Molecular and Functional Characterisation of SBP-box genes: The role of *SPL3* during the floral transition of *Arabidopsis thaliana*

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Zusammenfassung:

Die einwandfreie Entwicklung von Organismen basiert auf der präzisen Kontrolle der Genexpression: Die sowohl räumlich als auch zeitlich hoch spezifische Gen- bzw. Proteinexpression ist die Grundvoraussetzung für die korrekter Zelldifferenzierung. Transkriptionsfaktoren und microRNAs sind die Hauptkomponenten der transkriptionellen und translationalen Regulation der Genexpression. Von der *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) Genfamilie aus *Arabidopsis thaliana* weiß man, dass sie an verschiedenen Entwicklungsvorgängen der Pflanzen, wie der Blütenentwicklung und dem für einjährige Pflanzen irreversiblen Phasenwechsel vom vegetativen zum reproduktiven Wachstum, beteiligt ist. Dieser Wechsel ist essenziell, um der Pflanze die erfolgreiche Reproduktion während günstiger Umweltbedingungen zu ermöglichen. Für den durch MIR156/157 kontrollierten SBP-box Transkriptionsfaktor *SPL3* wurde bereits eine wichtige Rolle während der Blütenentwicklung in *Arabidopsis* diskutiert, da seine konstitutive Überexpression in einer microRNA unempfindlichen From (*SPL3OX*) zu verfrühter Blüte führt. Dennoch bleiben diese Pflanzen photoperiodisch sensitiv. Darüber hinaus konnten *in vitro* Bindestudien zeigen, dass SPL3 an das Kernsequenzmotifv CGTAC bindet.

Ziel der vorliegenden Doktorarbeit war, die Rolle von SPL3 während der Entwicklung vom vegetativen zum reproduktiven Wachstum durch Identifikation von Zielgenen zu ermitteln. Die Ergebnisse dieser Arbeit zeigen, dass das florale Meristemidentitätsgen *FRUITFULL* (*FUL/AGL8*) wahrscheinlich ein direktes Zielgen von SPL3 ist. Expressionsstudien zweier transgener Linien, die ein GUS-Reportergen trugen, zeigten, dass in einem *SPL3OX* Hintergrund *FUL* in Kotyledonen und Blättern verfrüht exprimiert wird und die Anwesenheit von Bindemotiven sowohl im Promotor als auch im ersten Intron von *FUL* von Bedeutung sind. Darüberhinaus konnte druch die Analyse der Gesamtgenexpression von SPL3 Überexprimierern ein Einfluss von SPL3 auf den Zuckermetabolismus, das „Red Light Signalling“ sowie die Circadiane Uhr festgestellt werden. Eine anschließende Analyse der diurnalen Expression der Gene, die in der Circadianen Uhr eine Rolle spielen, zeigte, dass die Periode der Biologische Uhr verkürzt ist, was zu einer verfrühten Aktivierung sog. "Abendgene" führt.

Abstract:

Proper developmental processes require a tight control of spatial and temporal gene regulation, since specific gene and protein expression is a prerequisite of cell differentiation. Transcription factors as well as microRNAs are major components for transcriptional and translational control of gene expression. In *Arabidopsis thaliana*, one of the plant specific transcription factor families is the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) gene family, which comprises 17 members, that have been shown to play important roles in several developmental processes.

A decisive step in plant development is the transition from vegetative to reproductive growth, as it has to happen during favorable conditions to ensure successful reproduction and is a "one-time decision", as this phase change is not reversible in the annual plant Arabidopsis. The MIR156/157-controlled SBP-box transcription factor *SPL3* has been shown to play a role during flowering in Arabidopsis, since its constitutive overexpression in a microRNA insensitive form results in early flowering plants, that nevertheless remain photoperiodically sensitive. Moreover, it has been shown that SPL3 binds *in vitro* to the sequence core motif CGTAC.

During this thesis the role of SPL3 during the development to reproductive growth should be elucidated through identification of target genes. The results of this work suggest the floral meristem identity gene *FRUITFULL* (*FUL*; *AGL8*) to be a direct target of SPL3. Expression studies of two transgenes carrying the reportergene GUS in combination with genomic *FUL* or the *FUL* promoter region revealed a precocious activation of *FUL* in cotyledons and leaves in an SPL3OX background. Moreover, these data indicate that both, the binding motifs in the promoter as well as in the *FUL* first intron, are required for proper activation of *FUL*. A global expression analysis revealed that sugar metabolism, red light signaling and the circadian clock are affected by overexpression of *SPL3*. Subsequent analysis of diurnal expression of clock genes as well as of leaf movement in *SPL3* overexpressing plants revealed a shortened period of the circadian clock and a precocious activation of so called "evening genes".

Contents

1 Introduction

Proper development in plants, as in all living organisms, depends on the precise spatial and temporal regulation of gene expression. This is cardinally achieved on the level of transcription through sequence specific DNA binding proteins. These so called transcription factors (TFs) are able to specifically interact with target genes and either activate or repress their expression in a particular developmental context (Chen and Rajewsky 2007). Thus, detailed knowledge on the role of transcription factors will contribute to a generally better understanding of development.

A well established model system for studying plant development is *Arabidopsis thaliana*. Its entire genome has been largely sequenced (The Arabidopsis Genome Initiative 2000) and approximately 6% of the total genome of *Arabidopsis thaliana* encode for transcription factors (Guo *et al.,* 2005, Ratcliff and Riechmann 2002). The more than 1500 transcription factors identified in Arabidopsis can be classified into 49 transcription factor families due to the type of DNA-binding domain encoded (Ratcliff *et al*., 2002; Guo *et al*. 2005, Riechmann *et al*., 2000). Many transcription factor families in Arabidopsis are large and incorporate more than 100 members. The largest transcription factor families are the *AP2/ERF* (APETALA2/ethylene responsive element), the bHLH (basic region helix-loop-helix) and the MYB-(R1)R2R3 family. Furthermore, whereas 53% of the Arabidopsis transcription factors belong to families that appear in other eukaryotic organisms like animals and fungi, as well, ca. 45% are plant specific (Riechmann *et al*., 2000). However, despite their assumed regulatory roles, less than 10% of the Arabidopsis transcription factors are currently known for their contribution to plant developmental processes.

1.1 The SBP-box gene family of transcription factors in *Arabidopsis thaliana*

One plant specific family of transcription factors is the SBP-box gene family. The first SBP-box genes have been identified in *Antirrhinum majus*, as they encode proteins able to bind *in vitro* a defined sequence motif in the promoter region of the floral meristem identity gene *SQUAMOSA* (Huijser *et al.,* 1992, Klein *et al.,* 1996). Therefore, members of this family are called SQUAMOSA-PROMOTER BINDING PROTEINS (SBP-box proteins) and share the highly conserved SBP-domain, which is responsible for DNA-binding.

Members of the SBP-box gene family are known from unicellular algae like *Chlamydomonas*, the moss *Physcomitrella* up to mono- and dicots like rice and poplar. For example, the *Chlamydomonas* SBP-domain protein *COPPER RESPONSE REGULATOR 1 (CRR1*, Kropat *et al*., 2005), is involved in copper homeostasis, whereas the maize SBP-box genes *LIGULELESS1* (*LG1*; Moreno *et al*., 1997) and *TEOSINTE GLUME ARCHITECTURE 1* (*TGA1*; Wang *et al*., 1995) control leaf and glume organogenesis, respectively.

A search for orthologs of the *A. majus* SBPs, *SBP1* and *SBP2*, revealed that in the model species *Arabidopsis thaliana* the SBP-box family today comprises 17 members, the *SPL*-genes (for *SBP- LIKE*; (Cardon *et al.,* 1999, Cardon *et al.,* 1997). Analysis of the SBP-domain of those proteins by heteronuclear NMR spectroscopy revealed the 76 amino acid residues spanning SBP-domain to contain two zinc-binding sites with eight Cys or His residues in a $Cys_3HisCys_2$-HisCys or $Cys_6HisCys$ sequence manner. The first four and the last four residues, in a novel constellation, both coordinate one zinc ion (Yamasaki *et al*., 2004, 2006). Furthermore, the SBP-domain harbors a conserved bipartite nuclear localization signal at its C-terminal end to target the transcription factor to the nucleus (Birkenbihl *et al*., 2005).

It has been shown that SBP-domain proteins generally recognize the tetranucleotide core motif GTAC, although for the individual members of this family a more specific sequence for DNA-binding seems to be required, i.e. SPL14 prefers CCGTAC(A/G), PpSBP2 GTACT and SPL3 CGTAC (Birkenbihl *et al*., 2005; Liang *et al*., 2008; Nagae *et al*., 2008).

SBP-box genes can be grouped according to their genomic structure and sequence similarities into three subfamilies or into 7 phylogenetical subfamilies (Riese *et al*., 2007). The group encoding the largest SBP-domain proteins comprises the members *SPL1, 7, 12, 14* and *16*, which are characterized by relatively large genomic loci with 10 or more exons. Downstream of the SBP-domain the protein products of this subfamily share ankyrin repeat-like sequences probably involved in protein-protein interaction and a transmembrane domain like feature at their C-termini. Moreover, they carry a possible AHA-like activation site upstream of the SBP-domain and an IRPGC motif of unknown function downstream the SBP-domain. Members of this subfamily are largely constitutively expresses during plant development according to the microarray database genevestigator (Zimmermann *et al*., 2004). Yet, a function has solely been described for *SPL14*, which is involved in the resistance against the

fungal toxin fumonosin B1 and in leaf development (Stone *et al.,* 2005). Moreover, recently a role for *SPL7*, which has shown to be a central regulator for copper homeostasis (Yamasaki *et al*., 2009), has been described.

The second subfamily comprises *SPL2, SPL6, SPL8, SPL9, SPL10, SPL11, SPL13a, SPL13b* and *SPL15* and represents rather mid-sized genes with three to four exons. In contrast to the subfamily of large *SPL* genes, expression of these genes is more spatially and temporally regulated. In particular, an increase towards the end of the vegetative growth phase is often observed as well as a strong response to photoperiodic induction of flowering (Cardon *et al*., 1997; Schmid *et al*., 2003; AtGenExpress). These observations already suggest that these SBP-box genes are involved in the transition from vegetative to reproductive growth and flower development. In agreement, *SPL9* and *SPL15* have recently been shown to control the juvenile-to-adult phase transition (Schwarz *et al*., 2008). Furthermore, their mutants affect plastochron, inflorescence architecture and branching (Schwarz *et al*., 2008; Wang *et al*., 2008). Interestingly, all members of this subfamily except *SPL8* are targets of the related microRNAs 156 and 157 (Gandikota *et al*., 2007; Rhoades *et al*., 2002). The one exception, *SPL8*, plays an important role in anther and ovule development (Zhang *et al*., 2007; Unte *et al*., 2003).

The remaining SBP-box genes in Arabidopsis, *SPL3, -4* and *-5* represent the smallest *SPL* genes in Arabidopsis. Like the mid-sized genes, they become upregulated during floral transition and at least *SPL3* seems to play an important role in the transition apex. Additionally, they are also targets for the microRNAs 156 and 157. However, whereas the other miR156/157 regulated *SPL* genes carry the miRNA response element (MRE) in the coding region, transcripts of *SPL3, -4* and *-5* carry the MRE in their 3'UTR (Rhoades *et al*., 2002). In association with the RNA-induced silencing complex (RISC), microRNAs mediate translational inhibition and/or mRNA cleavage through hybridization to the complementary MRE (Chen 2008 and references therein).

Reduced *SPL3* transcript levels in the floral transition apex of plants overexpressing the MIR156b locus indicate a post-transcriptional regulation of *SPL3* by miR156 (Schwab *et al*., 2005). Recently Gandikota and co-workers could show that the MRE in the 3'UTR of *SPL3* prevents detection of SPL3 protein before the presence of the

transition apex despite the presence of abundant mRNA as in *SPL3* overexpressing transgenic plants (Gandikota *et al*., 2007). Cardon and coworkers showed that *SPL3* is highly expressed in the transition and inflorescence apex, the floral meristem, as well as in primordia of leaf and floral organs. Constitutive overexpression of *SPL3* in a miRNA insensitive form leads to early flowering, nevertheless the plants remain photoperiodically sensitive, i.e. they flower later under shortday (SD) than under longday (LD) conditions (Cardon *et al*., 1999; Wu and Poethig 2006). Schmid and co-workers could show that *SPL3*, as well as its likely paralogs *SPL4* and *5*, dramatically respond to floral induction by LD treatment (Schmid *et al*., 2003).

Although *SPL3* is orthologous to *SBP1* from *A. majus*, and *APETALA1* (*AP1*, Mandel *et al*., 1992) the ortholog of *SQUA*, the assumption that constitutive overexpression of *SPL3* caused early flowering by precociously activating *AP1* turned out to be false. Despite the presence of putative SPL3 recognition sites in the *AP1* promoter region, plants overexpressing *SPL3* in the absence of functional *AP1* remain early flowering (Cardon *et al*., 1997).

Taken together, these results strongly suggest a role for *SPL3* (as well as for *SPL4* and *5*) in the phase change from vegetative to reproductive growth. Furthermore, post-transcriptional inhibition of gene expression mediated by miRNAs seems to play an important role in plant development, especially the floral transition.

1.2 Regulation of Flowering time in Arabidopsis

In the annual plant *Arabidopsis thaliana* the phase transition from vegetative to reproductive growth means a "one-time decision", since the phase change is not reversible. To ensure optimal use of resources, Arabidopsis has to time this switch in accordance to the most favorable period of the season. Therefore, the phase transition from vegetative to reproductive growth, i.e. flower and seed production, is tightly controlled in response to environmental cues.

For the facultative long-day plant Arabidopsis, flowering is accelerated by increasing day-length (Carré 2001). In addition to this photoperiod dependent pathway, at least three other signaling pathways that coordinally promote flowering are known: the autonomous, the vernalization and the gibberelin dependent pathways. Moreover ambient temperature is an important regulator for flowering (Lee *et al*., 2007).

The autonomous pathway involves the *FRIGIDA* gene (*FRI/FLA*; Lee *et al* 1993), which is a positive regulator of the flowering time repressor *FLOWERING LOCUS C*

(*FLC/FLF*; Michaels and Amasino 1999; Sheldon *et al*. 2006; Sheldon *et al*. 1999). *FLC* encodes a MADS-box transcription factor and its expression is epigenetically silenced by cold treatment, e.g. winter, and by several other components of the autonomous pathway, such as *FCA* and *LD*. *FLC* acts thus as an integrator for the signals from both the autonomous pathway (Alexandre and Henning 2008) and the vernalization pathway. Several genes have been identified, that act as repressors for *FLC* through modification of its chromatin. For example, *VERNALIZATION 2* (*VRN2*; Chandler *et al*., 1996) encodes a protein with similarity to PcG-proteins known to be involved in long-term maintenance of gene repression and regulation of chromatin structure (Gendall *et al*., 2001), while *PROTEIN ARGININE METHYLTRANSFERASE 5* (*PRMT5;* Sung *et al*., 2006; Schmitz *et al*., 2008) and *TERMINAL FLOWER 2 (TFL2*; Larsson *et al*., 1998; Mylne *et al*., 2006) have been shown to be required for epigenetic silencing of *FLC*.

Flowering in Arabidopsis also seems to be dependent on gibberellic acid in particular under SD conditions as the GA biosynthesis mutant *gibberellin insensitive1*-3 (*ga1*-3) never flowers under these conditions (Wilson *et al*., 1992). Blazquez et al. (2000) found that *ga1*-3 mutants lost *LEAFY* (*LFY*, Schultz *et al*., 1991) activity in SD conditions, but when *LFY* is overexpressed in these mutants (Carré and Kim 2002) the plants were able to flower under SD conditions (Blazquez and Weigel 2000). So it is likely that the GA signal is a positive regulator of *LFY* expression, which is a floral meristem identity gene required for flower formation and may integrate both photoperiod and GA pathways (Blazquez and Weigel 2000).

Probably the best studied flowering time controlling pathway involves photoperiodic induction in association with the circadian clock. *GIGANTEA* (*GI*; Park *et al*., 1999; Fowler *et al*., 1999) is a clock-controlled positive regulator of *CONSTANS* (*CO*; Putterill *et al*., 1995) and forms a complex with *FLAVIN-BINDING, KELCH REPEAT, F-BOX1* (*FKF1*, Nelson *et al*., 2000) protein to degrade the repressor of *CO, CDF1*, acting at the *CO* promoter (Sawa *et al*., 2007).

CO transcripts show daily oscillations with an accumulation in the afternoon or evening (Suárez-López *et al*., 2001). But *CO* transcript accumulation has to happen during the light phase, i.e. LD conditions, to upregulate the floral pathway integrator *FLOWERING LOCUS T* (*FT*, Araki *et al*., 1998) in the leaves, since the CO protein is stabilized by light (Valverde *et al*., 2004). This phenomenon is known as the external coincidence model and has been proposed by Erwin Brüning in 1936 because it

requires the coincidence of an external stimulus (light) with an internal rhythm of sensitivity to light (CO protein). Additionally, GI protein is post-transcriptionally regulated by light and dark (David *et al*. 2006). FT is a mobile signal, that moves through the phloem to the shoot apical meristem (SAM) to change the meristem from vegetative to reproductive phase (Corbesier *et al*., 2007). FT activates another floral pathway integrator, the MADS domain transcription factor *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*; Lee *et al*., 2000). The floral pathway integrators integrate signals from all four signaling pathways and activate floral meristem identity genes (Kobayashi *et al*., 1999; Samach *et al*., 2000), i.e. *AP1*, *CAULIFLOWER* (*CAL,* Kempin *et al*., 1995), *FRUITFULL (FUL/AGL8,* Gu *et al.* 1998*)* and *LFY*, which promote floral meristem development and flower architecture.

Additionally, flowering in Arabidopsis is known to be controlled by a few microRNAs. Of these, miR156 and the highly similar miR157 repress flowering time and are highly expressed in the transition and inflorescence apex (Schwab *et al*. 2005). Constitutive overexpression of *MIR156b* results in delayed flowering and faster initiation of rosette leaves. Furthermore, elevated levels of miR156 cause decreased apical dominance, so that the first flowers often arise from side shoots (Schwab *et al.,* 2005). Upon floral transition miR156 becomes downregulated. Another microRNA, miR172, promotes flowering posttranscriptionally by repressing a set of *APETALA2-Like* genes, such as *TARGET OF EAT* (*TOE1*), *TOE2* and *TOE3* (Aukerman and Sakai 2003). Overexpression of miR172 results in early flowering plants both under LD and SD, that is in a CO-independent way. The level of MiR172 is regulated by daylength. This daylength effect is lost in *ft* and *co* mutants (Schmid *et al*., 2003). Furthermore, GI regulates miR172 abundance at the miRNA processing level (Jung *et al*., 2007). Targets of miR172 are *SCHLAFMÜTZE* (*SMZ*) and *SCHNARCHZAPFEN* (*SNZ*), which repress flowering and become downregulated upon floral initiation (Schmid *et al*., 2003).

A third microRNA involved in flowering time control is miR159, which is upregulated by gibberellic acid and causes the repression of *LFY*, specifically under SD (Achard *et al*. 2004). MiR159 directs the cleavage of mRNA encoding GAMYB-proteins, which are involved in the activation of *LFY* and the regulation of anther development. Therefore overexpression of miR159 results in a delay of flowering under SD conditions and disturbed anther development (Achard *et al*. 2004).

Taken together it can be assumed that for proper floral induction it is necessary to repress miR156 / 157 and miR159 while miR172 has to be upregulated.

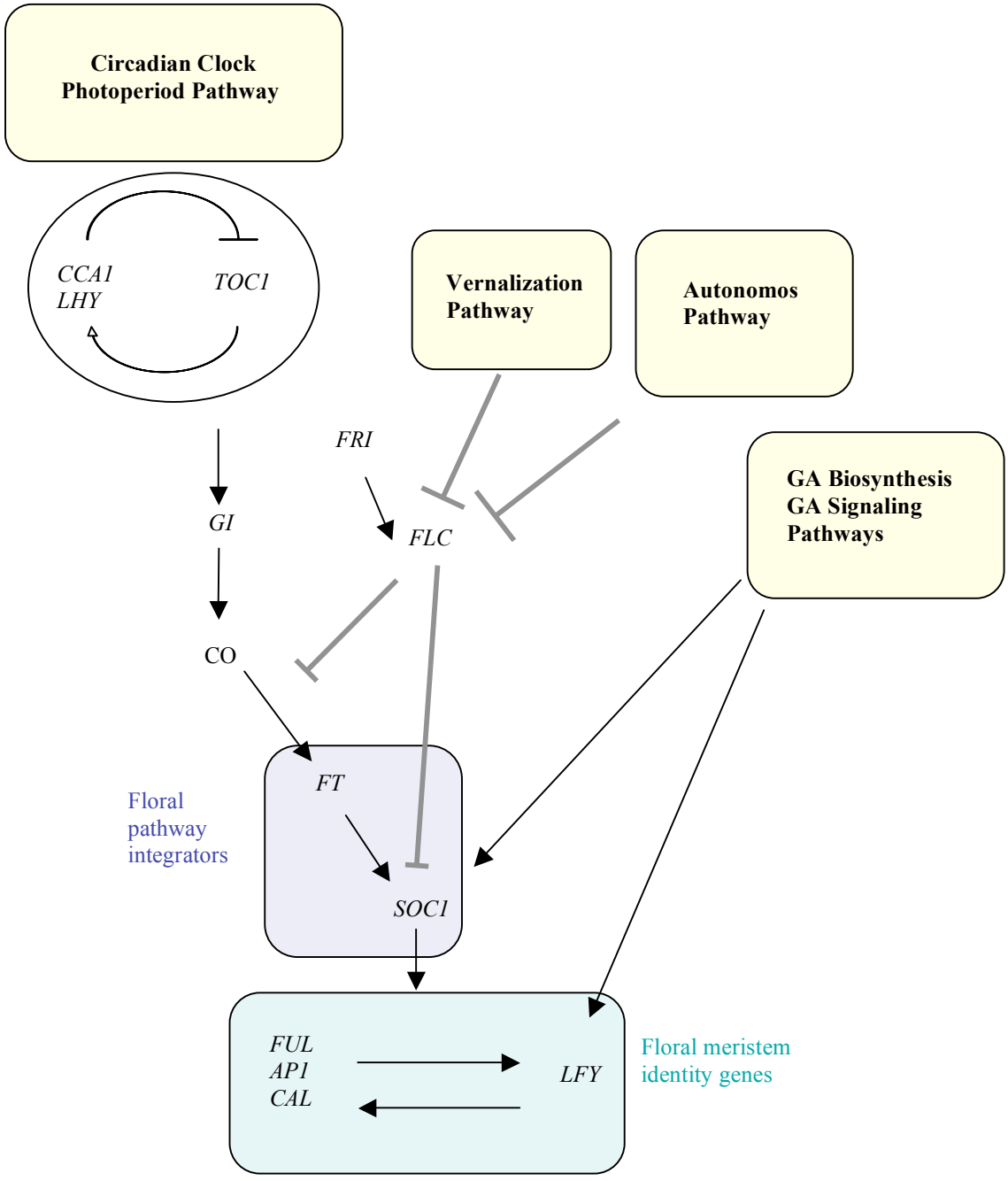


Fig. 1.1
Overview of the flowering time pathways with some key components.
The floral pathway integrators *SOC1* and *FT* integrate the signals coming from the photoperiod, vernalization and autonomous pathways as well as from the GA signalling pathway and pass these signals on to the floral meristem identity genes *AP1*, *CAL*, *FUL* and *LFY*.

