8 = (89{,}5 - b)^2 / 30 + (b - 90{,}5)^2 / 90$
$b^2 - 179{,}5\ b + 7969{,}75 = 0$
b = 80,5 and b = 99

Observed resistant seedlings must be between 80,5 and 99 to have 95% significant segregation of 3:1

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10. Erklaerung

"Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. George Coupland betreut worden."

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