1.3 Circadian Biological Clock in *A. thaliana*

Due to rotation of the earth around its axis, light and temperature oscillate with a period of 24h. It is advantageous for both sessile and motile organisms to synchronize their cellular activities to such an environmental cue in order to ensure successful growth, development and reproduction. Therefore, organisms have established an internal time-keeping mechanism, the biological clock, generating biological rhythms with a periodicity of about 24h, i.e. acting as a circadian clock (Bell-Pedersen *et al*. 2005). The circadian biological clock is capable of translating environmental signals into temporal information in order to rhythmically coordinate metabolism and physiology (Wijnen and Young 2006). Circadian biological rhythms are repeated once a day and persist in absence of environmental cues. Furthermore, they are entrained to local time and maintain over a range of physiologically permissible temperatures (Más, 2008). The circadian biological clock (in the following simply referred to as circadian clock) therefore is an excellent mechanism to enable an organism to measure both day-length and seasonal change and thus to adapt to environmental changes in light and temperature by timing important developmental processes to a biologically beneficial time of the day or year (Más, 2008). Some examples of circadian clock controlled processes in plants are the movement of cotyledons and leaves, the growth of the hypocotyl, the opening and closing of flowers, as well as the subcellular localization of chloroplasts and stomatal aperture size. Moreover, endogenous oscillations in gene and protein expression, posttranslational modification of proteins as well as rhythmic changes in chromatin structure and protein stability receive signals from the circadian clock and may feedback on it.

Molecular and genetic approaches have shown, that the genes involved in the circadian clock, so-called clock genes, are not conserved across kingdoms, but that the genetic components of the clock all contribute to positive or negative feedbacks in all organisms studied (Dunlap, 1996). The Arabidopsis core oscillator of the circadian clock involves the single MYB-like transcription factors *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*; Wang and Tobin 1998) and *LATE ELONGATED HYPOCOTYL* (*LHY*; Schaffer *et al*., 1998), which are partly redundant (Mizoguchi *et al*., 2002). It has been shown that constitutive overexpression of *LHY* leads to arrhythmia and the clock is unable to work (Wang and Tobin 1998), whereas in a loss-of-function mutant of *CCA1* or *LHY* the rhythmicity remains, but the period of

the clock is shortened (Schaffer *et al*., 1998; Green and Tobin 1999). Another core component of the clock is *TIMING OF CAB EXPRESSION 1* (*TOC1*, Strayer *et al*., 2000; Makino *et al*., 2000), which encodes for a protein, that contains a receiver domain similar to that found in classical Arabidopsis response regulators (ARRs). Together with five other members, *TOC1* belongs to the family of pseudo-response regulators (PRRs; *TOC1, PRR3, PRR5, PRR7* and *PRR9*). But the receiver domain in those proteins is atypical, since it lacks the conserved Asp residue and has glutamic acid instead (Hanano and Davis, 2005) and therefore the genes are called pseudo-response regulators (PRRs). Loss-of-function of *TOC1* leads to a shortened period of clock controlled gene expression. Moreover, flowering of those plants becomes day-length insensitive (Strayer *et al*., 2000). Similar to overexpression of *CCA1* or *LHY*, overexpression of *TOC1* results in an arrhythmicity of clock outputs (Makino *et al*., 2000; Murakami *et al*., 2005). *CCA1*, *LHY* and *TOC1* have been shown to be to be functionally connected, because *CCA1* and *LHY* can bind to a so-called evening element motif in the promoter region of *TOC1* leading to transcriptional repression of *TOC1* (Harmer *et al*., 2000; Alabadí *et al*., 2001). On the other hand, increased *TOC1* expression leads to an activation of *CCA1* and *LHY* transcription (Alabadí *et al*., 2001). So a reciprocal feedback loop of gene expression leads to rhythmical oscillation of *CCA1*/*LHY* and *TOC1*, with *CCA1* and *LHY* peaking at dawn, while *TOC1* peaks at dusk.

Since this single reciprocal regulation loop cannot explain all the rhythmicity in Arabidopsis alone, recent work has focused on the identification and characterization of new core components that in additional loops participate in the clock function. Additional members of the *TOC1* family (*PRR7* and *PRR9*) as well as the GARP-MYB-domain transcription factor *LUX ARRHYTHMO/PHYTOCLOCK1* (*LUX*/*PCL1*, Hazen *et al*., 2005), *GIGANTEA* (*GI*; Park *et al*., 1999; Fowler *et al*., 1999) and *EARLY FLOWERING 4* (*ELF4*; McWatters *et al*., 2007) have been found to be associated with the circadian clock. It still remains unclear how the biological clock generates a 24h rhythm, but mathematical and experimental research currently predict to a three loop network (see fig. 1.2).

GI expression is repressed by *CCA1* and *LHY*. *CCA1* and *LHY* also are responsible for the activation of *PRR7* and *PRR9* (morning oscillator), since they are able to bind directly to CCA1-binding sites in the promoters of the latter genes (Farré *et al*., 2005). Since loss-of-function mutants of both genes (*prr7; prr9*) leads to a delay in the

period of *CCA1* and *LHY* expression, a negative feedback of *PRR7* and *PRR9* on *CCA1* and *LHY* expression is likely.

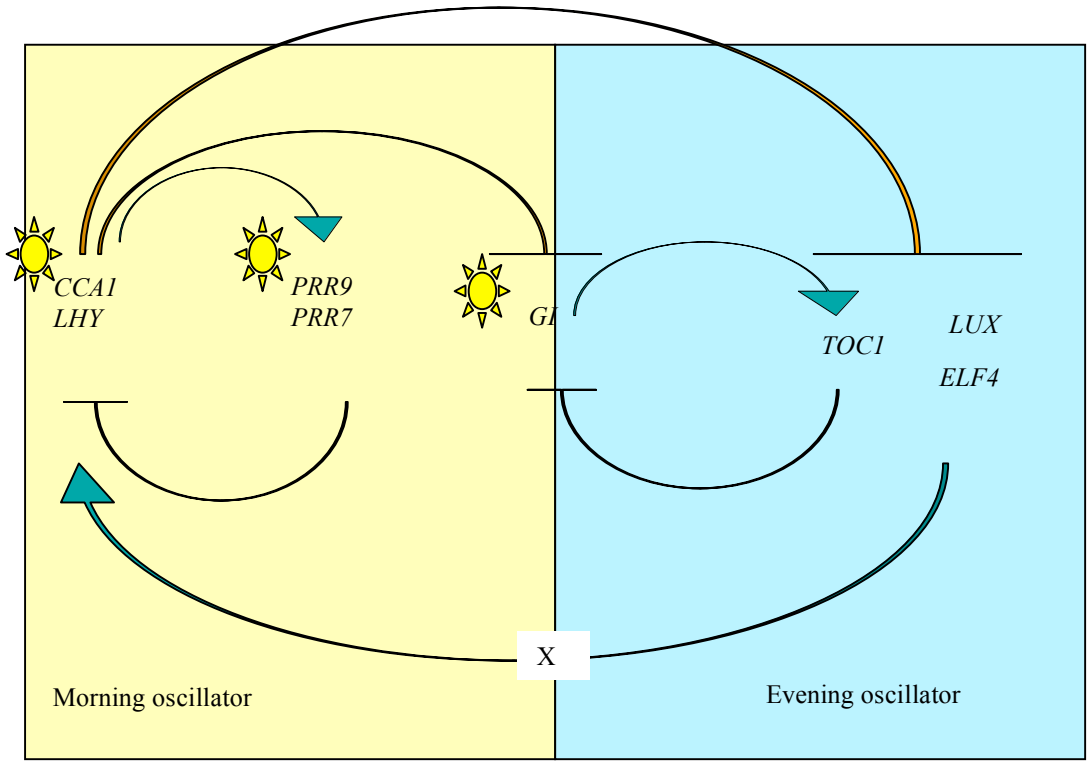


Fig. 1.2
Overview of the key components of the circadian clock in *A. thaliana* (after Mas 2008). Suns indicate an activation by light. A so far unknown component connects the evening oscillator with the morning oscillator through an activation of *CCA1* and *LHY* by *ELF4*.

1.6 Outline of the project

The experiments described hereafter were performed with the intention to come to a better understanding of the function and importance of SBP-box genes for developmental processes in plants using *Arabidopsis thaliana* as a model species. Particularly, the role of *SPL3* for the developmental phase transition from vegetative to reproductive growth should be elucidated.

Before the beginning of this thesis, it was known that overexpression of *SPL3* in a microRNA insensitive form leads to an early flowering phenotype. Furthermore, overexpression of the MIR156b locus results in a strong downregulation of *SPL3* and the other *SPL* genes targeted by microRNAs 156 / 157. Additionally, Schmid and coworkers showed, that *SPL3* and its close orthologs *SPL4* and *SPL5* strongly respond to photoperiodic induction. Taken together these observations strongly suggested a

role for *SPL3* (and *SPL4* and *5*) in the phase change from vegetative to reproductive growth. However, it remained unclear how the transcription factor SPL3 affects flowering.

In order to investigate how *SPL3* is involved in the process of flowering, it would be very helpful if direct targets for transcriptional regulation could be identified.

Firstly a candidate target gene approach, based on the knowledge that SPL3 is involved in the regulation of flowering and has been shown to bind to the core motif CGTAC, should be performed to identify putative target genes within the flowering time controlling pathways by means of transcript expression studies.

Subsequently, the technique of Cross-linked Chromatin Immunoprecipitation (X-ChIP) should be used to confirm identified candidates as direct targets for SPL3 *in vivo*. During this experiment, proteins are *in vivo* covalently linked to the DNA by infiltration of fresh plant material with formaldehyde. Then nuclei are extracted and the chromatin is sheared by sonication. The protein of interest then is immunoprecipitated along with the crosslinked DNA fragment(s) using a specific antibody. Specific enrichment of any DNA fragments can then be determined by quantitative real-time PCR.

Thirdly, the importance of spatial distribution of binding motifs for SPL3 in its target genes should be investigated with respect to spatial and temporal expression patterns of the target gene during plant development. Therefore, fusions of target gene genomic sequences to a GUS reporter should be introduces into *SPL3* overexpressing plants.

Finally, the function of *SPL3* should be integrated into the flowering time pathway, using a global transcript profiling of *SPL3* overexpressing plants compared to wild-type. On the one hand, further candidates for direct target genes for SPL3 could be expected from this approach, on the other hand more global effects of *SPL3* overexpression on metabolic pathways that are indirectly affected may become elucidated. The latter possibility may shed further light on the role of SPL3 during plant development.

2 Material and Methods

2.1 Material

2.1.1 Antibiotics

Table 1: Antibiotics in this study were used in the following concentrations:

Antibiotic	solvent	stock conc. [mg/ml]	final conc. [mg/l]	
			E. coli	*A. tumefaciens*
Gentamycin	H_2O	10	10	25
Kanamycin	H_2O	50	50	50
Rifampicin	MeOH	50	100	100
Spectinomycin	H_2O	100	100	100

Abbreviations used: conc.: concentration

2.1.2 Antibody

For X-ChIP the SPL3 specific antibody described in Gandikota *et al.*, 2007 was used.

2.1.3 Bacteria strains

E.coli strain:

DH5α (life technologies, USA)

Agrobacterium tumefaciens strains:

GV3101 (pMP90) Rifampicin and Gentamycin resistance

2.1.4 Chemicals

Chemicals and antibiotics used in this study were purchased from the following companies:

BioRad (USA), Clontech (Germany), Difco Lab (USA), Duchefa (Netherlands), Invitrogen (USA), Fermentas (Germany), Merck (Germany), Pharmacia (USA), Promega (Germany), Roche (Germany), Roth (Germany) and SigmaAldrich (Germany), Pierce (USA).

2.1.5 Enzymes

All Restriction enzymes were obtained from New England Biolabs (USA), T_4-ligase and RNase Inhibitor was purchased from Roche (Mannheim), Taq polymerase was purchased from Ampliqon (Denmark), Reverse transcriptase Superscript II was

purchased from Invitrogen (USA), ImmunoPure Immobilized Protein a sepharose beads were purchased by Pierce (USA).

Enzymatic reactions were performed according to the manufacturers' protocols if not explicitly stated otherwise in the methodological description.

2.1.6 General buffers, solutions and stocks

2.1.6.1 General Buffers

TE (Tris/EDTA)

10 mM Tris/HCL (pH 8.0 or pH 7.5)
1 mM EDTA (pH 8.0)

Tris/HCL (1M)

Tris-Base	121g
H_2O	in 1000 ml

EDTA stock (0.5M, pH 8.0)

EDTA	186.1g
H_2O	in 1000 ml

Sodium phosphate buffer (0.2M), pH 7.0

Solution I: 0.2M monobasic phosphate

NaH_2PO_4	27.6g / L

Solution II: 0.2M dibasic phosphate

Na_2HPO_4	53.65g /L

To obtain 0.2M buffer solutions I and II were mixed to 100 ml for the desired pH and then diluted with water to 200 ml.

Potassium phosphate buffer (0.1M)

Solution I: 0.2M KH_2PO_4

KH_2PO_4	27.2g / L

Solution II: 0.2M K_2HPO_4

K_2HPO_4	34.8 g / L

To obtain 0.2M buffer solution I and II were mixed to 100 ml for the desired pH and then diluted with water to 200 ml.

2.1.6.2 Solutions and stocks

Ethidium Bromide stock (10 mg/ml)

Ethidium bromide	0.2 g
H_2O	in 20 ml

Stored at 4°C in the dark.

DNA gel loading buffer (6x)

Bromphenol blue	0.25%
Xylen cyanol FF	0.25%
Glycerol	30% (v/v)

GUS histochemical Buffer

$NaPO_4$	0.20 M
$K_3Fe(CN)_6$	0.05 M
$K_4Fe(CN)_6$	0.05 M
EDTA	0.50 M
Triton X-100	10% (v/v)
in H_2O	

Stored at 4 °C.

X-Gluc stock

50 mg/ml in DMSO has to be prepared freshly.

GUS staining Buffer (10 ml)

X-Gluc stock (50 mg/ml)	0.12 ml
GUS histochem. Buffer	8.00 ml
Methanol	2.00 ml

2.1.6.3 Buffers for genomic plant DNA extraction

CTAB Buffer

Tris/HCL, pH 8.0	100 mM
NaCl	1.4 M
EDTA	20 mM
CTAB	2 %

Edwards Extraction Buffer

Tris/HCl pH 7.5	200 mM
NaCl	250 mM
EDTA	25 mM
SDS	0.5 %

2.1.6.4 Buffers for bacteria DNA extraction

TELT (DNA extraction buffer)

Tris/HCL pH 7.5	50 mM
EDTA	62.5 mM
LiCl	2.5 M
Triton X-100	0.4 % (autoclave)

Lysozyme solution

Lysozyme 10 mg/ml
Tris/HCL pH 7.5 10 mM
EDTA 0.1 mM

PBS (1 L)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g in 800 ml water

pH was adjusted with HCl to 7.4, water was added up to 1 L and buffer was autoclaved.

2.1.6.5 Buffers for X-ChIP

Fixation buffer (500 ml)

0.1M Na-Phosphate buffer pH 7.4

101.25 ml 0.2M Na_2HPO_4

23.75 ml 0.2M NaH_2PO_4

pH was adjusted to pH 7.4 with NaH_2PO_4; volume was adjusted to 250 ml with water. 50 ml Paraformaldehyde (final conc. 0.1%) was added and volume adjusted to 500 ml)

Washing buffer

0.1M Na-Phosphate buffer, pH 7.4
0.125 M Glycine

Buffer A

10 mM Hepes/NaOH pH 7.9
10 mM KCl
1.5 mM $MgCl_2$
0.5 mM DTT
0.1% NP-40
20% Glycerol

Sonication buffer

10 mM Hepes/NaOH pH 7.9
1 mM EDTA pH 8.0
0.5 % SDS

Dilution buffer

60 mM Hepes/NaOH pH 7.9
1 mM EDTA pH 8.0
175 mM NaCl
0.625% NP-40
0.18% NaDOC
156 mM Glycine

Buffer was used for 1:4 dilution of sonified samples to obtain a final concentration of 50 mM Hepes/NaOH pH 7.9; 1 mM EDTA pH 8.0; 140 mM NaCl; 0.5% NP-40; 0.15% NaDOC; 125 mM Glycine and 0.1% SDS in a final volume of 2.5 ml. 16.5 µl proteinase inhibitor were added after diluting the sample.

1X RIPA buffer

50 mM Hepes/NaOH pH 7.9
140 mM NaCl
1 mM EDTA pH 8.0
0.5 % NP-40
0.1% NaDOC
125 mM glycine

Glycine elution buffer (pH 2.8)

0.1 M Glycine 0.5 M NaCl 0.05 % Tween 20

pH was adjusted to pH 2.8

2.1.7 Media for bacteria

All media were sterilized by autoclaving at 121 °C for 20 Min.

YEB Medium

Beef extract	5g/l
Yeast extract	1g/l
Peptone	1g/l
Sucrose	5g/l, pH 7.5
MgSO4	2 ml/l (1M stock) after autoclaving

For solid medium 15g/l Agar is added prior autoclaving.

LB (Lauria Bertani)-Medium

Tryptone/peptone	1%
Yeast extract	0.5%
NaCl	0.5%

1.5-2% Agar is added to the above medium for solid medium. Antibiotics are added after autoclaving and cooling down to 55°C.

Infiltration Medium

1/2 MS salts (micro and macro) 2.205g/l
1/2 x B5 vitamins 50µl/l
Sucrose 50g/l
Surfactant SILWET L-77 0.005%

pH 5.7 (KOH)

2.1.8 Primers for PCR based amplification

All primers and oligos used were obtained from Operon (Germany). The sequences and efficiencies for qRT-PCR amplification are listed in appendix A.

2.1.9 Vectors

pGJ2148

Spectinomycin (75mg/l, bacteria) and Basta (0.1%, plant) as selection marker; source: Guido Jach, MPIZ

2.2 Methods

2.2.1 Plant material and growth conditions

For stratification seeds were kept either on moist paper or on soil at 4°C for at least 4 days to break dormancy and synchronize germination. Plants were grown on soil containing a 3:1 mixture of substrate and vermiculite. They were cultured under controlled environmental conditions with 18°C (night) to 22°C (day) temperature, 50% relative humidity and 99 to 229 µmol $m^{-2}s^{-1}$ light (fluorescent Sylvania F72T12 cool-white [75%] and incandescent Sylvania 100-W lamps [25%]) either under long day (LD) conditions (16 hours of light, 8 hours of darkness) or 204 to 290 µmol $m^{-2}s^{-1}$ under short day (SD) conditions (8 hours of light, 16 hours of light).

In case of testing for BASTA resistance seedlings were sprayed with 0.1% BASTA every second or third day for 3 to 5 times depending on age and sowing conditions.

Seedlings of any genotype were harvested at the age of 7 days for every experiment. For inflorescence material flowers of any age until anthesis were harvested.

2.2.2 DNA manipulation

2.2.2.1 Extraction of genomic plant DNA

CTAB Method

1 - 1.5g freshly harvested tissue is flash frozen in liquid nitrogen and ground. Ground material is resuspended in 10 ml of CTAB buffer and incubated at 60°C for 30 min. After adding the same volume of chloroform and vigorous shaking samples are centrifuged for 10 min, RT at 4000 rpm. Supernatant is transferred to a fresh tube and DNA is precipitated by adding 1/10 volume of 3M NaAc, pH 5.2 and 0.8 volume of isopropanol. After incubation for 5 min at RT DNA is pelleted by centrifugation for 15 min, RT at 4000 rpm. Pellet is washed with 70% EtOH and resuspended in 1880 µl TE buffer and 3.8 µl RNase (stock conc. is 10 mg/ml). Sample is incubated for 30 min at 37°C. Subsequently, 120µl of 5M NaCl is added and DNA is isolated using the QIAGEN TIP 20 column Kit according to the manufacturer's protocol.
DNA is resuspended in 50-100µl 1xTE buffer. DNA concentration is determined photometrically and sample is stored at a concentration of 1µg/µl at 4°C.

Edwards Method

For quick isolation of genomic DNA, a method from Edwards *et al.* (1991) was adapted. Harvested tissue is ground in 400µl Edwards Extraction Buffer and sterile sand with a pistil. After centrifugation for 2 min, 4°C 13.000 rpm the supernatant is carefully mixed with the same volume of isopropanol and incubated for 5 min at RT to precipitate DNA. DNA is pelleted (5 min, RT, 13.000 rpm) and washed with 70% EtOH 3 times. Pellet is resuspended in 100µl 1xTE buffer. For PCR a dilution of 1:100 is used.

2.2.2.2 Extraction of bacterial DNA

Plasmidminiprep with TELT-Method

To do a plasmidminiprep with the TELT-method 1 ml of a well grown overnight culture of bacteria is centrifuged 2 min, RT at 13.000 rpm.

Alternative: 1 ml of a well grown overnight bacteria culture was centrifuged 2min at RT at 13000rpm.

The pellet is resuspended in 200µl TELT buffer and 20µl lysozyme solution. After 3 min incubation at 95°C the sample is immediately put on ice for 5 min and subsequently centrifuged for 15 min, 4°C at 13.000 rpm. The pellet is removed with a toothpick and discarded. The supernatant is mixed with 100µl isopropanol and immediately centrifuged for 15 min, 4°C at 13.000 rpm. The formed pellet is washed with 70% EtOH, air-dried and resuspended in 50µl TE buffer (+RNase 10µg/ml). In case of a high copy number plasmid 2µl are used for digesting; in case of a low copy number 4 µl are used.

Isolation of plasmid DNA from gels

In order to isolate DNA fragments out of an agarose gel the NucleoSpin® extraction Kit (Macherey-Nagel) was used according to the manufacturer's protocol.

2.2.2.3 Precipitation of DNA

DNA was precipitated by adding 1/10 volume of 3M NaAc pH 2.8 and 2 volumes of 70% EtOH and putting samples on ice for 1 hour or at -20°C overnight. Subsequent

centrifugation for 45Min at 4°C, twice washing with 70% EtOH. Pellet was air-dried and resuspended in 50-100µl of 1xTE buffer or 10mM Tris.

2.2.2.4 PCR reactions

Standard PCR reaction

All standard PCR reactions were done on a Biometra T 3000 thermocycler. For standard PCR reactions Taq DNA polymerase from Amplicon (5 Unit/µl) with the 10x standard buffer (15 mM $MgCl_2$) was used. A standard PCR reaction was carried out in a total volume of 25µl, the reaction solution contained the following ingredients:

dNTPs	(25µM)	0.5 µl
Primer forw.	(20µM)	0.5 µl
Primer rev.	(20µM)	0.5 µl
10X buffer		2.5 µl
Taq		0.3 µl
DNA template		1.0 µl
H_2O		ad 25µl

Annealing temperature: 55-65 °C, depending on Primers.
Elongation time: 1 min / 1 kb
Cycles: 28-35 cycles

Quantitative RT-PCR (qRT-PCR; real-time PCR)

qRT-PCR was carried out on iQ5 Multicolor Real-time PCR Detection System (BioRad) using the IQ SYBR Green Supermix (BioRad). Wellfactors were collected during the run.

A standard qRT-PCR reaction was carried out in a total volume of 25µl, the reaction solution contained the following ingredients:

SYBR-Green Master Mix	12.5 µl
Primer forw. (2.5 mM)	1.25 µl
Primer rev. (2.5 mM)	1.25 µl
cDNA	10 µl

qRT-PCR program (3 step Amplification & Melting curve):

Cycle 1: (1x)

Step 1:	95.0°C	for 3:00 min

Cycle 2: (45x)

Step 1:	95.0°C	for 00:30 min
Step 2:	58°C	for 00:30 min

Data collection enabled

Step 3:	72°C	for 00:45 min

Data collection and real-time analysis enabled.

Cycle 3: (1x):

Step 1:	95°C	for 1:00 min

Cycle 4: (1x)

Step 1:	55°C	for 1:00 min

Cycle 5: (81x)

Step 1:	55.0°C-95.0°C	for 00.10 min

Data were analyzed using the BioRad software provided with the iQ5 thermocycler and program.

2.2.3 RNA manipulation

2.2.3.1 Extraction of total RNA from plant tissue

In order to extract total RNA from plant tissue the Qiagen RNeasy Plant Mini Kit was used. Extraction was performed according to the manufacturer's protocol, but on-column DNA digestion was skipped. For DNA digestion 4µl DNase (Roche) and 4 µl 10x buffer were used in a total volume of 40µl. The incubation was performed for 30 - 60 min at 37°C, then 10 min at 65°C after elution of RNA from the columns and a subsequent centrifugation step (15-20 min, RT, 13.000rpm) to get rid of eventual column material.

Success of the DNA digestion was assured by performing a PCR with 40 cycles on the RNA samples using Primers for intronic sequences (usually NB 4 and NB 2 for *FUL* 1st intron). The RNA concentration was performed photometrically in a dilution of 1:59 against water

2.2.3.2 Precipitation of RNA

RNA was precipitated by adding 1/10 volumes of 3 M NaAc pH 2.8 and 2 volumes of 100% EtOH and putting it on ice for 1 hour or at -20°C overnight.

2.2.3.3 Reverse Transcription

Reverse transcription of RNA was done by using Superscript II (Invitrogen) according to the manufacturer's protocol.

2.2.3.4 Hybridization of Affymetrix Arabidopsis ATH1 arrays

Hybridization of Affymetrix Arabidopsis ATH1 arrays was performed at the University of Münster at the Institute of integrated and functional genomics, Röntgenstraße 21, 48149 Münster.

2.2.4 Protein manipulation

2.2.4.1 Crosslinked Chromatin immunoprecipitation (X-ChIP) (modified after Orlando 2000 and Lauri 2005)

Fixation of plant material

3 g of plant material were washed in 40 ml H_2O and dried on paper. 40 ml of fixation buffer were added and samples were vacuum infiltrated for 20 min. Fixation was stopped by adding 2.5 ml of 2M glycine (final conc. 0.125M). Material was washed three times with washing buffer, dried on paper, flash frozen in liquid nitrogen and stored at -80°C.

Sonication

Fixed plant material was ground and powder was collected and homogenized in 30 ml ice cooled buffer A with 200 µl proteinase inhibitor added. Material was filtered through 300 - 75 - 20 - 10 µm membranes by centrifugation at 3000 rpm for 20 min at 4°C. Flow-through was collected in 50 ml falcon tubes and nuclei were pelleted by centrifugation at 1700 rpm for 10 min at 4°C. The pellet was washed with 10 ml of washing buffer until it was light white and supernatant was clear. Then it was resuspended in 1 ml buffer A and transferred to a 1.5 ml tube. After centrifugation at 13000 rpm for 10 min at 4°C in was resuspended in 500 µl freshly prepared sonication buffer and incubated for 30 min slightly shaking at 4°C. Nuclei were sonified with Dr. Hielscher UP50H sonicator for 12 x 10 sec with a cyclic control of 0.5 and a amplitude between 50 - 60%. Under these conditions DNA was sheared to fragments between 350 and 650 bp, which can proven on a 1% agarose gel. A 10-

20µl aliquot was transferred into a fresh 1.5 ml tube and used as the "input" for PCR later on to calculate the amount of precipitated chromatin. The rest of the supernatant was diluted 1:4 with dilution buffer to reduce the amount of SDS to under 0.1%, which might disturbed the subsequent immunoprecipitation. Water was added up to a final volume of 2.5 ml. 16.5µl of proteinase inhibitor were added as well.

Immunoprecipitation

2.5 ml of the supernatant were transferred in equal amounts of 833µl to fresh tubes. The SPL3-specific antibody was added in 1:200 fold dilution (i.e. 4 µl). Samples were incubated over night at 4°C with slight shaking.

50 µl of protein A sepharose beads were washed 4 times with 1 X RIPA buffer (including proteinase inhibitor) and added to the samples to precipitate the chromatin-protein complex with the immunoprecipitated antibody. After incubation of the samples for two hours at 4°C beads were centrifuged for one minute at 3800g at 4°C. Supernatant was kept and stored at -20°C .

Elution

Beads were washed three time with ice cooled 1X RIPA buffer. Elution of immunoprecipitated protein-DNA-complexes was performed by adding 100 µl glycine-elution buffer, vortexing for 15 sec and centrifugation at 13000 rpm for 1 minute at RT. The supernatant was neutralized with 10 µl of 1M Tris pH 8.0. Elution was repeated twice, the fractions were pooled and centrifuged at 13000 rpm for two minutes at RT. 420µl of the supernatant were transferred to a fresh tube, 10.5 µl of 10% SDS and 8 µl of proteinase K (40 mg/ml stock) were added and sample was incubated at 37°C overnight.

De-crosslinking

1µl of RNase (40 mg/ml stock) was added to the samples and incubated for one hour at 37°C. A subsequent incubation at 65°C for 6 hours or overnight removes the formaldehyde induced crosslinks.

Afterwards DNA was extracted by phenol-chloroform extraction and precipitated using isopropanol and NaAc (see 2.2.2.3).

Samples were resuspended in 20µl 10 mM Tris pH 8.0.

2.2.5 Determination of circadian rhythms with leaf movement measurement

In order to investigate circadian rhythms the leaf movement of 7d old seedlings under free running conditions was measured following Edwards and Millar 2007.

2.2.5.1 Preparation of seeds and growth conditions

About 100 seeds were surface sterilized by soaking in 98% Ethanol for 4 min, followed by soaking in 10% hypochloride for 10 min at RT. Hypochloride was removed with a pipette and seeds were washed 3 times in aqua dest., before resuspending them in 1 ml 0.1% agar and stratifying at 4°C for 4 days.

Seeds were plated on MS plates containing 2% agar and 3% sucrose. Plates were kept in 12h light/12h dark growing cabinets for 5 days. Seedlings were then transferred to fresh plates cut into 1 cm^2 and transferred randomly into 25-well tissue culture plates (Bibby Sterilin Ltd, UK). After another 24 hours in the growing cabinets, plates were placed in front of cameras in a continuous light (CL) growing chamber. Pictures were taken every 30 min for 6 days.

2.2.5.2 Data analysis of leaf movement measurement

For data collection as well as measuring the leaf position the MetaMorph software package was used according to Edwards and Miller (2007) and the manufacturer's protocol. In order to analyze the leaf movement rhythms the MS Excel interface Biological Rhythm Analysis Software System (BRASS) package was used, which is available at www.amillar.org and which is used to perform a fast-Fourier transform-nonlinear least squares analysis of the period, phase and amplitude of circadian rhythms (see Edwards and Miller 2007 for a detailed description).

2.2.6 Remaining Techniques

2.2.6.1 Cloning

In order to investigate the binding site specificity of SPL3 protein to GTAC motifs in *FUL* promoter and / or *FUL* 1st intron, a *FUL* genomic fragment of a size of 2748 kb was amplified using a forward primer with an artificial *Xma*I site (SH 250) and a reverse primer with an artificial *Nco*I site (SH 246). Subsequently the fragment was cloned in the *Xma*I / *Nco*I site of the pGJ2148 vector. The construct was called pSH56 and included the putative promoter of *FUL* with a size of 1616 bp (before ATG), *FUL* 1st exon, *FUL* 1st intron and 42 bp of *FUL* 2nd exon.

In a second construct (pSH57) the same *FUL* fragment that was lacking the 1st intron was cloned into pGJ2148. Both constructs were further modified, so that the promoter motif CCGTACGCGTAC (5'-3' orientation) 461 bp upstream of the ATG was changed to CACTAGTGAAG (5'-3' orientation) and concurrently an artificial *Spe*I site was inserted.

Therefore, from the respective constructs a fragment of 1178 bp was amplified using the forward primer SH 250 with the artificial *Xma*I site and the reverse primer NB 52, which inserted the artificial *Spe*I site. And a second fragment of 1010 bp was amplified using NB 51 as a forward primer that included the artificial *Spe*I site and NB 3 as a reverse primer. The second fragment was digested with *Bgl*II and then fused to the first fragment via the *Spe*I site. The resulting fragment was cloned into the *Xma*I / *Bgl*II site of pSH56 and pSH57. The constructs were transformed into Col-0 plants by means of Agrobacterium mediated transfection.

2.2.6.2 Sequencing

All sequencing reactions were done by the ADIS core facility at the MPIZ in Cologne.

2.2.6.3 Arabidopsis transformation

For transformation of Arabidopsis plants competent agrobacteria were prepared. Therefore 5 ml YEB media was inoculated with 5 ml of agrobacteria stock and incubated at 28°C for two days. Subsequently the culture was transferred into 500 ml YEB media and incubated for four to six hours at 28°C and transferred on ice afterwards to cool down. After centrifugation at 4°C for 15 min at 4200 rpm the resulting pellet was resuspended in 100 ml ice cooled H_2O. Cooling, centrifugation and resuspension was repeated three times, with resuspension in 50 ml ice cooled H_2O, 5 ml ice cooled 10% glycerol and 800 ml ice cooled 10% glycerol respectively. Cells were snap-frozen in liquid nitrogen in aliquots and stored at -80°C until usage for electroporation.

Arabidopsis plants were grown under SD conditions and used for transformation using the floral dip method after Clough and Bent 1998. Transgenic plants were selected by spraying seedlings three times with 0.1 % BASTA.

2.2.6.4 GUS staining

For GUS staining tissue was harvested and submerged completely in freshly prepared GUS staining buffer. After vaccuum infiltration for 20 min material was incubated overnight at 37°C. Chlorophyll was removed by incubation in several changes of 70% ethanol until chlorophyll was bleached. Stained material was then analysed and recorded using a LEICA MZFL III stereomicroscope equipped with a digital camera.

2.2.7 Data analysis of affymetrix arrays

The normalized raw data from the hybridization of the affymetrix arrays were analyzed using GCOS, Cyber T and MS Excel.

First of all, for all expression values PPDE values (p-values) were calculated using the online program Cyber T (http://cybert.microarray.ics.uci.edu/). From the whole data set only those genes were further analyzed, that had more than a 1.5 fold change in expression level in a mutant compared to wt and a confidence level of above 0.95. Genes, having an absence call according to GCOS in all of the samples were not further analysed. Subsequently those genes were ranked according to their p-values. From this list the genes were categorized according to their Gene Ontology (GO) annotations using a tool found on http://www.arabidopsis.org/tools/bulk/go/index.jsp , the Arabidopsis Information Resource (TAIR) web site. Afterwards a more detailed GO term analysis was performed with the web-based tool AmiGo (http://amigo.geneontology.org/cgi-bin/amigo/term_enrichment.

Each not described method was performed according to Sambrook *et al.* 1989.

3. Results

3.1 Identification of putative SPL3 target genes following a candidate gene approach

To answer the question on how the SBP-box transcription factor SPL3 promotes the transition from vegetative to reproductive growth, a first attempt was to identify target genes following a candidate gene approach. Such a candidate target gene should fulfil several criteria.

First of all, *SPL3* has been shown to be negatively regulated by the microRNA miR156 (Schwab *et al*., 2005; Wu and Poethig, 2006; Gandikota *et al*., 2007). If SPL3 acts as a transcriptional activator, its target genes are likely to be among the most downregulated genes in plants overexpressing MIR156b - particular at stages where normally SPL3 becomes upregulated, i.e. the transition apex (Schwab *et al*., 2005).

Secondly, it has been shown that constitutive overexpression of *SPL3* in a microRNA insensitive form results in early flowering (Cardon *et al*., 1999). Therefore it is reasonable to assume that target genes of SPL3 are also involved in the process of flowering.

Thirdly, Birkenbihl *et al*. (2005) showed that SBP-domain proteins prefer GTAC in general and SPL3 CGTAC in particular, as core DNA recognition sequence. A direct target gene of SPL3 is thus expected to carry (C)GTAC motifs in its promoter region or close vicinity. Additional support for the assumptions above comes from the fact that *SPL3* is the presumed ortholog of *SBP1* from *A. majus*. SBP1 has been shown to bind *in vitro* to a regulatory sequence in the promoter region of the *Antirrhinum* floral meristem identity gene *SQUA*. Interestingly, the Arabidopsis ortholog of *SQUA* is *AP1*, but despite the conservation of the SPL3 recognition sequence in its promoter region, *AP1* turned out not to be essential for the early flowering phenotype of the constitutive *SPL3* overexpressing transgenic Arabidopsis plants (Cardon *et al*., 1997). However, *AP1* and *SQUA* are closely related to several other members within the core eudicot *AP1*/*FUL* lineage of MADS-box transcription factors (Litt and Irish, 2003) and it is not unlikely that in Arabidopsis the *AP1* paralog *CAULIFLOWER* (*CAL*; Kempin *et al*., 1995) or *FRUITFULL* (*FUL*; Gu *et al*., 1998) represent actual targets of SPL3. I finally came up with a list of six candidate genes fulfilling the criteria discussed above (fig. 3.1, *AP1* is shown for comparison).

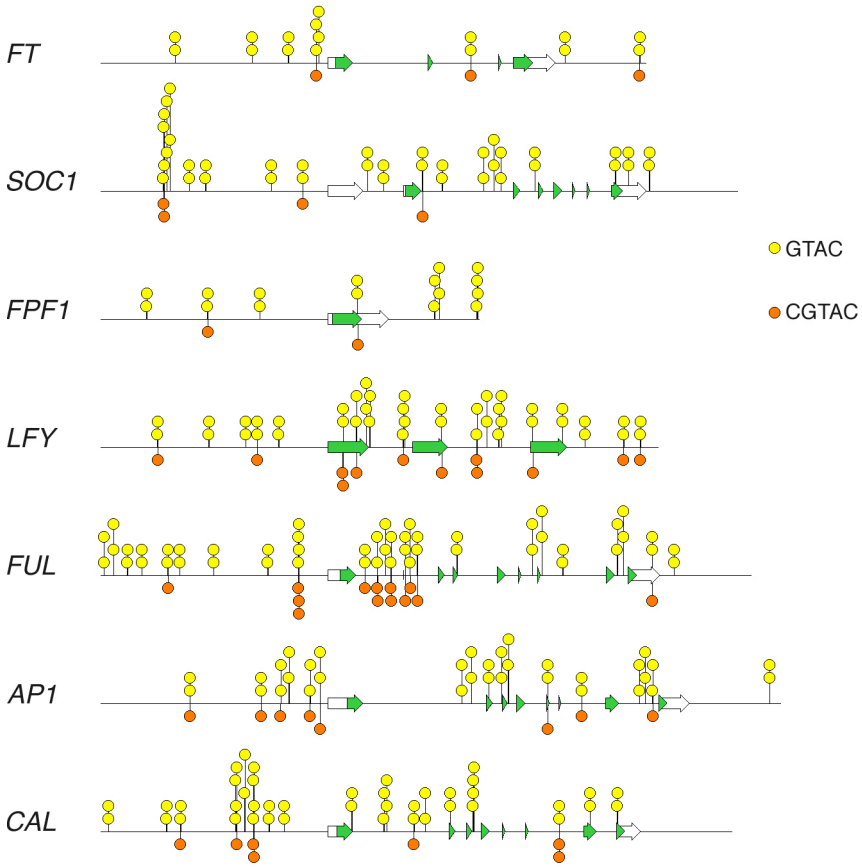


Figure 3.1
A list of putative target genes for SPL3. Shown is the genomic locus of the respective genes based on annotated coding sequences (green) and mRNA (in white). Depicted are 2.5kb upstream and 1 kb downstream of the respective gene. Any intron-exon structure of any flanking loci, if present, were deleted. Yellow dots designate the binding motif GTAC on both strands, orange dots display SPL3's preferred motif CGTAC. *AP1* shown for comparison.

With the exception of *AP1*, all of the listed genes are among the most differentially expressed genes of the global expression analysis of plants overexpressing MIR156b (Schwab *et al.*, 2005). Furthermore, the listed genes are all involved in flowering time and are key regulators of this pathway as the floral pathway integrators *SOC1* and *FT* integrate the signals coming from the photoperiodic pathway, the vernalization pathway and the autonomous pathway. Moreover, the floral meristem identity genes *FUL*, *CAL*, *LFY* define the meristem identity. *FPF1* is also involved in the floral transition and might act on the same level as it integrates the signal coming from the GA biosynthesis and signalling pathway. But since it displays only two of the SPL3 binding motifs, it was not further analysed.

Birkenbihl et al. (2005) noticed an underrepresentation of both the CGTAC and GTAC motif in the Arabidopsis genome. The frequency with which these motifs appear is only half of what could be expected for a random DNA sequence with a GC content of about 40%.

As illustrated by yellow dots in figure 3.1 all of the listed genes display an accumulation of the binding motif GTAC in either promoter or intronic regions. Furthermore, the SPL3-

binding motif CGTAC is overrepresented in the *FUL* genomic sequence, especially in the first intron. Therefore the listed genes may well be putative target genes of SPL3.

3.1.1 Expression analysis of floral pathway integrators and floral meristem identity genes early in *SPL3OX* and wild type development

Precocious activation of any of the floral pathway integrators or the floral meristem identity genes, identified above as candidate SPL3 target genes, could help to explain the early flowering phenotype of *SPL3* overexpressing (*SPL3OX*) plants. Therefore, I determined the relative transcript levels of these genes in 7-day-old seedlings grown in LD with the help of quantitative real-time PCR (qRT-PCR). From three independent *SPL3OX* lines, the two lines with the most different *SPL3* expression levels, i.e. *SPL3OX*-2342 and *SPL3OX*-2350 (Fig. 3.2A), were chosen for the analysis. The normalized expression levels of the candidate target genes were compared to wild-type expression levels. As expected, transcript levels of all tested genes are relatively low in wild-type seedlings (Fig. 3.2B), since they become only upregulated upon the floral transition. In comparison to wild type, however, transcript levels of the floral pathway integrator *FT*, which becomes induced by LD conditions, were clearly upregulated in *SPL3OX* seedlings. In contrast, the transcript levels of *SOC1*, which acts together with *AGL24* (Liu *et al*., 2008) to promote flowering and the inflorescence architecture (Lee *et al*., 2008), were not differentially expressed between *SPL3OX* and wild-type plants. Furthermore, whereas expression of the floral meristem identity genes seemed largely unaffected in the case of *AP1* and *CAL* and only a slightly elevated for *LFY*, *FUL* transcript levels were strongly raised in the transgenics (Fig. 3.2C).

Interestingly, in the context of the findings above, an early flowering phenotype has previously been described for plants constitutively overexpressing *FT* (Teper-Bamnolker and Samach, 2005). Furthermore, the phenotype of these plants mimics that of *SPL3OX* plants in other aspects too, e.g. relatively small with curled leaves. Teper-Bamnolker and Samach found an accumulation of *FUL* transcripts in response to *FT* overexpression in seedlings as well as of the MADS-box transcription factors *AP1* and *SEPALLATA3* (*SEP3,* Mandel and Yanovsky 1998). Therefore, I additionally determined the expression of *SEP3* and found it slightly increased in *SPL3OX* compared to wild type (Fig. 3.2D). It should be noted, however, that the relative increase in expression levels of both *FT* and *SEP3* in *SPL3OX* seedlings were found to be much lower in comparison to that of *FUL*. The big differences in *FT* expression

might be due to different sampling time points of the analyzed material, since *FT* shows a strong diurnal expression pattern.

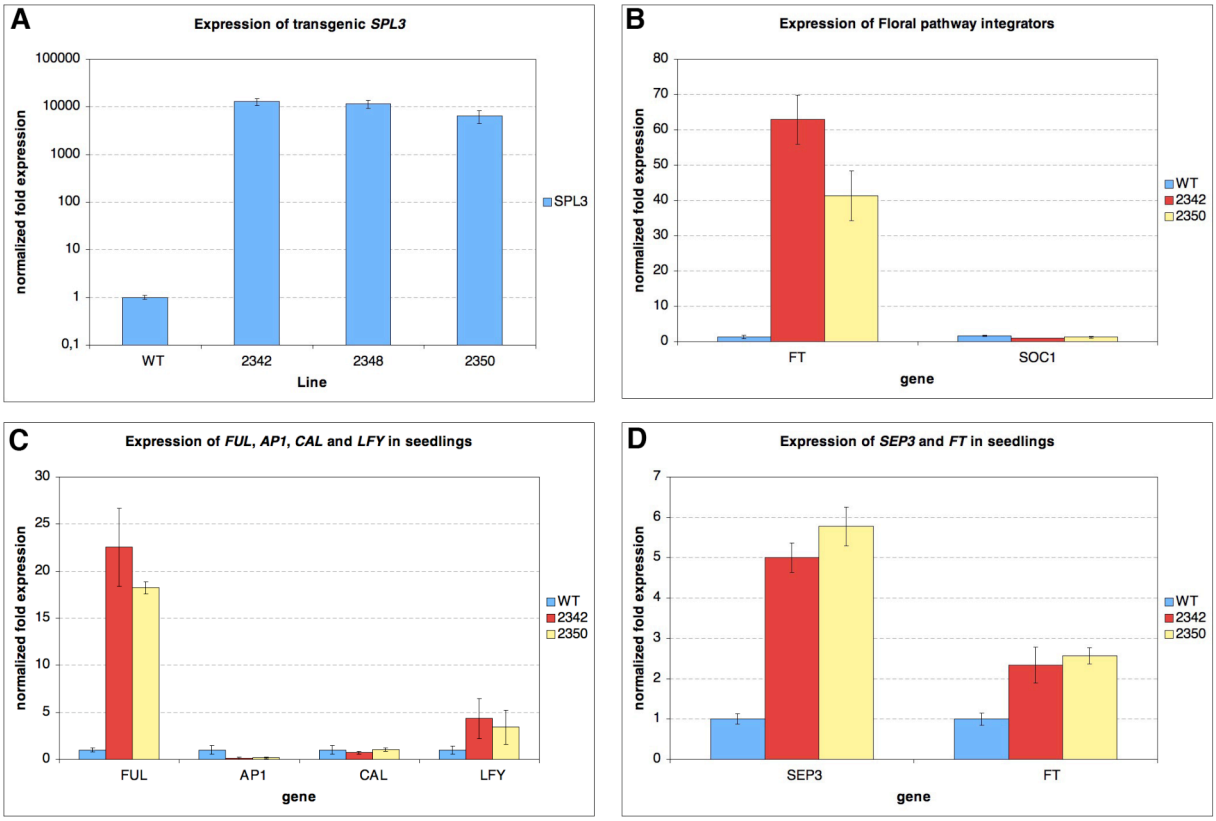


Figure 3.2
Comparative qRT-PCR expression analysis of wt and *SPL3OX* transgenic seedlings.
A) Normalized expression levels of *SPL3* in 7-d-old seedlings in three independent lines of *SPL3OX* (lines 2342, 2348 and 2350) compared to wild type.
B) Normalized expression levels of the floral pathway integrators *SOC1* and *FT* in 7-d-old seedlings of *SPL3OX* (lines 2342 and 2350) compared to wild type.
C) Normalized expression levels of the floral meristem identity genes *AP1*, *CAL*, *FUL* and *LFY* in 7-d-old seedlings of *SPL3OX* (lines 2342 and 2350) compared to wild type.
D) Normalized expression levels of *FT* and *SEP3* in 7-d-old seedlings of *SPL3OX* (lines 2342 and 2350) compared to wild type.
Arbitrarily the expression level of the respective genes in wild-type is set at one and was chosen for reference.

3.1.2 Diurnal expression of *FT* and *FUL* in *SPL3OX* under LD conditions

Previous studies showed that plants constitutively overexpressing the microRNA insensitive form of *SPL3* remain photoperiod sensitive (Cardon *et al*., 1997; Gandikota *et al*., 2007). A key gene in photoperiodic induction of flowering in Arabidopsis is *CONSTANS* (*CO*; Putterill *et al*., 1995) a direct regulator of the floral pathway integrator *FT* (Samach *et al*., 2000). *CO* expression is closely associated with the circadian clock through *GIGANTEA* (*GI*; Fowler *et al*., 1999; Park *et al*., 1999), which is involved in the degradation of repressors of *CO* transcript (Sawa *et al*., 2007). Furthermore, CO protein is rapidly degraded in darkness but stabilized by light under LD conditions. As a result of the complex regulation of *CO* on both

transcriptional and post-translational level, *FT* expression shows a diurnal rhythm with a strong peak at the end of the light period in LD conditions (Mizoguchi *et al.*, 2005). Therefore, the time of sampling may become decisive in uncovering altered transcriptional behaviour of genes with circadian-dependent expression.

To investigate the possibility that a single sampling at a particular time of the day did not reveal the true extent of *SPL3* overexpression on target gene transcript levels, seedlings grown under LD conditions were sampled after 7 days in 4-h intervals over a 24-hour period. *FT* as well as *FUL* transcript levels were determined by qRT-PCR.

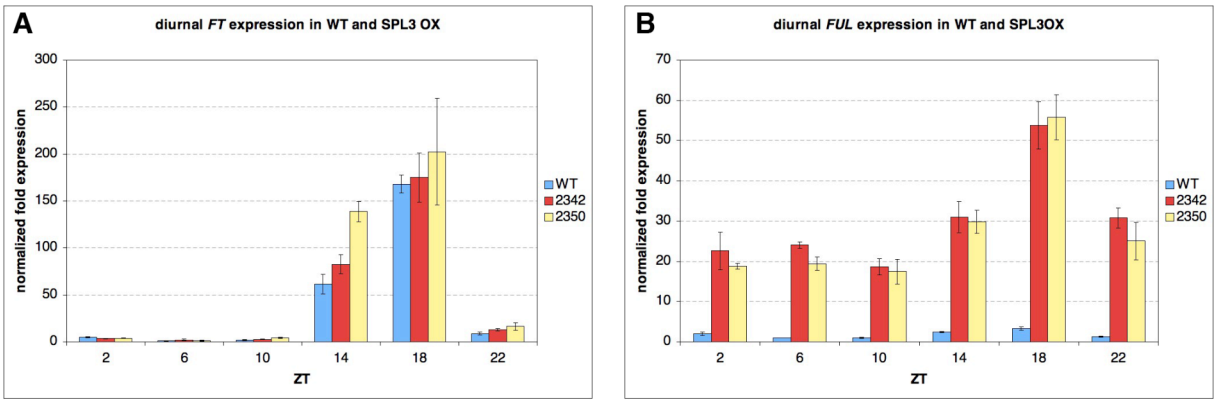


Figure 3.3
Expression analysis over a 24-h period in seedlings of wt and *SPL3OX* transgenics (lines 2342 and 2350) grown for 7 days under LD conditions of *FT* (A) and *FUL* (B).
Expression levels were normalized against *PP2A*. ZT 0 represents the beginning of the light period. Expression of *FT* (A) or *FUL* (B) in wt at ZT 6 (i.e. 6 h after the beginning of the light period) was chosen as a reference and set arbitrarily at one.

Figure 3.3A shows the *FT* transcript-level variation over 24 h in *SPL3OX* seedlings in comparison to wild type with the wild-type sample at ZT 6 chosen as reference with value one. From dawn until 6 h (ZT 10) before the end of the light period, *FT* transcript levels remained relatively low in wild type as well as in the *SPL3* overexpressors. Thereafter, *FT* transcript levels strongly raised in wild type and this could also be observed in the *SPL3* overexpressors resulting in levels being even, albeit moderately, higher than in wild type.

As shown in figure 3.3B the *FUL* transcript levels in the same samples stayed low over the whole day in wild type, while they were significantly upregulated in the *SPL3* overexpressors during the light and dark period. Interestingly, *FUL* seemed to behave like *FT,* i.e. becoming higher expressed towards the end of the light period in the *SPL3* overexpressing lines. Therefore, it appeared reasonable to assume the possibility that *SPL3* regulated *FUL* expression through *FT*.

3.1.3 Phenotypic characterization of *ful SPL3OX* and *ft SPL3OX* double mutants

It has previously been shown that *AP1* does not affect the earliness of flowering caused by a constitutively overexpressed *SPL3* transgene (Cardon *et al*., 1997). Also, additional loss-of-function of the *AP1* paralogous and functionally redundant gene *CAL* (*35S::SPL3 ap1*-1 *cal*-1) did not attenuate early flowering and the same holds true for the non-MADS-box floral meristem identity gene *LFY* (P. Huijser, personal communication). These observations are in agreement with the largely unaffected transcript levels of these genes in *SPL3OX* transgenics as reported above. In contrast, the candidate gene *FUL,* acting redundantly with *AP1* and *CAL* to control floral meristem identity (Ferrándiz *et al*., 2000), was found to be strongly upregulated in these transgenics. The role of *FUL* in the *SPL3OX* transgenic phenotype, however, had not been studied before. Furthermore, *FUL* expression in LD grown *SPL3OX* transgenics positively correlated with the circadian rhythm in *FT* expression. This all led to the obvious question whether *FUL* and/or *FT* are involved in mediating the effect of *SPL3* on flowering. Thereto, two independent *SPL3* transgenic lines were crossed respectively to a *ful*-2 and a *ft*-10 mutant, both in Col-0 background. For *ful SPL3OX* and *ft SPL3* plants, homozygous double mutants were selected from an F2 population and analyzed for their flowering time by determining the average total leaf number (TLN, i.e. number of rosette and cauline leaves formed by the primary meristem). In addition, the time to bolting (shoot 0.5 cm over the rosette) and anthesis were recorded. For comparison, the same parameters were determined for parental mutant and transgenic lines, and for Col-0 wild type, all grown under the same conditions.

As shown in Fig. 3.4A the phenotype of the *ful SPL3OX* double mutant resembled much more that of the parental *ful*-2 mutant line or wild type than that of the *SPL3* overexpressing parents. For instance, rosette and cauline leaves of the double mutant did not curl, as is often observed in *SPL3* overexpressing plants (Gandikota *et al*., 2007). Interestingly, the *ful SPL3OX* plants flowered later than *SPL3OX* parental lines (5.3 and 5.6 rosette leaves respectively) with about 9.6 and 8.5 rosette leaves respectively (table 3.1). The number of rosette leaves resembled much more the *ful*-2 flowering time (10.8 rosette leaves) than wild type (14.0 rosette leaves). Also the number of cauline leaves in *ful SPL3OX* plants (3.6 and 3.3 respectively) resembled more the wild type (4.3) or *ful*-2 plants (4.5) than the *SPL3OX* (1.4 and 1.6 respectively). The number of days to bolting was reduced in *SPL3OX* plants (17.0 and 18.1 days respectively) compared to wild type (20.6 days) and resembled in *ful SPL3OX* plants (23.0 and 22.0 days respectively) more the *ful*-2 plants (23.4 days). Also the number of days from bolting until anthesis was somewhat longer in *ful SPL3OX* plants (5.1 and 6.0 days

respectively) compared to wild type (5.0 days) and resembled more the *ful*-2 parent (5.1 days). *SPL3OX* parents though also took about 0.5 days more for anthesis than wild type. Therefore, concerning the total leaf number (TLN) *ful SPL3OX* plants flowered a little earlier than wild type under LD conditions, but concerning the days to bolting were as late as wild type. Interestingly, *SPL3OX* as well as *ful SPL3OX* plants seemed to take a little longer to open the first flower (table 3.1).

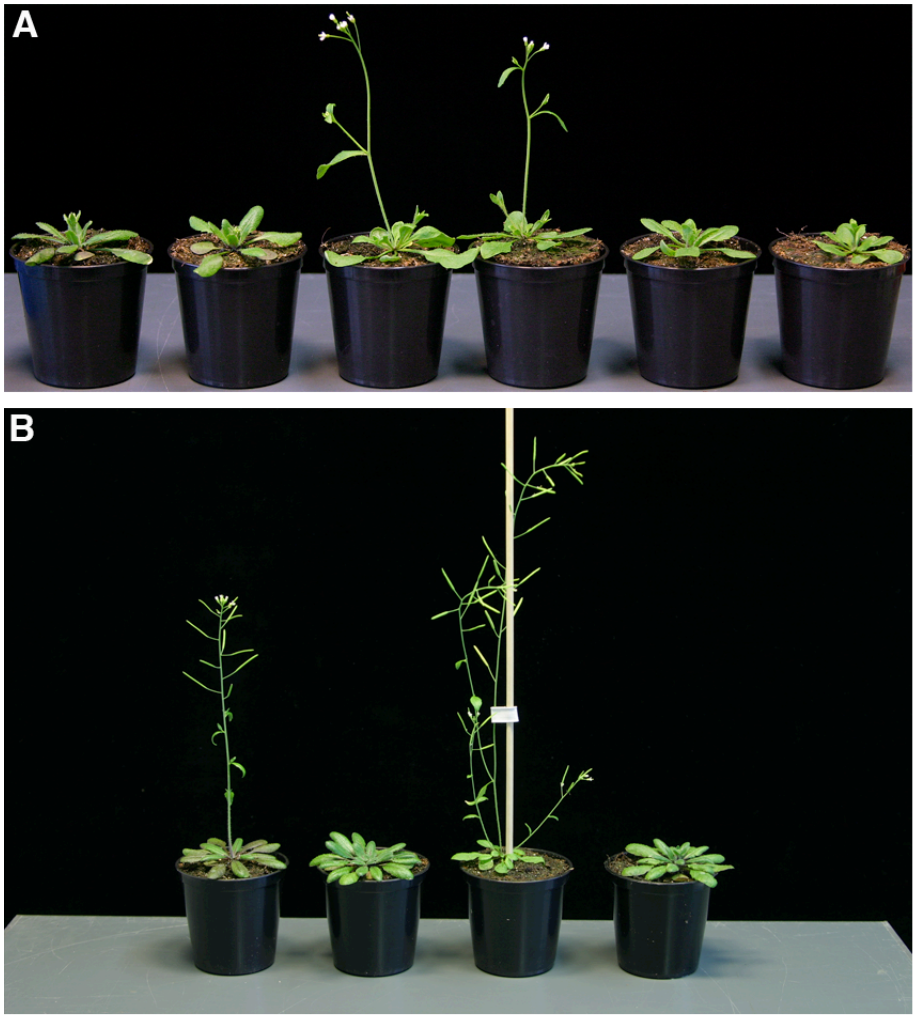


Figure 3.4
Phenotypes of, from left to right, wild-type, *ful*-2, *SPL3OX*-2342, *SPL3OX*-2350, *ful SPL3OX*-2342 and *ful SPL3OX*-2350 plants grown under LD conditions
A) Phenoytpes of wild-type, *ful*-2 and *SPL3OX*-2342, *SPL3OX*-2350, *ful SPL3OX*-2342 and *ful SPL3OX*-2350 plants at the same age of about 3.5 weeks after sowing grown under LD conditions.
B) Phenotype of wild-type, *ft*-10, *SPL3OX*-2342 and *ft SPL3OX*-2342 plants at the same age of about 8 weeks after sowing grown under LD conditions.

Finally, in contrast to the effect of ectopic *SPL3* expression in *ap1* and *ap1 cal* mutant backgrounds (personal communication P. Huijser), the *ful*-2 mutant flower phenotype with short siliques that fail to dehisce seemed not attenuated in the double mutant.

Under SD conditions wild type flowered, on average, with about 44.8 rosette leaves, while *SPL3OX* made between 18.7 and 20.3 leaves (Table 3.1). The *ful*-2 parent made 35.5 rosette leaves, while the *ful SPL3OX* double mutants made 26.3 and 27.0 rosette leaves. Interestingly, the number of cauline leaves was significantly increased in a *ful*-2 mutant background (*ful*-2 plants 14.3; *ful SPL3OX* plants 13.8 and 12.6) in comparison to wild type (7.0) and *SPL3OX* (7.8). Also under SD conditions the days until bolting and the days from bolting to anthesis were increased in *SPL3OX*, *ful*-2 and *ful SPL3OX* plants compared to wild type. Nevertheless, *SPL3OX* as well as *ful SPL3OX* plants seemed to remain photoperiodically sensitive. Plants grown under SD conditions are shown in appendix B.

Table 3.1
Flowering behaviour in long day and short day conditions of wt, *SPL3OX* transgenics (lines 2342 and 2350), *ful*-2 and *ft*-10 single and *ful SPL3OX* and *ft SPL3OX* (crossed to 2342) double mutant plants as determined by the average number of days to anthesis, bolting (shoot 0.5 cm above rosette) and number of rosette and cauline leaves formed. DAS, days after sowing; SD, standard deviation; TLN, total leaf number; n, number of plants.

	Genotype	Rosette leaves ± SD	Cauline leaves ± SD	TLN ± SD	Bolting (DAS) ± SD	Anthesis (DAS) ± SD	n	Bolting to Anthesis
Long Day	Col-0	14,0 ± 1,2	4,3 ± 0,9	18,3 ± 1,6	20,6 ± 1,0	25,6 ± 1,6	22	5,0
	ful-2	10,8 ± 1,4	4,5 ± 0,8	15,3 ± 2,1	23,4 ± 2,7	28,4 ± 2,7	16	5,1
	2342	5,3 ± 0,8	1,4 ± 0,6	6,7 ± 1,0	17,0 ± 0,9	22,0 ± 0,9	24	5,0
	2348	5,3 ± 0,7	1,4 ± 0,6	6,6 ± 0,9	17,3 ± 1,2	22,8 ± 1,2	24	5,5
	2350	5,6 ± 0,8	1,6 ± 0,6	7,2 ± 1,1	18,1 ± 1,5	23,6 ± 1,9	29	5,5
	ful-2 2342	9,6 ± 1,5	3,6 ± 0,5	13,2 ± 1,6	23,0 ± 2,1	29,0 ± 1,6	5	6,0
	ful-2 2350	8,5 ± 1,3	3,3 ± 0,7	11,9 ± 1,6	22,0 ± 1,6	27,0 ± 1,9	28	5,1
Short Day	Col-0	44,8 ± 1,9	7,0 ± 1,3	51,9 ± 2,0	54,5 ± 3,9	62,6 ± 4,3	23	8,0
	ful-2	35,5 ± 3,5	14,3 ± 2,2	49,8 ± 3,9	60,9 ± 5,0	70,8 ± 5,6	12	9,9
	2342	18,7 ± 1,2	7,8 ± 0,9	26,4 ± 1,6	37,2 ± 1,6	46,7 ± 2,2	23	9,4
	2348	20,9 ± 2,8	7,9 ± 1,3	28,7 ± 3,7	39,3 ± 2,4	49,2 ± 3,8	23	10,0
	2350	20,3 ± 2,0	7,8 ± 0,8	28,0 ± 2,1	37,9 ± 1,9	48,2 ± 2,9	24	10,3
	ful-2 2342	26,3 ± 3,0	13,8 ± 1,3	40,0 ± 3,6	75,0 ± 2,2	85,5 ± 2,5	12	10,5
	ful-2 2350	27,0 ± 2,5	12,6 ± 3,6	39,6 ± 3,9	55,2 ± 5,8	67,4 ± 8,7	16	12,3
Long Day	Col-0	14,9 ± 1,2	4,54 ± 1,1	19,5 ± 1,8	26,1 ± 1,5	32,3 ± 1,3	34	6,1
	ft-10	36,8 ± 2,3	6,8 ± 1,0	43,7 ± 2,7	48,9 ± 2,8	56,4 ± 2,8	35	7,6
	2342	5,6 ± 0,5	0,81 ± 0,5	6,4 ± 0,5	18,3 ± 0,8	24,7 ± 0,9	36	6,5
	ft SPL3	23,3 ± 2,7	6,78 ± 1,9	30,1 ± 4,3	37,7 ± 4,0	46,9 ± 6,2	18	9,2

The *ft SPL3* plants flowered significantly later than wild type (14.9 rosette leaves) or *SPL3OX* plants (5.6 rosette leaves) under LD conditions with 23.3 rosette leaves on average but yet earlier than the *ft*-10 parental line (36.8 rosette leaves). Differences in flowering time values for wild-type and *SPL3OX*-2342 under the two LD conditions listed in table 3.1 are due to two different light intensities (229 vs 99 µmol $m^{-2}s^{-1}$). Therefore, the values cannot be compared directly with the flowering time values of the *ful SPL3* mutants, but the *ft SPL3OX* plants would presumably be later flowering than the *ful SPL3* mutants when a difference in flowering time between the two LD conditions of 0.9 rosette leaves for wild-type are

considered. Yet, it should be noted, that the days until bolting are increased by about 5.5 days under the weaker light intensities.

The results from the flowering time experiments show that in both backgrounds, *ful*-2 and *ft*-10 respectively, the effect of overexpression of *SPL3* is decreased, but the double mutants nevertheless flower significantly earlier than their single mutant knock-out parent. Since *FT* as a floral pathway integrator acts upstream of *FUL* it cannot be excluded that the later flowering of the *ft SPL3* double mutant is a consequence of a loss of activation of other *FT*-dependent flowering time components rather than a loss of *FUL* activation. The results clearly indicate though, that there is a certain dependency on *FT* in an *SPL3OX* plant. But rather than being directly under the control of *FT*, the effect of *SPL3* seems to be additive to *FT* since the *ft SPL3OX* mutant is still significantly earlier flowering. Therefore, it could be assumed that SPL3 may activate *FUL* in an *FT* independent manner.

3.1.4 Circadian expression of *FUL* and flowering behaviour in the absence of *FT*

From the double mutant analysis described above, it seemed reasonable to assume that earliness of *SPL3OX* transgenics is indeed largely mediated through *FUL*. Furthermore, constitutive overexpression of *SPL3* in an *ft*-10 mutant background resulted in much earlier flowering in comparison to the *ft*-10 parental line. Therefore, the possibility that *SPL3* promotes *FUL* expression via *FT* had become less likely. To further analyse the role of *FT* in mediating the effect of *SPL3* overexpression on *FUL*, the *FUL* diurnal expression in *SPL3OX* and wild type seedlings was determined under SD conditions. Under these conditions, *FT* transcript levels were expected to stay low during the entire day. Additionally, flowering behavior of *SPL3OX* and *ful SPL3OX* plants under SD conditions was investigated.

Indeed, as shown in Fig. 3.5A, *FT* mRNA was virtually undetectable in 7-d-old seedlings grown under SD conditions, i.e. qRT-PCR required cycle numbers as high as 50 to detect any signal (and caused large standard deviations). At the same time *FUL* transcripts remained at significantly higher levels in the *SPL3* overexpressors compared to wild type. Furthermore, *FUL* expression peaked at the end of the day independently of the presence of light (Fig 3.5B). Thus, remarkably, *FUL* transcript levels in SD grown *SPL3OX* seedlings showed the same diurnal rhythm as found before under LD conditions (compare Fig. 3.3B).

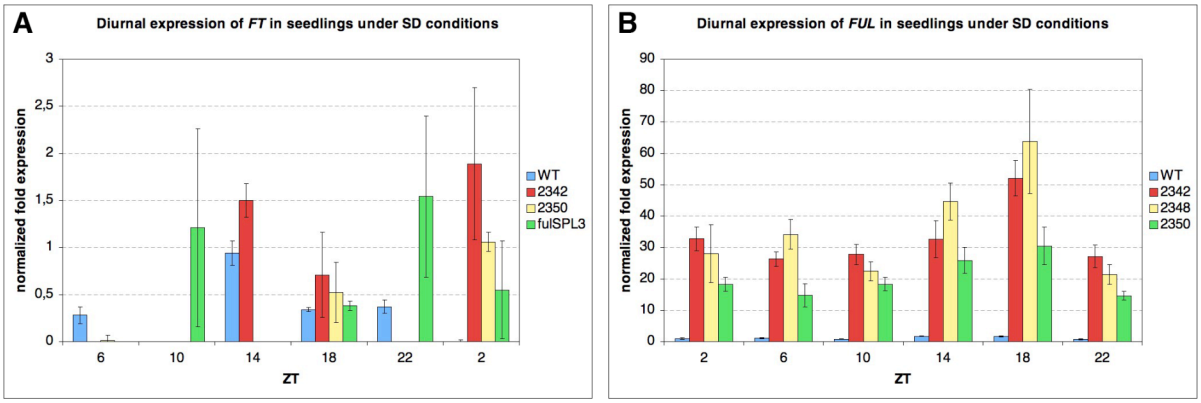


Figure 3.5
Expression analysis over a 24-h period in seedlings of wt and *SPL3OX* transgenics (lines 2342 and 2350) grown for 7 days under SD conditions of *FT* (A) and *FUL* (B). Expression levels were normalized against *PP2A*. ZT 0 represents the beginning of the light period. Expression of *FT* (A) or *FUL* (B) in wt at ZT 2 (i.e. 2h after the beginning of the light period) was chosen as a reference and set arbitrarily at one.

Therefore, the temporal increase of *FUL* expression at the end of a day seemed to be independent of the light period and independent of *FT*.

3.1.5 Expression profiling of other known factors capable of affecting *FUL* expression

Furthermore, possible interaction partners of FUL were tested, i.e. the MADS-box genes *AGL24* and *SOC1*. As depicted in Fig. 3.6A, the expression of *AGL24* in *SPL3OX* as well as in *SPL3OX ful* plants grown under LD conditions did not differ much from wt. At ZT 2, expression levels seemed to be a little higher in the *SPL3OX* line 2350 and in the *SPL3OX ful* double mutant (line 2350 crossed to *ful*-2). However, since this elevation could not be not be detected in the other *SPL3OX* transgenic line (line 2342) it is unlikely that the observed change in expression was due to the overexperssion of *SPL3*. At ZT 18 in both *SPL3OX* lines the expression of *AGL24* was not as high as in wt, but nevertheless, the difference in expression remained small. It is therefore unlikely that *AGL24* expression is directly influenced by elevated *SPL3* and/or *FUL* levels.

Although the expression of *SOC1* had already been tested in *SPL3OX* once at day time, its expression levels were monitored over a 24-h period in 7-d-old seedlings grown under LD conditions. Fig. 3.6B shows, that *SOC1* expression was slightly upregulated in both *SPL3OX* lines as well as in the *SPL3OX ful* double mutant. The largest recorded difference, i.e. a 3.5-fold increase, was found for line 2342 in comparison to wt. It is unlikely, that elevated expression of *SPL3* and/or *FUL* is of much relevance to the expression of *SOC1* in seedlings.

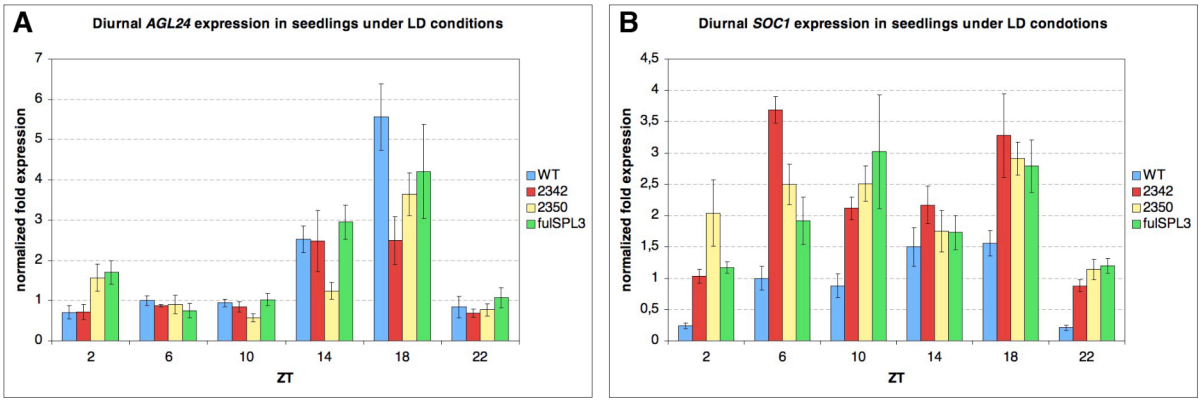


Figure 3.6
Expression analysis over a 24-h period in seedlings of wt and *SPL3OX* transgenics (lines 2342 and 2350) grown for 7 days under LD conditions of *AGL24* (a) and *SOC1* (b).
A) *AGL24* expression over a 24-h period in 7-d-old seedlings of wt and *SPL3OX* (lines 2342 and 2350) grown under LD conditions
B) SOC1 expression over a 24-h period in 7-d-old seedlings of wt and *SPL3OX* (lines 2342, 2348 and 2350) grown under LD conditions.
Expression levels were normalized against *PP2A*. ZT0 represents the beginning of the light period. Expression of *AGL24* (A) or *SOC1* (B) in wt at ZT 2 (i.e. 2h after the beginning of the light period) was chosen as a reference and set arbitrarily at one.

3.2 Characterization of the interaction of SPL3 with its putative target gene *FUL*

3.2.1 Crosslinked Chromatin Immunoprecipitation

In vitro DNA-binding assays showed that SBP-domain proteins require the nucleotide sequence GTAC as core motif for high affinity DNA binding (Birkenbihl *et al*., 2005; Liang *et al*., 2008). As mentioned before, SPL3 prefers the motif CGTAC for DNA binding (Birkenbihl *et al*., 2005). Additionally, functional studies *in planta* seem to indicate that multiple copies of the core motif are required as cis-element for target gene responsiveness (Quinn *et al*., 2003; Nagae *et al*., 2008; Yamasaki *et al*. 2009). In order to investigate if *FUL* could be a direct target of SPL3 and fulfil these requirements, a 30kb region around the *FUL* genomic locus was analyzed for the distribution of both GTAC and CGTAC motifs. The positional distribution was compared to the situation in the genomic regions surrounding *AP1* and *CAL* loci. As depicted in Fig. 3.1, both motifs were found in the genomic regions of *FUL*, *CAL* and *AP1*. However, whereas in and around *AP1* and *CAL* both motifs seemed to be uniformily distributed, the *FUL* promoter and the first intronic region showed an enrichment. In particular, the CGTAC motif was found to be enriched in the first intron of *FUL*.

In order to investigate if *FUL* is a direct target of the SPL3 protein, I performed a Crosslinked Chromatin Immunoprecipitation (X-ChIP) experiment using an SPL3 specific antibody. In brief, material from two independent *SPL3OX* lines as well as wild type, representing either 7-d-old seedling or inflorescence tissues, was fixed with formaldehyde to induce protein-

DNA and protein-protein crosslinking. Subsequently, nuclei were extracted and their chromatin sheared by sonication to yield fragments of about 200 bp. Before incubation with the SPL3 specific antibody, a 50µl aliquot was taken from each sample and saved as "input DNA" control. From the remaining of the sample, the SPL3 protein was immunoprecipitated along with any associated DNA, i.e. "output DNA" (for experimental protocol details, see Material and Methods). Finally, the purified DNA was analyzed by quantitative real-time PCR, using several primer pairs that covered the *FUL* genomic region enriched for the CGTAC motif. Primer pairs allowing to amplify *FUL* fragments that did not include any (C)GTAC motifs, served as negative controls, i.e. expected not to be enriched due to interaction with SPL3 protein.

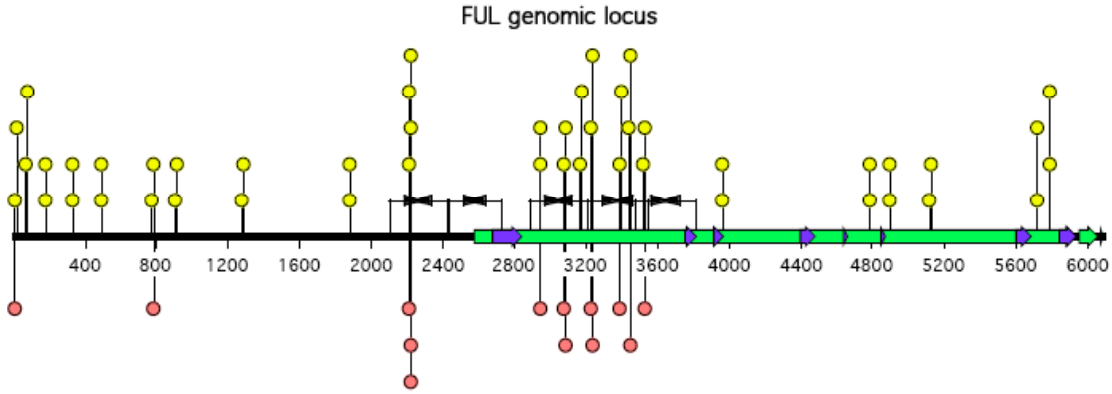


Fig 3.7
The *FUL*-fragments obtained with the promoter primer pair as well as with the primer pairs for the 1st intron include several CGTAC motifs.
Exons are depicted in blue, introns and non-translated regions in green. *FUL* genomic locus showing the positions of the GTAC (yellow circles) and CGTAC (red circles) motifs. The thin black arrows indicate the primer pairs used to amplify selected *FUL* fragments in the SPL3 X-ChIP assay.

The housekeeping gene *PP2A*, a constitutively expressed protein phosphatase, assumed not to be targeted by SPL3 protein and indeed not affected in *SPL3* overexpressing transgenics (P. Huijser and S. Höhmann, personal communication), was used for normalization of input and output DNA quantity.

FUL promoter fragments were found to be relatively enriched in immunoprecipitated chromatin derived from inflorescences of both *SPL3OX* transgenics and wild type (Fig. 3.8A). Furthermore, the 3' region of the first *FUL* intron also seemed to be enriched in comparison to both the first exon and the 5' region of the first intron. The same results were obtained with chromatin isolated from *SPL3OX* transgenic seedlings (Fig. 3.8B). As in both *SPL3* transgenic seedlings and wild-type inflorescences *FUL* and *SPL3* are known to be highly

expressed, this observation could have been an argument in support of a physical interaction between SPL3 and *FUL*. However, a contradictory observation was made, when immunoprecipitated chromatin of 7-d-old seedlings of wild-type plants was analyzed. In this material both the *FUL* promoter region and the 3' region of the first intron showed an enrichment as well. The contradiction arises, because SPL3 protein cannot be detected by Western blot in 7-d-old seedlings of wild type plants (P. Huijser personal communication; Gandikota et al., 2007). Therefore, the X-ChIP results didn't allow a final conclusion, but may lend support to the suggestion that *FUL* is a direct target of the SPL3 protein and that the first intron of *FUL* might play an important role in this interaction. A more thorough evaluation of this experiment follows in the discussion part of this thesis.

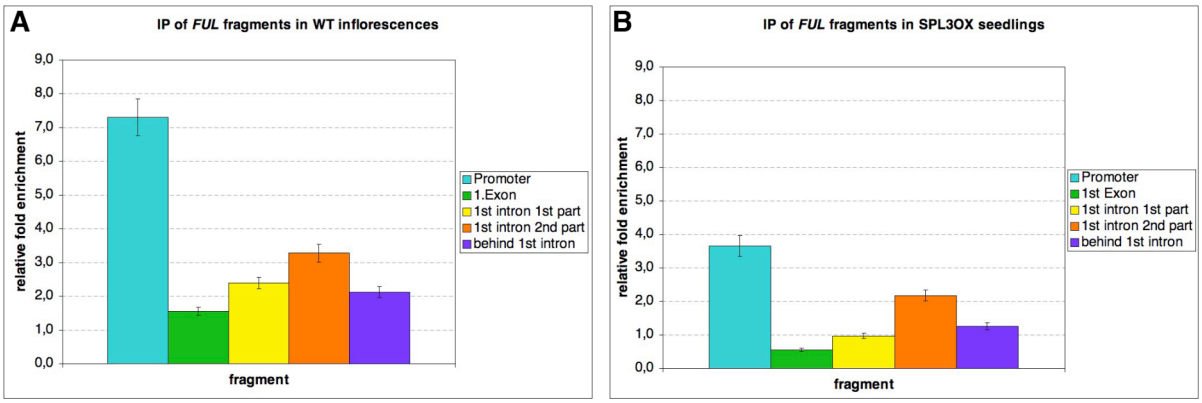


Figure 3.8
Enrichment of *FUL* fragments after chromatin immunoprecitpitation
Immunoprecipitated *FUL* fragments in Col-0 wt inflorescences (A) and SPL3OX seedlings (B) obtained by using several primerpairs covering the promoter region, the first exon, the 5' part and 3'part of the first intron and a fragment behind the first intron, that did not display any binding motifs.

3.2.2 Functional analysis of putative regulatory motifs in *FUL* genomic region

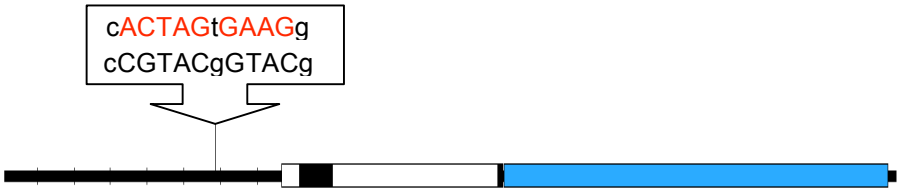
In order to test if the CGTAC motifs in both the first intron of *FUL* and the *FUL* promoter region are of functional relevance for transcriptional regulation, a reporter gene approach was set up. In a first construct, 1616 bp of the putative *FUL* promoter upstream of the ATG start codon as well as the *FUL* genomic region until 42bp of the second exon were fused to a *GUS* reporter gene (*E. coli* beta-D-glucuronidase gene). In a second construct, the same promoter region of *FUL* and a *FUL* cDNA truncated after 42bp of the second exon were fused to the *GUS* reporter. In comparison to the first construct, the second construct lacked the first intron. Additionally, variants of both constructs were made in such a way, that the CGTAC motifs covering region 461bp upstream of the ATG were modified from CCGTACGGTAC to

CACTAGTGAAG on the forward strand. The final four resulting GUS reporter gene constructs are depicted in Fig. 3.9.

A) *pFUL::FUL::GUS* (+ promoter, +intron)

B) *pFUL::ΔFUL::GUS* (+promoter, - intron)

C) *ΔpFUL::FUL::GUS* (- promoter, + intron)



D) *ΔpFUL::ΔFUL::GUS* (-promoter, + intron)

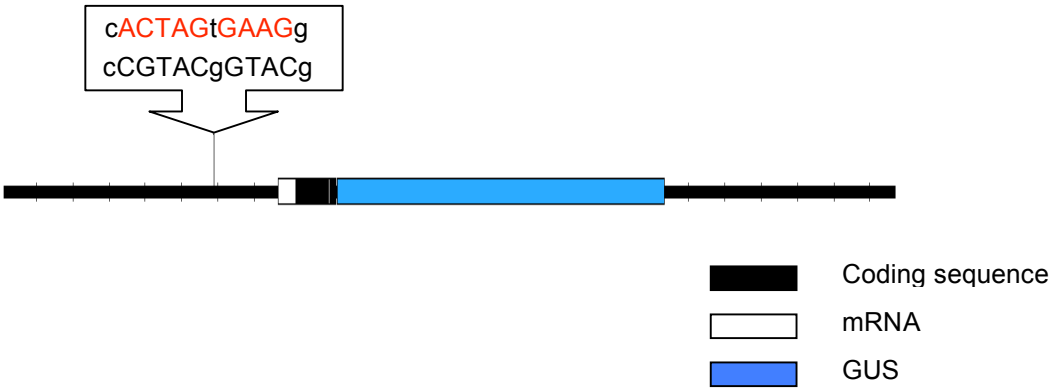


Figure 3.9
A) *pFUL::FUL::GUS* (+ promoter, +intron)
B) *pFUL::ΔFUL::GUS* (+promoter, - intron)
C) *ΔpFUL::FUL::GUS* (- promoter, + intron)
D) *ΔpFUL::ΔFUL::GUS* (-promoter, + intron)
"+" indicates that the CGTAC motifs and / or the intron is present, "-" indicates that CGTAC motifs and / or the first intron are absent

After sequencing confirmed that the GUS reporter was correctly fused in frame with the *FUL* coding sequence, wild-type plants were transformed with the constructs using agrobacterium-mediated transfection. For each of the constructs, homozygous transgenics carrying a single

insertion were isolated and analyzed for expression of the *GUS* reporter gene. Thereto, inflorescences as well as cauline leaves, in which *FUL* is known to reach its highest expression values (see appendix C) were harvested and stained for GUS activity. Unfortunately for none of the plants carrying one of the four constructs a GUS signal could be detected.

A problem here could be that *FUL* expression is generally low in wild type as data from AtGenExpress, visualized with the eFP Browser (see appendix C), indicate. As mentioned before, Teper-Bamnolker and Samach (2005) observed an upregulation of *FUL* in a *35S::FT* transgenic background. These authors used the *ful*-1 (*agl8*-1) mutant, which carries a GUS reporter inserted in the *FUL* 5' untranslated leader (Gu *et al*., 1998) and reported a visible, albeit weak, GUS signal in a this background (Teper-Bamnolker and Samach, 2005). Therefore, it was decided to analyse the response of this *ful*-1 allele to constitutive overexpression of *SPL3*. Since the *ful*-1 mutation is in a Landsberg *erecta* (L*er*) background, homozygous *ful*-1 plants were crossed to two independent lines both homozygous for the *SPL3OX* transgene and of the L*er* ecotype, i.e. lines *L35S::SPL3-UTRΔ2*-4 and *L35S::SPL3-UTRΔ2*-10 (Gandikota *et al*., 2007). Seven-d-old seedlings, inflorescences, cauline leaves and siliques at different ripening states of plants hemizygous for the *SPL3OX* transgene and heterozygous for *ful*-1 were again analyzed for GUS expression. Furthermore, to exclude dosage effects when comparing signal strength, homozygous *ful*-1 plants were also crossed to L*er* wt in order to obtain heterozygous F1 plants.

For comparison the transgenic NASC line N8847, which carries a fusion construct of a 2.3kb fragment of the *FUL* promoter region fused to a GUS reporter, was ordered and also stained in this expression series. Although this line is in a NOSSEN background, it was assumed that it would be elucidating the importance of intragenic sequences, e.g. the first intron. Moreover, the promoter region of this line, fused to the GUS reporter, was about 684bp longer than the fusion constructs that I had made and therefore might cover regulatory elements of the promoter region, that I missed in my constructs.

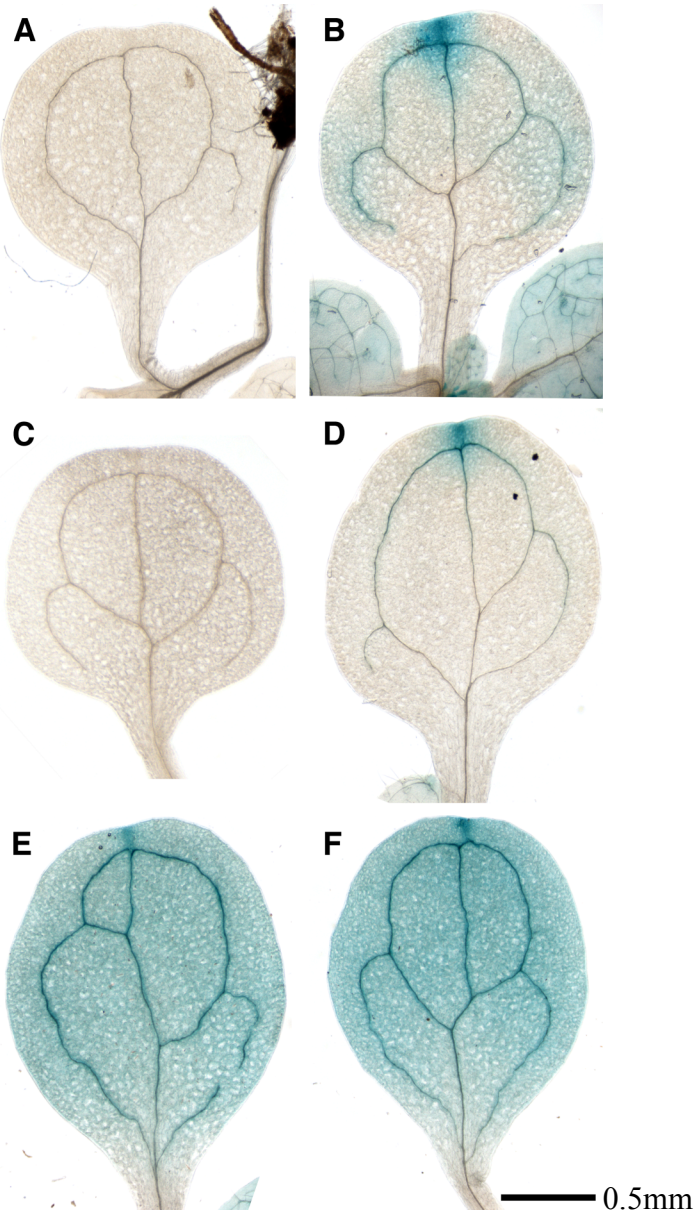


Fig. 3.10
GUS expression in cotyledons of hemizygous plants. L*er* (A); *ful*-1 homozygous (B); N8847 (C); L*er* x *ful*-1 (D); *ful*-1 x *L35S::SPL3-UTRΔ2*-4 (E); *ful*-1 x *L35S::SPL3-UTRΔ2*-10 (F).

In both, the parental GUS line as well as the F1 plants of the crosses a GUS signal was detectable (Fig. 3.10-13). In the cotyledons of the *ful*-1 mutant and the backcross of *ful*-1 to L*er* wt the GUS signal is prominent at the distal tip and weakly detectable in the lateral parts. In particular the vasculature in these areas show a GUS signal (Fig 3.10B). In the F1 plants from the crosses of *ful*-1 to *SPL3OX* the GUS signal was much stronger than in the parental lines (Fig 3.10D to F) and is detectable ubiquitously in the cotyledon and particularly strong in the veines. Interestingly, GUS activity was limited to the laminar area, the petiole of the cotyledons remained unstained. Also, the cotyledons of *SPL3OX* seemed to be less roundish and the vasculature seemed to be a bit more advanced, which could indicate that the phase

transition is accelerated and leads to a more “adult” phenotype. Also, in the N8847 line, no GUS expression was detectable in cotyledons, which might be due to the lacking of the first intron (Fig. 3.10C).

Furthermore, GUS expression in inflorescences, cauline leaves and siliques of the same crosses were analyzed.

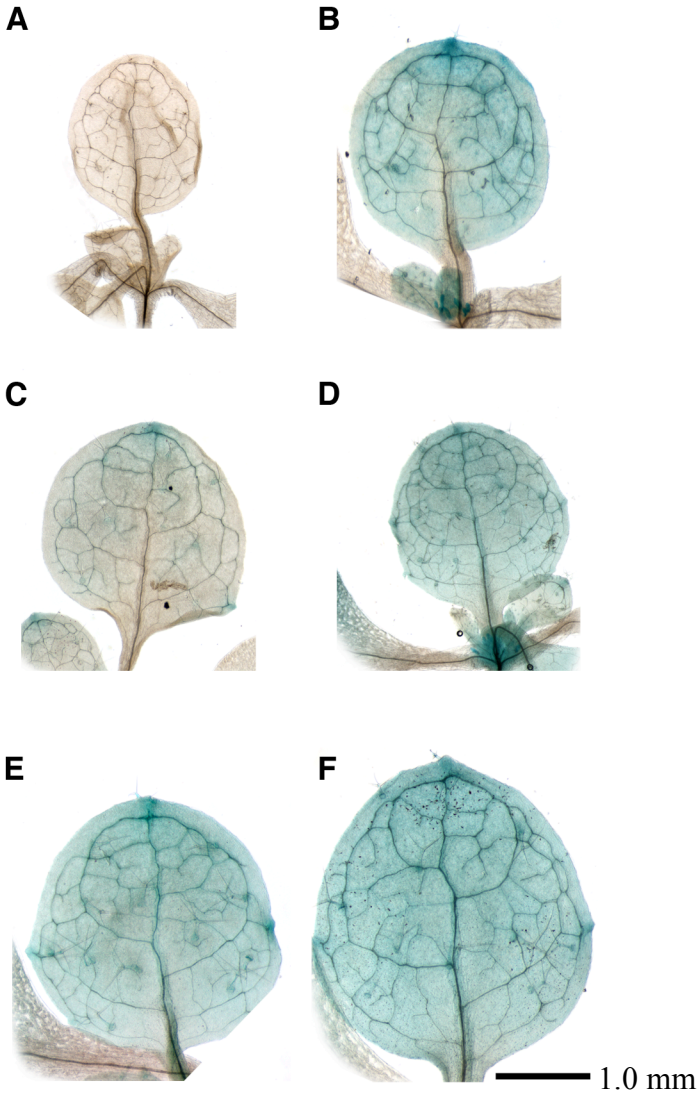


Fig. 3.11
GUS expression in the first true leaves of hemizygous plants. L*er* (A); *ful*-1 homozygous (B); N8847 (C); L*er* x *ful*-1 (D); *ful*-1 x *L35S::SPL3-UTRΔ2*-4 (E); *ful*-1 x *L35S::SPL3-UTRΔ2*-10 (F).

The staining pattern of the first true leaves of the same plants resembled that of the cotyledons. GUS staining in the presence of the *SPL3OX* transgene (Fig 3.11 D to F) was again much stronger than in *ful*-1 homo- or hemizygous mutants and in particular in the central vein (Fig 3.11 B and C). Also the petioles of the true leaves remained unstained. But,

differently from the cotyledons, the distal tip of the true leaves displayed a less prominent GUS signal. Again, the staining of N8847 was less strong.

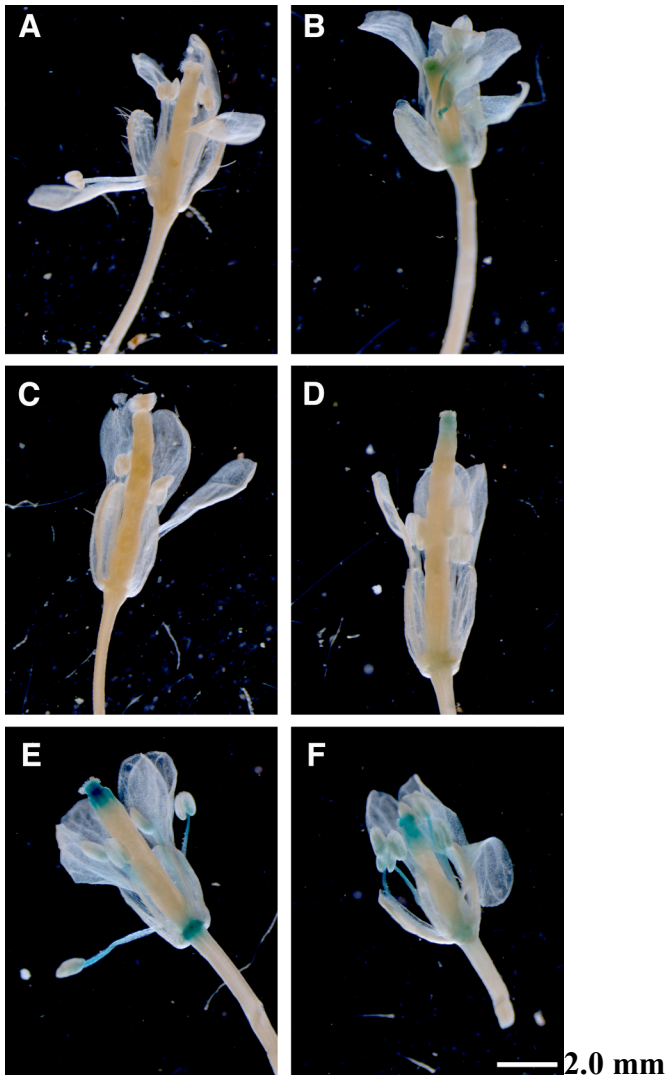


Fig. 3.12
GUS expression in inflorescences of hemizygous plants in dark field. L*er* (A); *ful*-1 (B); N8847 (C); L*er* x *ful*-1 (D); *ful*-1 x *L35S::SPL3-UTRΔ2*-4 (E); *ful*-1 x *L35S::SPL3-UTRΔ2*-10 (F).

While the inflorescences of wild-type did not show a GUS activity (Fig. 3.12A) the inflorescences of the *ful*-1 mutant showed a staining in pistil tips, filaments of stamens and in the receptacle. To a lesser extend also a weak staining was detectable in petals. Interestingly, the N8847 line did not show GUS activity in the inflorescence (Fig. 3.12 C), while the hemizygous mutants (Fig. 3.12 D to F) did show GUS activity in the same organs as the parental *ful*-1 mutant with a stronger expression.

Fig. 3.13
GUS expression in cauline leaves. L*er* (A); *ful*-1 homozygous(B); N8847 (C); L*er* x *ful*-1 (D); *ful*-1 x *L35S::SPL3-UTRΔ2*-4 (E); *ful*-1 x *L35S::SPL3-UTRΔ2*-10 (F).

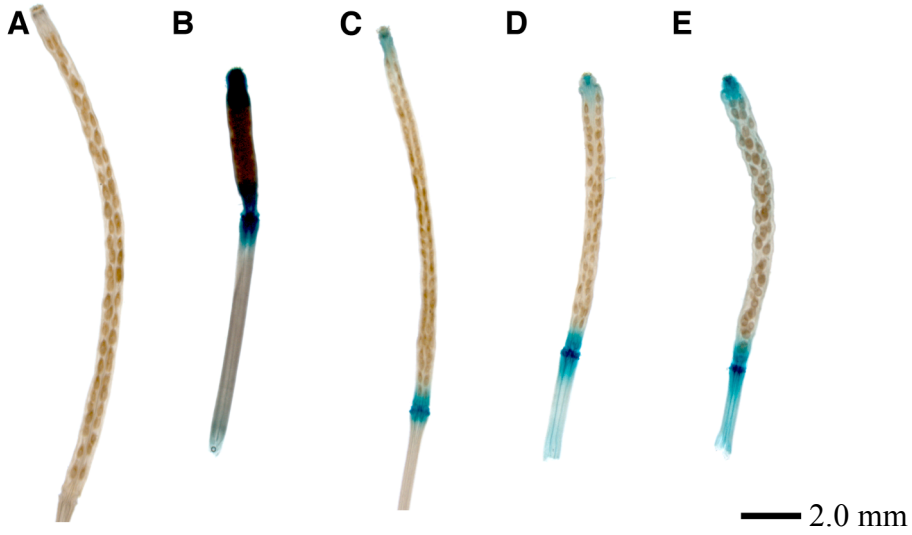


Fig. 3.14
GUS expression in siliques. L*er* (A); *ful*-1 (B); L*er* x *ful*-1 (C); *ful*-1 x *L35S::SPL3-UTRΔ2*-4 (D); *ful*-1 x *L35S::SPL3-UTRΔ2*-10 (E).

The GUS expression in cauline leaves was again not detectable in wild-type, while in the *ful*-1 mutant it was clearly visible (Fig. 3.13 A and B). The staining of the hemizygous mutants was weaker than in the *ful*-1 parent, but detectable throughout the leaf, although it seems that the GUS signal was not uniformily distributed (Fig. 3.13 D to F) and not visible in the vasculature as it was in the cotyledons. Interesting to mention is that the shape of cauline leaves differed much in an *ful*-1 background, for it was more roundish compared to the much more lanceolated shape of the L. *er* parent. The leaf shape of the hemizygous mutants seemed to be intermediate.
GUS expression in siliques was detectable at the tips in both *ful*-1 mutants and the hemizygous mutants (Fig. 3.14 B-F) as well as around the floral organ abscission zone at its base.

Taken together, the GUS expression patterns confirm that overexpression of *SPL3* leads to higher *FUL* expression levels. Furthermore, these data indicate that SPL3 requires (an) additional factor(s) to upregulate *FUL* expression. Otherwise, the GUS expression should have followed the ubiquitous CaMV 35S promoter activity driving the *SPL3* transgene. Nevertheless, the GUS expression only becomes ectopic in cotyledons, where it covers more of the leaf blade than in the *ful*-1 parent.

3.3 A global expression analysis using affymetrix ATH1 arrays

It has been shown that many transcription factors not only regulate multiple genes but also have dual functions in activating or repressing these in a context depended manner. Therefore, it is well conceivable that also *SPL3* positively or negatively regulates multiple target genes during plant development. To identify developmental processes possibly affected by *SPL3*, and also as an alternative to the candidate gene approach, a global expression analysis by means of microarray technology was performed. On the Arabidopsis ATH1 array (Affymetrix) that was used for the experiment, each gene is represented by a probe set of several oligomers, i.e. 25-mers. In this way, 22,810 such probe sets cover approximately 90% of all Arabidopsis genes.

Candidates for possibly *SPL3* regulated genes were first selected according to their degree of differential expression (above 0.95 confidence level) between *SPL3OX* with wild type. Furthermore, expression values were compared within the genotypes for the two different timepoints over the day and also between genotypes for the two timepoints. Comparison for the lines with themselves was performed to track the endogenous diurnal expression of genes in order to distinguish them from *changes* in diurnal gene expression caused by the overexpression of SPL3. The same comparisons were done for *ful SPL3*. The cut-off level was arbitrarily set at 1.5 fold. This resulted in a large number of up- and downregulated genes. In a second step, the identified genes were categorized according to their Gene Ontology (GO) annotations using a tool found on http://www.arabidopsis.org/tools/bulk/go/index.jsp , the Arabidopsis Information Resource (TAIR) web site. This tool sorts the genes according to their molecular function, biological processes involved and cellular distribution.

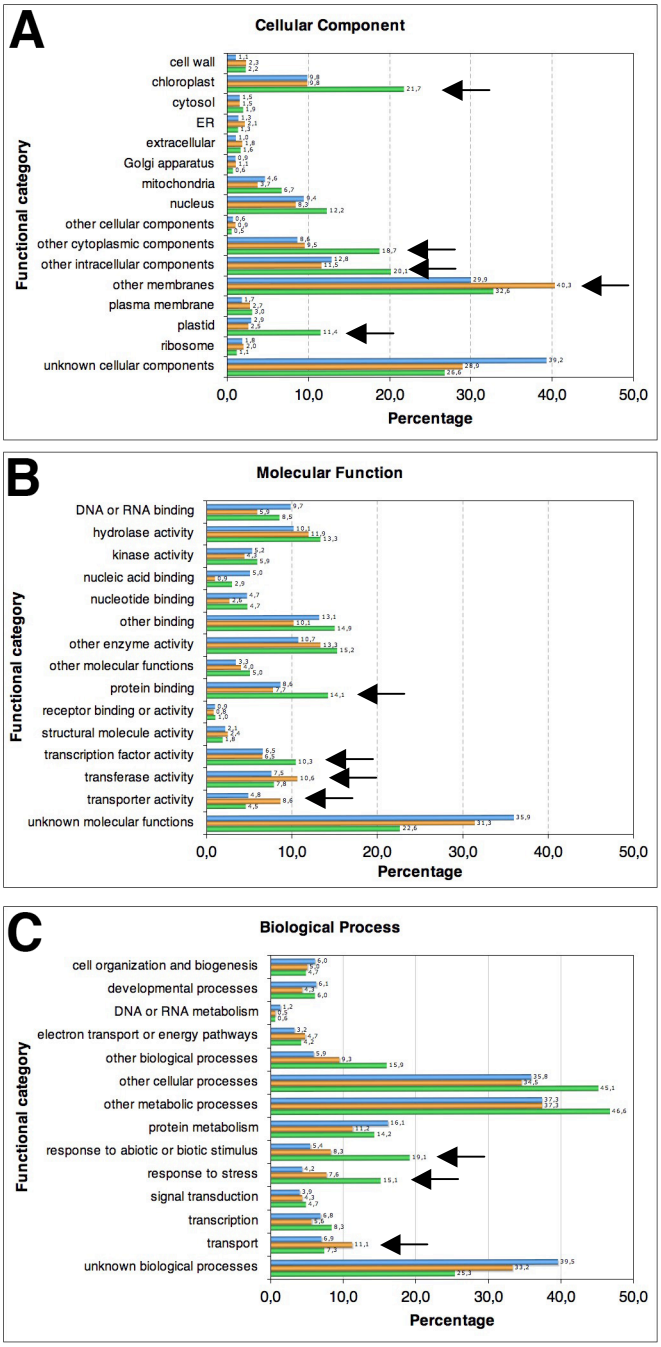


Figure 3.15
GO annotation analysis of genes that are differentially expressed between *SPL3OX* line 2350 and wild type. Samples being taken at the mid of the light period (ZT6 = 12:00h)
Percentile distribution of GO terms assigned to the differentially expressed genes at the middle of the light period, functionally categorized as cellular component (A), Molecular function (B) and biological process (C). Blue bars, percentile distribution for the whole genome; Green bars: percentile distribution for the upregulated genes; Red bars: percentile distribution for the downregulated genes

From Fig. 3.15A it can be seen, that of the GO Cellular Component terms assigned to the upregulated genes at 12:00h, 11.4% are related to 'plastid'. This is ca. four times as much as for the whole genome (i.e. 2.9%). Obviously, also the term 'chloroplast', a 'part_of' the GO term 'plastid', is found to be overrepresented in the upregulated genes The rest of the

overrepresented upregulated genes are primarily clustered under 'other cytoplasmic components' and 'other intracellular components'. For the downregulated genes the GO term 'other membranes' is somewhat overrepresented. Concerning the GO terms related to Molecular Function (Fig. 3.15B), the upregulated genes at 12:00h seem to be somewhat overrepresented in 'protein binding' (14.1% vs. 8.6%) and 'transcription factor activity' (10.3% vs. 6.5%) while the downregulated genes cluster in 'transferase activity' and transporter activity'. Finally, GO terms related to Biological Processes and overrepresented in the upregulated genes in the 12:00h sample (Fig. 3.13C), refer predominantly to abiotic or biotic stress responses, while again the downregulated genes are related to 'transport'.

Though this analysis of the affymetrix data was quite rough, it can be conducted that the most upregulated genes have functions in cell organization and biogenesis as well as play a role in developmental processes. However, after this coarse categorization it was not immediately obvious what processes were particularly affected due to constitutive overexpression of *SPL3*. Therefore, a more detailed GO term analysis was performed with the help of the web-based tool AmiGo (http://amigo.geneontology.org/cgi-bin/amigo/term_enrichment). Moreover, the available microarray data were analysed in a more comprehensive way. Thereto, samples representing both different time points of the day were first compared per genotype, e.g. *SPL3OX* at 12:00h compared to *SPL3OX* at 21:30h. Then, the results were compared between the genotypes. In fact, this comparison should not primarily detect quantitative differences in gene expression levels between the genotypes but rather qualitative differences in daily expression patterns, i.e. between 12:00h and 21:30h. Finally, genes that varied at least a factor of 1,5 between *SPL3OX* line 2350 and Col-0 wild type were subjected to GO term analysis.

Table 3.3: Selected results from the AmiGO GO term analysis

Further results from the AmiGo analysis could not be included in the appendix due to very large data sets

A) Cellular component:

GO Term	P-value	Sample freq.	Background freq.	Genes
GO:0030076 light-harvesting complex	1.05e-14	11/436 (2.5%)	25/31842 (0.1%)	AT5G54270 AT2G05100 AT2G05070 AT1G29910 AT3G47470 AT3G27690 AT2G34430 AT1G29930 AT3G54890 AT2G34420 AT1G29920
GO:0009535 chloroplast thylakoid membrane	2.76e-05	22/436 (5.0%)	592/31842 (1.9%)	AT3G45140 AT1G15820 AT2G39730 ATCG00720 AT5G54270 AT2G05100 AT2G05070 AT1G29910 AT3G47470 ATCG00470 AT3G27690 AT1G29395 AT2G34430 AT3G16000 AT1G29930 AT4G20360 AT3G54890 ATCG00480 AT3G47860 AT3G01500 AT2G34420 AT1G29920

Table 3.3 continued
B) Molecular Function

GO Term	P-value	Sample freq.	Background freq.	Genes
GO:0016168 chlorophyll binding	2.79e-13	11/436 (2.5%)	32/31842 (0.1%)	AT1G15820 AT2G05100 AT2G05070 AT1G29910 AT3G47470 AT3G27690 AT2G34430 AT1G29930 AT3G54890 AT2G34420 AT1G29920
GO:0003700 transcription factor activity	5.36e-10	56/436 (12.8%)	1661/31842 (5.2%)	AT5G39660 AT4G16610 AT5G62020 AT1G80840 AT3G06160 AT4G37260 AT1G01060 AT4G00050 AT4G17880 AT5G04340 AT4G23810 AT5G49330 AT1G69490 AT3G20810 AT4G16780 AT1G32640 AT3G56400 AT3G54390 AT4G14560 AT3G15210 AT1G73830 AT1G21910 AT1G01720 AT3G50060 AT5G61600 AT4G38620 AT5G48250 AT4G26150 AT1G68840 AT5G24120 AT3G49530 AT2G26150 AT2G33810 AT1G71030 AT5G59780 AT5G05410 AT1G22640 AT5G51190 AT3G16770 AT2G40750 AT2G40140 AT1G13260 AT3G24500 AT2G40970 AT5G47220 AT2G25930 AT4G17500 AT5G59570 AT1G66390 AT5G04240 AT3G47500 AT3G07650 AT3G46640 AT1G78600 AT5G54470 AT4G37180
GO:0030246 carbohydrate binding	4.61e-04	7/436 (1.6%)	100/31842 (0.3%)	AT3G04720 AT1G65390 AT4G27300 AT1G63090 AT1G16900 AT1G12710 AT3G52180
GO:0015144 carbohydrate transmembrane transporter activity	1.01e-03	7/436 (1.6%)	114/31842 (0.4%)	AT1G11260 AT1G19450 AT4G36670 AT3G47420 AT2G29650 AT3G54700 AT2G38940
GO:0005975 carbohydrate metabolic process	9.59e-03	13/436 (3.0%)	449/31842 (1.4%)	AT4G26270 AT5G51820 AT1G10760 AT1G13700 AT2G32610 AT2G32530 AT4G17090 AT5G26570 AT3G52180 AT3G51160 AT1G60590 AT2G32540 AT4G33440

C) Biological process

GO Term	P-value	Sample freq.	Background freq.	Genes
GO:0050896 response to stimulus	2.54e-38	147/436 (33.7%)	3426/31842 (10.8%)	AT5G13930 AT4G38860 AT3G23120 AT5G52310 AT3G04720 AT1G66270 AT4G22880 AT3G23110 AT3G45140 AT1G15820 AT5G54490 AT5G62020 AT2G42540 AT2G15080 AT1G80840 AT1G29460 AT3G23560 AT4G24960 AT4G37260 AT1G01060 AT2G21220 AT5G12020 AT2G39730 AT2G37030 AT3G57040 AT1G27330 AT4G30270 AT4G23810 AT1G32920 AT1G29430 AT1G07400 AT5G66070 AT2G40000 AT1G17420 AT1G33970 AT2G44080 AT5G14920 AT4G27320 AT2G22300 AT1G65390 AT3G55120 AT2G11810 AT5G51820 AT1G13930 AT5G15960 AT1G10760 AT4G16780 AT1G32640 AT3G56400 AT4G14560 AT2G41010 AT1G72940 AT2G19310 AT2G46240 AT3G15210 AT2G41100 AT1G76650 AT4G23600 AT2G40080 AT1G29450 AT5G20230 AT3G17790 AT1G01720 AT5G44420 AT3G50060 AT4G34150 AT1G20450 AT5G01220 AT3G44260 AT1G74310 AT1G29395 AT1G77760 AT5G02810 AT2G04795 AT2G21200 AT2G28900 AT4G17090 AT3G62550 AT4G38620 AT1G22770 AT2G42530 AT1G66160 AT4G26150 AT5G24120 AT2G26020 AT3G22231 AT3G49530 AT2G26150 AT1G54050 AT4G32770 AT2G29650 AT1G71030 AT5G59780 AT2G14560 AT1G29440 AT4G19030 ATCG00480 AT4G21830 AT5G05410 AT1G80420 AT1G22640 AT2G29500 AT3G45640 AT5G51190 AT3G22370 AT3G09870 AT1G21250 AT3G16770 AT2G40750 AT2G40140 AT1G13260 AT1G72520 AT3G24500 AT5G54770 AT4G14400 AT5G47220 AT4G12400 AT1G73500 AT5G45340 AT5G20410 AT2G25930 AT3G01500 AT4G17500 AT5G06320 AT4G16860 AT5G59320 AT2G47730 AT5G20630 AT3G10020 AT5G04240 AT2G21660 AT2G46440 AT5G15970 AT3G07650 AT5G54110 AT3G46970 AT5G59080 AT5G27780 AT1G78600 AT1G59860 AT2G17840 AT3G61460 AT3G46620 AT3G23150 AT3G13790 AT3G12580 AT5G12030
GO:0009416 response to light stimulus	1.77e-15	33/436 (7.6%)	430/31842 (1.4%)	AT5G13930 AT1G15820 AT2G42540 AT1G01060 AT2G39730 AT3G55120 AT5G15960 AT4G16780 AT2G19310 AT2G46240 AT2G41100 AT2G40080 AT5G20230 AT1G74310 AT1G77760 AT5G02810 AT4G38620 AT1G22770 AT5G24120 AT2G26150 AT1G54050 AT4G32770 AT5G05410 AT2G29500 AT4G12400 AT5G45340 AT2G25930 AT5G04240 AT3G07650 AT1G78600 AT2G17840 AT3G12580 AT5G12030
GO:0010228 vegetative to reproductive phase transition	4.18e-06	8/436 (1.8%)	68/31842 (0.2%)	AT1G01060 AT2G46240 AT2G40080 AT1G22770 AT2G25930 AT5G04240 AT2G21660 AT3G07650
GO:0007623 circadian rhythm	5.92e-06	7/436 (1.6%)	51/31842 (0.2%)	AT3G57040 AT2G40080 AT5G02810 AT1G22770 AT2G25930 AT2G21660 AT3G46640
GO:0009648 photoperiodism	1.65e-05	6/436 (1.4%)	40/31842 (0.1%)	AT1G01060 AT2G40080 AT1G22770 AT2G25930 AT5G04240 AT3G07650
GO:0010017 red or far red light signaling pathway	4.26e-05	5/436 (1.1%)	29/31842 (0.1%)	AT2G42540 AT5G15960 AT4G16780 AT2G40080 AT5G02810
GO:0005982 starch metabolic process	8.14e-05	5/436 (1.1%)	33/31842 (0.1%)	AT5G51820 AT1G10760 AT4G17090 AT5G26570 AT3G52180

From the GO terms related to Cellular Component, 'light harvesting complex' and 'chloroplast thylakoid membrane' were significantly enriched (Table 3.3A). This fitted well with the previous determined association with the chloroplast. Also related to these findings could be the enrichment of the term 'chlorophyll binding' and terms related to carbohydrate metabolism and transport among the GO terms for Molecular Function (Table 3.3 B). Similarly, this could be linked to the enrichment of the Biological Process GO term 'starch metabolic process'. Furthermore, among the Biological Process GO terms enriched in the pool of selected genes were many related to 'response to stimulus'. These stimuli encompassed elements of abiotic and biotic stresses. Of particular interest were those associated to 'response to light stimulus' like 'phase transition', 'photoperiodism', 'circadian rhythm' and 'light signalling' (Table 3.3.C) as flowering time is well known to be influenced by the photoperiod and the circadian clock. Therefore, with a renewed focus on genes being involved in circadian rhythm, phototransduction and regulation of circadian rhythms, the expression data of *SPL3*, *ful SPL3* and wild-type were again compared at both specific time points of the day, i.e. at 12:00 h and 21:30 h (see tables 3.4 and 3.5).

The fold change values were sorted into two tables according to the roles of the genes in circadian rhythm and phototransduction processes (Table 3.4 and 3.5). For comparison of wtild-type gene expression also the wild-type data from both sampling timepoints were included. Positive values display a higher expression of the gene in the mutant, i.e. *SPL3OX* or *ful SPL3* compared to wild-type.

Table 3.4

Ratios for differential gene expression in 12:00 and 21:30h samples for genes involved in circadian rhythm. positive values: upregulated in numerator; negative values: downregulated in numerator. Only present called genes with PPDE(p)>0.950 and a fold change up/down>1.5 are listed.

		SPL3OX / WT ratio	***ful*SPL3 / WT ratio**	***ful*SPL3 / SPL3OX ratio**	WT evening / WT midday ratio	WT evening / WT midday ratio	**SPL3OX / WT ratio**	***ful*SPL3OX / WT ratio**	***ful*SPL3OX / SPL3OX ratio**
		12:00h	**12:00h**	**12:00h**	21:30h / 12:00h	all values	**21:30h**	**21:30h**	**21:30h**
circadian rhythm									
AT5G37260	RVE2/CIR1				40,1	40,1			
AT5G60100	PRR3				11,8	11,8			
AT2G21660	ATGRP7/CCR2	1,5	2,2		5,6	5,6			
AT5G61380	TOC1/PRR1		1,7		5,4	5,4			
AT5G15840	CO					5,1			
AT5G59570	LUX/PCL1	7,9	7,6		4,7	4,7			
AT2G25930	ELF3	1,9	2,5		4,5	4,5			
AT5G23410	FKF1		-1,5		3,3	3,3			
AT2G18170	ATMPK7				1,6	1,6			
AT3G22380	TIC					1,4			
AT5G24470	PRR5/APPR5	1,9	2,9			1,2			
AT5G51810	GA20OX2					1,2			
AT3G04910	WNK1/ZIK4					1,2			
AT1G09340	CRB					1,2			
AT2G18790	PHYB/OOP1					1,1			
AT3G22170	FHY3			-1,5		1,0			
AT5G59560	SRR1					1,0			
AT2G18915	LKP2/ADO2					-1,0			
AT1G10470	ARR4/IBC7					-1,1		1,6	
AT2G44680	CKB4					-1,1			
AT5G52910	ATIM					-1,1			
AT5G57360	ZTL/LKP1/FKL2					-1,1			
AT5G10140	FLC/FLF/AGL25		-5,5	-4,0		-1,1		-6,1	-4,6
AT1G59940	ARR3					-1,1			
AT2G21070	FIO1					-1,1			
AT4G18020	APRR2		-1,5			-1,3			
AT3G60250	CKB3					-1,3			
AT4G08920	CRY1/HY4				-1,7	-1,7			
AT4G25100	FSD1	-3,5	-3,4		-1,8	-1,8	-3,3	-1,6	2,1
AT4G18290	KAT2	-2,2	-2,9		-1,9	-1,9			
AT1G68830	STN7				-2,0	-2,0			
AT1G09530	PIF3/PAP3	-1,8	-2,1		-2,0	-2,0			
AT1G22770	GI		1,5		-2,9	-2,9		-2,3	
AT5G15850	COL1		-1,9	-1,8	-4,2	-4,2			
AT5G02810	PRR7/APRR7				-8,6	-8,6			
AT1G01060	LHY1	-2,0	-2,7		-10,2	-10,2			
AT2G46670	PRR9/APRR9/TL1		-1,3		-26,0	-26,0			
AT2G46830	CCA1		-2,7	-2,6	-64,1	-64,1			

Table 3.5
Ratios for differential gene expression in 12:00 and 21:30h samples for genes involved in phototransduction. positive values: upregulated in numerator; negative values: downregulated in numerator. Only present called genes with PPDE(p)>0.950 and a fold change up/down>1.5 are listed.

		SPL3OX / WT ratio	***ful*SPL3 / WT ratio**	***ful*SPL3 / SPL3OX ratio**	WT evening / WT midday ratio	WT evening / WT midday ratio	**SPL3OX / WT ratio**	***ful*SPL3OX / WT ratio**	***ful*SPL3OX / SPL3OX ratio**
phototransduction		**12:00h**	**12:00h**	**12:00h**	21:30h/12:00h	all values	**21:30h**	**21:30h**	**21:30h**
AT2G40080	ELF4				25,1	25,1	-3,4	-4,3	
AT2G42540	COR15A	2,4	4,3	1,8	8,3	8,3			
AT5G15960	MUR1;KIN1	1,8	2,1		6,4	6,4			
AT2G25930	ELF3	1,9	2,5		4,5	4,5			
AT5G11260	HY5				2,6	2,6			
AT4G16250	PHYD					1,3			
AT5G35840	PHYC					1,3			
AT4G15090	FAR1					1,2			
AT2G18790	PHYB/OOP1					1,1			
AT2G02950	PKS1					1,1			
AT3G22170	FHY3			-1,5		1,0			
AT5G59560	SRR1					1,0			
AT3G62090	PIL2					-1,0			
AT5G63310	NDPK2/NDPK1A					-1,1			
AT1G10470	ARR4/IBC7					-1,1		1,6	
AT1G59940	ARR3					-1,1			
AT5G20730	NPH4/MSG1/BIP					-1,1			
AT2G46370	JAR1	-1,5				-1,1			
AT2G42810	PAPP5/PP5					-1,2			
AT5G49230	HRB1					-1,2			
AT2G26670	HY1					-1,2			
AT5G63870	PP7					-1,2			
AT2G24790	COL3					-1,3			
AT4G02440	EID1					-1,3			
AT2G37970	SOUL-1					-1,4		1,7	
AT5G64330	NPH3/RPT3				-1,5	-1,5			
AT2G46340	SPA1		-1,5	-1,8	-2,0	-1,6			
AT4G08920	CRY1/HY4				-1,7	-1,7			
AT1G09570	PHYA/FRE1/HY8				-1,8	-1,8			
AT1G09530	PIF3/PAP3	-1,8	-2,1		-2,0	-2,0			
AT4G25350	SHB1					-2,7			
AT1G02340	HFR1	-2,5	-2,5		-2,7	-2,7			
AT4G16780	ATHB-2/HAT4		1,7			-3,8			
AT5G02810	PRR7/APRR7				-8,6	-8,6			
AT2G43010	PIF4/SRL2	-1,6	-1,5		-8,7	-8,7			
AT3G59060	PIL6				-12,4	-12,4		-3,1	

When comparing the fold changes of *SPL3OX* and wild-type at sampling time point 12:00h to the wild-type changes at timepoints 12:00h and 21:00h it was obvious that in *SPL3OX* transgenics genes, that usually are upregulated towards the end of the day, were already higher expressed at 12:00h. Among those were genes known to be involved in circadian rhythm, e.g. *CCR2* (1.5), *LUX* (7.9), *ELF3* (1.9) and *PRR5* (1.9). On the other hand there were also genes downregulated in *SPL3OX* in this comparison. Among those were *FSD1* (-3.5), *KAT2* (-2.2) and *LHY1* (-2.0). When comparing the fold changes in the 21.30 samples of *SPL3OX* the differences were not as strong as in the 12.00 samples. Only *FSD1* (-3.3) and *ELF4* (-3.4) were strongly missregulated. Genes involved in phototransduction and found to be upregulated in this comparison are *COR15A* (2.4), *KIN1* (1.8) and *ELF3* (1.9), while downregulated genes are *PIF3* (-1.8), *PIF4* (- 1.6) and *HRF1* (-2.5).

The same comparison was done for *ful SPL3*. Genes that were additionally (to the ones already identified in *SPL3OX*) upregulated at 12:00h in *ful SPL3* compared to wild-type were *TOC1* (1.7) and *GI* (1.5), while additionally downregulated were *ZTL* (-5.5), *PRR2* (-1.5), *FLC* (-5.5), *COL1* (-1.9) and *CCA1* (2.7). Again, when this comparison was done for the 21:30h sampling, there weren't many differences in gene expression. Only *ARR4* (1.5) and *SOUL*-1 (1.7) were differentially expressed (Table 3.5). Generally it seemed that gene missregulation was even stronger in the *ful SPL3* mutant, because the fold change differences in comparison to wild-type was often even stronger for genes that were affected also in the *SPL3OX* in the *ful SPL3* mutant.

When comparing the fold changes in *SPL3OX* to *ful SPL3* at 12:00h and 21:30h *FHY3* (-1.5) was missregulated in the 12:00h sample. Other genes involved in circadian rhythms or phototransduction were not significantly missregulated.

Taken together these data show, that in *SPL3OX* transgenics, genes at the core of the circadian clock are missregulated in the first half of the light period. So called 'evening genes', like *LUX*, seemed to be upregulated too early during the day and expression levels of 'morning genes' like *LHY* remained too low. Moreover, this effect seemed to be dependent on overexpression of *SPL3*, since in the comparison *ful SPL3 / SPL3OX* those genes did not occur anymore. Furthermore, a direct output signal of the circadian clock, *GI*, seemed to be missexpressed as well. Therefore, it was hypothesized that overexpression of *SPL3* affects the circadian clock. Interestingly, the missregulation of *FLC* seemed to be dependent on *FUL*, since in the comparison of *ful SPL3/* WT and *ful SPL3 / SPL3OX* the values of *FLC* differ, while in the comparison of *SPL3OX /* WT *FLC* was not missregulated. But since the expression levels of *FLC*, being a negative regulator of flowering time, these results seemed to be contradictory to the later flowering phenotype of the *ful SPL3* mutant.

3.4 Effects of *SPL3OX* on the circadian clock

The circadian clock in Arabidopsis represents a major genetic trait for perception and interpretation of environmental conditions that affect the transition from vegetative to reproductive phase. Furthermore, the clock regulates a suite of developmental and metabolic processes to optimize plant behaviour in response to regular environmental changes. In fact, around 6% of the Arabidopsis genome is believed to be under clock control (Salomé and McClung 2004). The endogenous circadian clock is entrained by external cues, called *Zeitgebers* of which daylight is a major one. As such the circadian clock is known to be part

of the photoperiodic flowering pathway. Plants being defective in correct interpretation of day-night cycles often show flowering time phenotypes.

One way to test if the circadian clock is affected is determining the periodicity of leaf movement under free-running conditions in plants that have been entrained by a distinct dark-light rhythm before (Edwards and Miller, 2007).

3.4.1 Circadian leaf movement of *SPL3OX*

In order to investigate the possibility of *SPL3* affecting the circadian clock, the vertical movement of cotyledons of seedlings of wild-type and two independent lines of *SPL3* overexpressors was recorded for 7 d under free-running conditions, i.e. continuous white light and a constant temperature of 22°C. Before, the plant were entrained to a 12h dark / 12h light rhythm. Additionally, the leaf movement of *ful SPL3* seedlings was compared to wild-type.

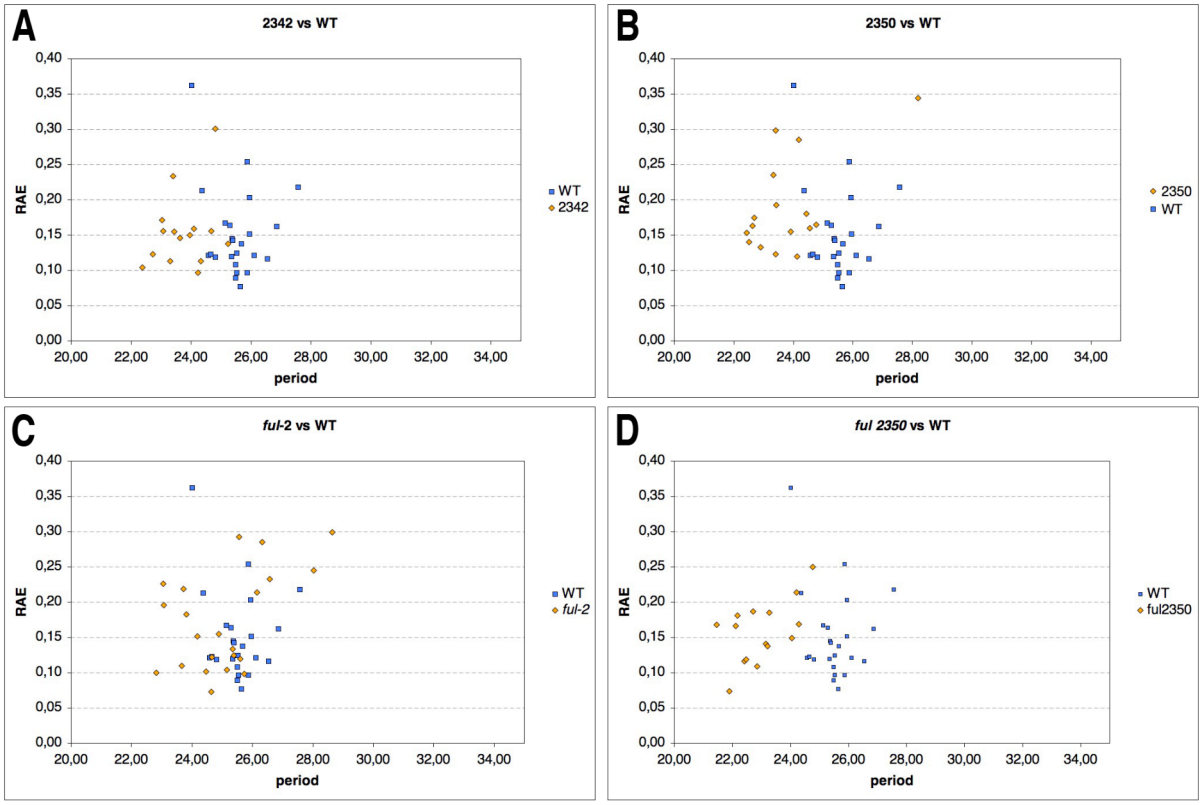


Fig. 3.16
Scatterplots indicating the vertical movement of cotyledons of wild-type, the two independent *SPL3OX* lines 2342 and 2350, *ful*-2 mutant and the *ful SPL3* mutant. The period of movement was plotted against the relative amplitude error (RAE), which is a value indicating the robustness of the movement. The lower the RAE value is the more robust the system / movement was, i.e. values below 0.4 are considered as robust and indicate a rhythmic movement.

The scatter plots in Fig. 3.16A and B indicate that, on average, the periodicity of leaf movement in *SPL3OX* transgenics was shortened in comparison to wild type, i.e. from 25.54 h for wt to respectively 23.75 h and 23.81 h for both transgenics. Thus the circadian clock in *SPL3OX* seemed to run faster in comparison to wild type. Interestingly, the *ful SPL3* seedlings had almost the same period as those of the *SPL3OX* transgenic lines (Fig. 3.16C and D; 2350: 23.81h; *ful SPL3*: 23.00h).

3.4.2 Expression of circadian clock genes in *SPL3OX* and *ful SPL3* seedlings

In order to investigate how SPL3 affects the circadian clock the diurnal expression of the core components of the circadian clock in Arabidopsis has been investigated. Thereto, 7d old seedling of two independent *SPL3OX* lines, *ful SPL3* and wt were grown under LD and SD conditions and harvested every 4 hours over a period 24h. RNA was extracted and expression of circadian clock transcripts was tested by quantitative real-time PCR. Figure 3.17 shows the expression of the core components of the circadian clock under LD and SD conditions, that showed the most dramatic changes in expression. All other clock associated genes, that showed only a marginal change in expression are shown in appendix E.

Figure 3.17A and B show the expression levels of *GI* over 24h under LD and SD conditions. In wt growing in LD, *GI,* being the output of the circadian clock, reached the highest expression level at ZT10 (LD) and ZT6 (SD) respectively. The expression values were significantly higher in both the *SPL3OX* and the *ful SPL3* mutant. Especially under SD conditions the expression of *GI* seemed to decrease slower towards the end of the light period in the *ful SPL3* mutant compared to wild-type.

The *TOC1* expression levels peaked at ZT18 in wild-type under LD conditions and SD conditions. In the *SPL3OX* lines the expression at ZT10 and ZT14 was already increased, which was also true for the *ful SPL3* mutant with the strongest expression levels (Fig. 3.17C). Interestingly, the expression increased earlier during the light period (LD) but did not exceed the maximum expression of wild-type in the *SPL3OX* and probably also not (much) in the *ful SPL3* mutant. Under SD conditions, the *TOC1* expression also peaked at ZT18 in the SPL3OX lines and the *ful SPL3* mutant. Nevertheless, while the expression stayed low in the SPL3OX during the day, it was earlier upregulated in the *ful SPL3* mutant (ZT14; Fig. 3.17D).

Figure 3.17 E and F show the expression of *ELF4* over 24h under LD and SD conditions in wild-type, *SPL3OXs* and *ful SPL3*. In wild-type *ELF4* expression peaked at ZT10 under LD conditions, while under SD the expression maximum was reached at ZT14 and then decreased

again. Interestingly, in both *SPL3OX* lines the levels of wild-type expressions were not reached. Under LD conditions the expression levels were about half of wild-type levels while under SD conditions about 2/3 of wild-type levels were reached. Interestingly, the expression of *ELF4* in *ful SPL3* seemed to follow the wild-time expression both under LD and SD conditions, although it seemed to decrease a little slower under SD conditions.

The diurnal expression of *PRR7* is shown in Fig. 3.17 G and H. Under LD conditions the maximum expression was reached at ZT10, while under SD it was reached a little earlier, at ZT6.

Under LD conditions the expression levels also were highest at ZT10, but they significantly exceeded the wild-type levels. The same holds true for *ful SPL3*, which showed the highest expression levels (Fig. 3.17G). Under SD conditions, the expression levels were not as high as under LD conditions. Moreover, the relative increase of expression in the *SPL3OXs* was not as much as under LD conditions. In the *ful SPL3* line, the highest levels were reached at ZT10, not as in wild-type at ZT6, and decreased slower than in wild-type (Fig. 3.17H).

In the two *SPL3* overexpressor lines as well as in *ful SPL3, LHY* expression levels at ZT2 were increased (Fig. Appendix D, A) under LD conditions, but otherwise did not show significant differences to wild-type expression under LD conditions. The same holds true for the expression of *CCA1* (Fig. C in Appendix D). Taken into account that the *TOC1* expression was altered in these samples as well this is not surprising, since these genes are the core components of the clock and connected through a positive-negative feedback loop. Under SD conditions the expression of *LHY* and *CCA1* did only show an increase in 2350 (Fig. B and D in Appendix D) at ZT22, while the expression was not altered any other time of the day.

The diurnal expression of *LUX* and *ELF3* was not changed in the *SPL3OXs* or *ful SPL3* under both LD and SD conditions compared to wild-type (Fig. E to H in appendix D).

Taken together the diurnal expression data of clock associated genes confirm the affymetrix data concerning the genes tested and indicate that overexpression of *SPL3* might result in a changed expression of clock associated genes.

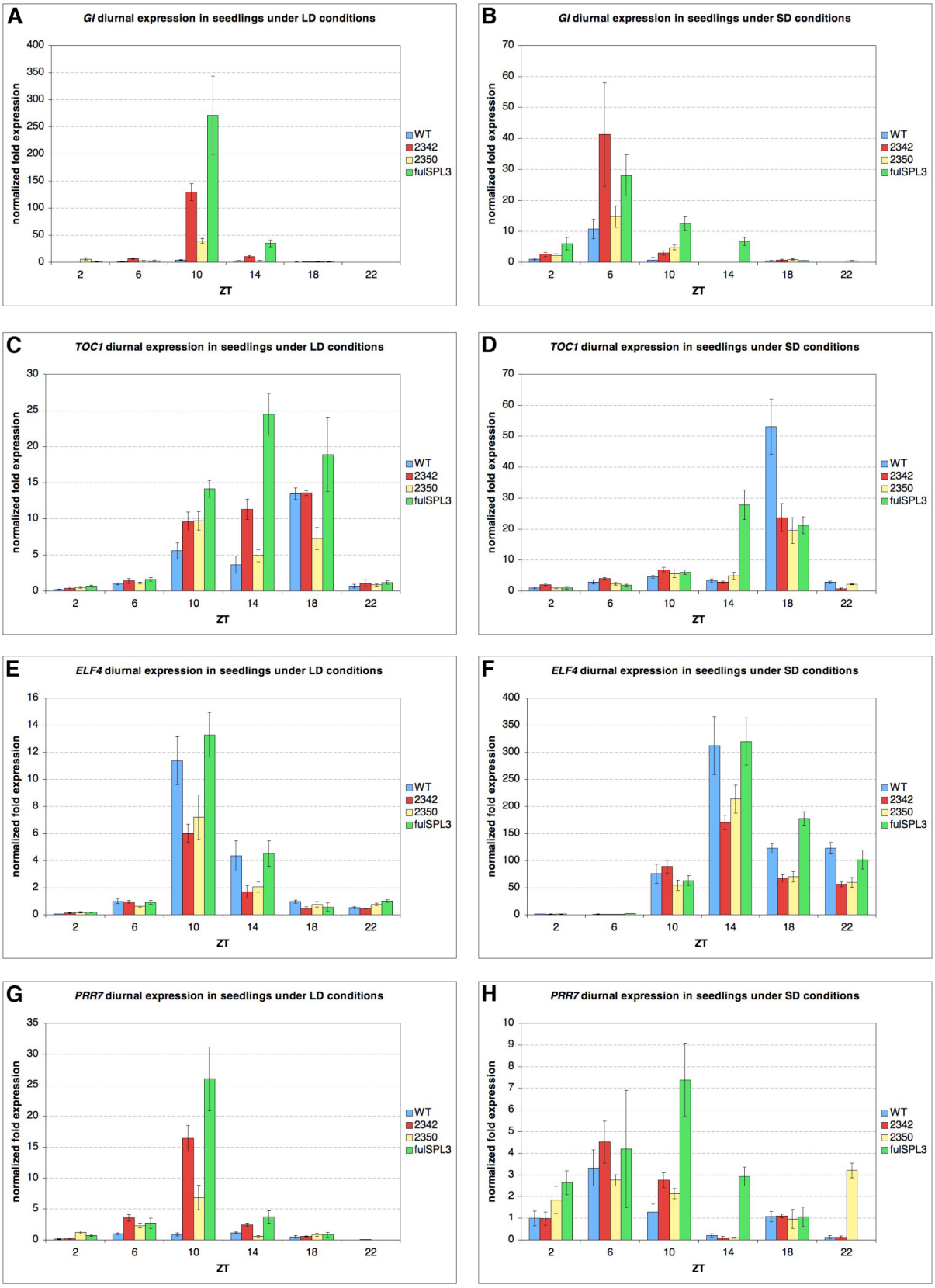


Fig. 3.17
Confirmation of diural expression of several clock associated genes in 7 d old seedlings under LD and SD conditions. Diurnal Expression of *GI* (A and B), *TOC1* (C and D), *ELF4* (E and F) and *PRR7* (G and H) under LD and SD conditions. All genes were normalized against *PP2A* and ZT2 of WT was set arbitrarily set as one. blue = WT, red =*SPL3OX* line 2342, yellow = *SPL3OX* line 2350, green = *SPL3OX* line 2350 *ful*-2. ZT0 represents the beginning of the light period.

4. Discussion

The SBP-box transcription factor *SPL3* from Arabidopsis has been shown to be post-transcriptionally regulated by miR156 (Gandikota *et al*., 2007, Schwab *et al*. 2005, Wu and Poethig 2006) and constitutive overexpression of a microRNA insensitive form results in early flowering (Cardon *et al*. 1999). Furthermore, upon photoperiodic induction of flowering, *SPL3* is among the first and strongest responding genes (Schmid *et al*., 2003). Moreover, SPL3 is the presumed ortholog of SBP1 from *A. majus*. Both proteins are able to bind *in vitro* to a conserved sequence in the promoter region of the orthologous floral meristem identity genes *SQUA* from Antirrhinum and *AP1* from Arabidopsis (Cardon *et al*., 1997, Klein *et al*., 1996). *AP1*, however, has been shown not to be essential for the earliness of the *SPL3* overexpressing transgenics (Cardon *et al*., 1999).

The different lines of experimental evidence suggest that *SPL3* promotes flowering and the data collected in this study may shed light on how this is achieved.

4.1 Identification of putative SPL3 target genes following a candidate gene approach

The floral pathway integrators *SOC1* and *FT* as well as the floral meristem identity genes *FUL*, *CAL* and *LFY* and *FPF1* fulfilled the previously described criteria for putative *SPL3* target genes. Therefore their expression levels were compared between *SPL3* transgenic seedlings and wild type.

4.1.1 Precocious expression of *SPL3* causes upregulation of *FT* and *FUL* in seedlings

As expected, transcript levels of both the floral pathway integrators were low in wild-type seedlings. In *SPL3OX* seedlings transcript levels of *FT*, but not those of *SOC1*, were clearly upregulated. Also, expression levels of the floral meristem identity genes were found generally low in both wild-type and *SPL3OX* transgenic seedlings with the exception, however, of *FUL*. *FUL* transcript levels were found to be significantly higher expressed in the transgenics. With respect to the upregulation of both *FT* and *FUL* it is particularly interesting to note that the phenotype obtained through constitutive overexpression of *FT* as described by (Teper-Bamnolker and Samach 2005) mimics the phenotype of *SPL3OX* plants, i.e. relatively small and early flowering plants with curled leaves. Moreover, an accumulation of *FUL* transcripts in seedlings was found in response to *FT* overexpression. However, in contrast to *SPL3OX* transgenic seedlings, Teper-Bamnolker and Samach also found higher transcript levels for of the floral meristem identity gene *AP1*. Similarly, *SEP3*, capable of enhancing earliness through interacting with *AP1* (Pelaz *et al.,* 2001) and strongly upregulated in

35S::FT, showed only slightly increased expression in *SPL3OX* transgenic seedlings. Furthermore, the relative increase in expression levels of both *FT* and *SEP3* in *SPL3OX* seedlings were found to be much lower in comparison to that of *FUL*. Therefore, it is concluded that the molecular genetic mechanisms underlying the early flowering phenotype of *SPL3OX* transgenics are not merely a copy of those in the *FT* overexpressors. It remains, however, reasonable to speculate that upregulation of *FUL* in *SPL3OX* transgenics is a subsequent consequence of enhanced *FT* expression. Alternatively, *FUL* may represent the primary target of *SPL3* with the upregulation of *FT* as secondary, i.e. feedback, effect.

4.1.2 Diurnal expression analysis reveals that SPL3`s effect on *FUL* expression is partly independent on *FT*

FT expression shows a diurnal rhythm with a strong peak at the end of the light period in LD conditions (Mizoguchi *et al*., 2005) as a result of the complex regulation of *CO* on both transcriptional and post-translational level. Therefore, the time of sampling may become decisive to uncover altered transcriptional behaviour when studying *FT* or any other gene showing a diurnal expression rhythm. The expression analysis over a 24 h period revealed that the diurnal expression profile of *FT* was not notably altered in *SPL3OX* seedlings in comparison to wild-type. *FT* transcript levels in *SPL3OX* seedlings were, however, slightly higher than in wild-type. In contrast, *FUL* transcription was significantly upregulated in the *SPL3* overexpressors during the light and dark period. Interestingly, *FUL* expression levels raised even more towards the end of the light period in the *SPL3* overexpressing lines, what could reflect a certain dependency on *FT*.

If *SPL3* would regulate *FUL* expression through *FT*, *FUL* transcript should be barely detectable in *SPL3OX* transgenics grown under SD conditions, because *FT* transcript is expected to stay low during the entire day then. However, *FUL* transcripts remained at significantly higher levels in the *SPL3* overexpressors compared to wild-type and remarkably also peaked at the end of the day independently of the presence of light, i.e. showed the same diurnal rhythm as found before under LD conditions. *FT* mRNA on the other hand indeed remained undetectable in the same SD samples. Therefore, the temporal increase of *FUL* expression at the end of a day seemed to be independent of the light period and independent of *FT*. It is thus concluded that the promotion of *FUL* is unlikely to happen through *FT*. At least *SPL3* is probably not promoting *FUL* expression through upregulation of *FT*. Final proof though can only come from testing the *FUL* expression in *ft SPL3* transgenics.

4.1.3 *FUL* represents a major mediator in *SPL3* induced earliness

To determine to what extend *FUL* and *FT* actually mediate the effect of *SPL3* overexpression on flowering, the *SPL3OX* transgene was analysed against both a *ful*-2 and a *ft*-10 mutant background. Concerning bolting and anthesis, under LD conditions *ful SPL3OX* plants resembled very much wild-type, but when compared on bases of their TLN, *ful SPL3OX* plants were still slightly earlier flowering than wild-type. This means that in a *ful*-2 mutant background the earliness of flowering accomplished by *SPL3* overexpression is almost completely abolished. Determination of flowering time under SD conditions revealed that again *ful SPL3OX* plants flowered earlier than wild-type when counting the number of rosette leaves, but not when expressed in days to bolting and anthesis. In fact, *ful SPL3OX* plants flowered even somewhat later. Nevertheless, both *SPL3OX* and *ful SPL3OX* remained photoperiodically sensitive, since the flowering times under SD conditions were longer than under LD. Remarkably, under SD conditions the number of cauline leaves of both *ful*-2 and *ful SPL3OX* plants were greatly increased.

As recently published, *35S::FT ful*-2 plants flower later than *35S::FT* plants both under LD and SD conditions (Melzer *et al*., 2008), which gives additional evidence for a decisive role for FUL presence during the floral transition.

Taken together these results suggest, that constitutive overexpression of *SPL3* results in early flowering primarily because of a precocious upregulation of the floral meristem identity gene *FUL*.

4.1.3 *AGL24* and *SOC1* are not affected in their expression by SPL3

MADS-domain proteins like FUL are known to function as homo- or heterodimers (Kaufmann *et al*., 2005). Among the MADS-domain proteins capable of forming heterodimers with FUL and known to promote flowering are SOC1 and AGL24 (deFolter *et al*., 2005). Moreover, these latter MADS-box genes are believed to act upstream of *FUL* in integrating flowering signals (Liu *et al*., 2008). With respect to *FUL* mediated early flowering, *SOC1* and *AGL24* expression levels could be important factors in *SPL3* overexpressor plants. However, RT-PCR transcription profiling over 24 h revealed that the expression of both *AGL24* and *SOC1* in *SPL3OX* as well as in *ful SPL3* seedlings did not differ much from wild-type. Therefore it is unlikely that *AGL24* or *SOC1* expression are directly influenced by elevated *SPL3* or *FUL* levels. Furthermore, although *SOC1* levels seemed to be slightly elevated in response to *SPL3* overexpression, it is unlikely to be

responsible for the high expression levels of *FUL*. More likely, through a so far unknown feedback mechanism on more upstream acting flowering time determining genes, high *SPL3* or *FUL* expression levels may lead to elevated *SOC1* expression levels. As discussed later, this feedback mechanism may involve the circadian clock and could thereby also explain the elevated *FT* levels observed in *SPL3* overexpressing plants.

Another possibility of promoting flowering time is the precocious repression of flowering time repressors such as *SVP*. SVP is a MADS domain protein that is controlled by the autonomous, thermosensory and gibberellin pathway (Lee *et al*. 2007) and is expressed in vegetative tissue before the floral transition (Hartmann *et al*., 2000) It is independently of photoperiod repressing flowering time in a dosage dependent manner (Hartmann *et al*., 2000 Yu *et al*., 2002, Michaels *et al*., 2004) and has shown to be epistatic to *AGL24* (Gregis *et al*., 2006). An interaction of *FUL*-likes with *SVP*-likes has been found in rice, petunia (deFolter *et al*., 2005) and *Lolium penne* (Ciannamea *et al*., 2006). Moreover, FUL has been shown to be able to interact with SOC1 in Arabidopsis (deFolter *et al*., 2005). It has already been stated that there might be positive and negative crosstalk between the induction of flowering and the formation of floral organ identity (deFolter *et al*., 2005) and that proteins involved in both processes might have an early and a late function (Mandel and Yanovsky, 1992; Ferrandiz *et al*., 2000).

This could mean that the elevated expression levels of *FUL* in *SPL3OX* transgenic plants could titer out the repressing protein SVP either through a so far undiscovered direct interaction of SVP and FUL or through the slightly elevated levels of *SOC1*. This could then result in an early flowering phenotype.

Therefore, it is conceivable that *SPL3* overexpression not only promotes flowering by upregulation of *FUL* on the transcriptional level, but also by repressing flowering time repressors such as SVP at a post transcriptional level. Through direct interaction on the protein level slightly elevated expression of both FUL and SOC1 protein might titer out the repressor SVP. This of course could not be detected in a transcriptional profiling.

4.2 Characterization of the interaction of SPL3 with its putative target gene *FUL*

In vitro DNA-binding assays showed that SBP-domain proteins require the nucleotide sequence GTAC as core motif for high affinity DNA binding (Birkenbihl *et al*. 2005, Liang *et al*., 2008) and that functional studies *in planta* indicate that multiple copies of the core motif have to be present as cis-elements for target gene responsiveness (Nagae *et al*., 2008, Quinn *et*

al., 2003, Yamasaki *et al.,* 2009). The expression data presented in this study pointed to *FUL* as representing a direct target of SPL3. In agreement, the GTAC motif was found abundantly in and around the *FUL* genomic locus. Also, the motif CGTAC, shown to be preferred by SPL3 (Birkenbihl, *et al*., 2005), was found to be enriched particularly in the first intron of *FUL*. With respect to theses features, the *FUL* locus clearly differed from the evolutionary and functional related *AP1* and *CAL* loci (Purugganan 1997, Ferrandiz *et al.*, 2000). Expression of the latter was also not found to be upregulated in *SPL3OX* transgenic seedlings.

4.2.1 Definite proof for physical interaction between SPL3 and *FUL* remains elusive

The Crosslinked Chromatin Immunoprecipitation (X-ChIP) technique is a powerful tool to identify direct targets of a transcription factor, when an adequate antibody against the transcription factor is available (Orlando 2000). Initial analysis indicated that fragments of the promoter region of *FUL* and in particular of the 3' region of its first intron, could be enriched after X-ChIP with an SPL3 antibody. Since in both *SPL3OX* and wild-type inflorescences *FUL* and *SPL3* are known to be expressed (Mandel and Yanovsky 1995a, Hempel *et al*., 1997, Cardon *et al*., 1996), this finding would be clearly in favor of a physical interaction between SPL3 and *FUL.* Furthermore, *FUL* promoter region as well as first intron sequences could also be enriched after X-ChIP on *SPL3OX* seedlings. However, when immunoprecipitated material of wild-type seedlings was analyzed a similar enrichment was obtained. The latter observation seems to offer a contradiction. Albeit *SPL3* mRNA is detectable in wild-type seedlings it is believed to be translationally repressed by miR156 and therefore SPL3 protein remains undetectable by Western blot analysis (Gandikota *et al*., 2007).

It is unknown to what extend the SPL3 antiserum discriminates between native and denatured protein. Western blot analysis could thus be less sensitive in comparison to X-ChIP in combination with quantitative real-time PCR. Low SPL3 protein levels in seedlings would remain unnoticed on Western blots but detectable in X-ChIP. However, it remains difficult to understand why then, in comparison to wild-type, the high SPL3 protein levels obtained in transgenic seedlings (Gandikota *et al*., 2007) did not result in relative higher enrichments.

Therefore, the X-ChIP experiment does not allow to draw the univocal conclusion that *FUL* is a direct target of SPL3 although the results seem to suggest this. Furthermore, it indicates that that the first intron of *FUL* might play an important role in its regulation.

4.2.2 Remote cis-regulatory sequences are required for upregulation of *FUL* in *SPL3OX* transgenics

Several different *GUS* reporter gene constructs were generated to determine the functional relevance of the CGTAC motifs in the promoter region and the first intron of *FUL*. None of the corresponding transgenic plants showed detectable GUS expression, even not in an *SPL3OX* background. Therefore a direct conclusion on the functional relevance of these motifs can not be drawn. This is quite unexpected, because GUS activity derived from a *GUS* reporter inserted in the endogenous *FUL* genomic locus, i.e. having generated the *ful*-1 mutant allele (Gu *et al*., 1998), was detectable. Moreover, when crossing this line with *SPL3OX* plants, the *GUS* reporter responded clearly to the *SPL3OX* transgene with a stronger signal and a wider distribution. It is reasonable to assume that all *FUL* regulatory sequences (unless the insertion of the *GUS* reporter destroyed one) are preserved in the *ful*-1 allele. Moreover, *SPL3* affected the GUS activity also in the NASC line N8847, which carries a *GUS* reporter driven by 2.3 kb promoter region from upstream of the *FUL* ATG translation start codon. In contrast to the NASC N8847 transgene, however, my constructs did not cover a CGTAC motif 2.2kb upstream of the *FUL* ATG. However, in the NASC line, the GUS activity also remained very low and, in contrast to *ful*-1 mutant plants, was undetectable in coteyledons. So despite of the regulatory motifs in the promoter region, that might have been missed in my constructs but covered in the NASC line, the low GUS signal indicates that downstream intragenic sequences, i.e. in the 1st intron, play a role in the regulation of *FUL*. On the other hand, the presence in the first intron, carrying several CGTAC motifs, is insufficient for an activation of the GUS reporter. It therefore must be concluded that regulatory motifs in both the promoter region and the first intron are necessary for *FUL* expression and the response to SPL3 *per se*. Moreover, the motifs within the first intron positively affect expression levels and might contribute to a tissue specific expression.

Notably, it has been recently reported that transcriptional regulators bind target genes not only in the promoter, but also in the first intron (Helliwell *et al*., 2006, Schauer *et al*. 2008, Rosea *et al*., 2008)

4.2.3 Precocious expression of *SPL3* not sufficient to activate *FUL* in all tissues

All expression data obtained in this study confirm that constitutive over-expression of a miR156 resistant *SPL3* transgene results in precocious activation of *FUL*. Notably, the *SPL3* transgene is under the control of the 35S CaMV promoter and thus expected to be more or less expressed ubiquitously in all tissues (Benfey and Chua 1990). However, upregulation of

FUL, as determined *in planta* with a *GUS* reporter, seems to be largely restricted to the leaves and there predominantly to the veins. Therefore, this strongly suggests that *SPL3* does not activate *FUL* alone but that it needs (a) partner(s). As discussed before, such a partner could be *FT*. *FT* transcript levels remained largely unaffected in *SPL3OX* transgenics but flowering of *SPL3OX* transgenics was strongly delayed against an *ft* mutant background but yet not as late as in the *ft* mutant. Furthermore, spatial expression of *FT* in wild-type (Takada and Goto, 2003, Abe *et al*., 2005.) resembles that of *FUL* in *SPL3OX* transgenics, i.e. largely restricted to the veins of the leaf blade. Nevertheless, FT cannot be the only partner required by SPL3 to activate *FUL* as upregulation of *FUL* since the earliness in *SPL3OX* transgenics is not completely lost under SD conditions and *ft SPL3OX* transgenics flower earlier than *ft*-10 transgenics. *TWIN SISTER OF FT* (*TSF*, Yamaguchi *et al*., 2005) acts redundantly to *FT* as a floral pathway integrator (Yamaguchi *et al*., 2005) with a similar diurnal and spatial expression, although with lower expression levels. Therefore one could speculate that TSF could fulfil the role as an interaction partner of SPL3 as well. Thus it would be interesting to study the *SPL3OX* transgene in an *ft tsf* double mutant background with respect to *FUL* expression.

The expression pattern of *FUL*, as observed in *SPL3OX* transgenics, raises an important question with respect to how this is translated into an early flowering phenotype. If, in response to *SPL3*, *FUL* becomes primarily upregulated in leaves how then could this promote flowering?

It is known that upon photoperiodic induction hormonal and metabolic signals are send from the leaves to the apex (Bernier *et al*. 1993). Notably, recent data strongly suggest that part of the multifactoral signal, transported through the phloem, is the 20 kDa FT protein (Corbesier *et al*., 2007; Jaeger and Wigge 2007). Arrived at the apex, FT interacts with the bZIP transcription factor *FLOWERING LOCUS D* (*FD*) to promote flowering (Abe *et al*., 2005).

As in *SPL3OX* transgenic plants, *FUL* and *FT* expression merge in the veins of cotyledons and early leaves, it is interesting to speculate that *FUL* promotes flowering through facilitation of loading and/or transporting FT protein through the phloem. In this context, the observation that expression of MIR156 in early leaves is also linked to the veins (Schwab 2006) and that miR156 has also been detected in the phloem sap (Yoo *et al*., 2005) may be of relevance to maintain juvenility. It is believed that the leaves of juvenile plants are incapable of producing (sufficient) floral stimulus in response to an inductive photoperiod (Zeevaart 1985). MiR156 as well as several of SBP-box gene targets have been associated with the

juvenile to adult phase transition in both Arabidopsis and maize (Wu and Poethig, 2006; Schwarz *et al*., 2008; Chuck *et al*., 2008)

4.3 Global expression profiling of *SPL3OX* transgenics revealed alterations of the circadian clock, in sugar metabolism and light perception

With a global expression analysis using Affymetrix ATH1 arrays more putative target genes for SPL3 should be identified. Having chosen two timepoints of the day for sampling gave the unique opportunity to follow the impact of overexpression of SPL3 over a day and gave a more dynamic picture of gene expression than a one time sampling would have provided. This was particularly interesting since an additional alteration of *FUL* expression towards the evening was previously discovered. Fluctuation of transcripts can be caused by the adaptation of an organisms' physiology to the earth rotation via the circadian clock. Research on the biological clock has revealed that up to 90% of all transcripts in Arabidopsis show a rhythmic expression pattern (Michaels *et al*., 2008) and that most of the physiological processes are influenced by the circadian clock.

As the GO annotations and AmiGo analysis revealed, an increased number of missexpressed genes in SPL3OX seedlings was associated with the chloroplast. Moreover, the AmiGo data indicated that overexpression of SPL3 affects the circadian clock, sugar metabolism and red and far red light perception. This is particularly interesting, since the circadian clock is involved in photoperiodic induction of flowering (Salomé and McClung 2004 and references therein).

It has been shown that the levels of sucrose is a stimulus for flowering because sucrose in the phloem sap and/or soluble sugars in the apical bud increase early upon photoperiodic floral induction in both LD and SD plants (Bodson and Outlaw 1985, Houssa *et al*., 1991). Moreover, upon photoperiodic induction of flowering, the ratio of C:N in the leaf exudates as well as in the shoot apical meristem greatly and early increases, which led to the suggestion that an imbalance of C:N in favour for C positively affects the transition to flowering (Corbesier *et al.,* 2002). Interestingly, the starchless mutant *phosphoglucomutase* (*pgm*, Caspar *et al*., 1985) as well as the *starch-in excess1* mutant (*sex1*, Caspar *et al*., 1991), which are both late flowering in days shorter than 16h, always show a large, early and transient increase of carbohydrate export from leaves under LD inductive conditions, whereas a displaced short day (DSD), which does not alter the period for photosynthesis, does not increase the export of carbohydrates from leaves (Corbesier *et al*., 1998). Therefore, the authors concluded that floral induction increases the capability of the phloem loading system

(Corbesier *et al*., 1998) and suggested an increased sink activity of the SAM for carbohydrates during the floral transition (Corbesier *et al*., 2002). This is particularly interesting since also FT protein is exported from the leaves through the phloem sap.
Notably, the *pgm* mutant not only showed a large change of diurnally expressed sugar-responsive genes but also a subset of circadian-regulated genes were found to be affected when a global expression analysis was conducted (Bläsing *et al*., 2005). The authors found that for a quarter of the circadian regulated genes, the diurnal change of sugars in a light/dark cycle reinforced the circadian regulation and therefore concluded that sugars modify the phase of a subset of the clock-regulated genes (Bläsing *et al*., 2005).

4.4 Leaf movement analysis and expression analysis of clock genes confirmed the missregulation of the circadian clock in *SPL3OX* seedlings

In order to investigate if and how the clock is affected by overexpression of *SPL3*, the circadian movement of cotyledons in two *SPL3OX* lines, *ful SPL3* and *ful*-2 seedlings was tested under free-running conditions after the seedlings had been entrained to LD conditions. This experiment uncovered that in the two *SPL3OX* lines the period of the circadian clock is shortened by about 2 hour compared to wild-type. Nevertheless the movement of the cotyledons was not arrhythmic.
The expression of the core components of the circadian clock as well as genes known to be closely associated with circadian rhythms were subsequently analysed by means of quantitative real time PCR under both LD and SD conditions. Expression of transcripts known to be associated with the circadian clock indicated that indeed the diurnal expression of the clock genes *TOC1*, *ELF4*, *PRR7* and *GI* is altered in *SPL3OX* as well as in *ful SPL3OX* whereas the diurnal expression pattern of *LUX* and *ELF3* though remained largely unaffected.
The altered expression of *TOC1* could be responsible for the slightly elevated *CCA1* and *LHY* expression at the beginning of the light period in both *SPL3OX* and *ful SPL3OX*, since the genes are believed to be connected via a feedback loop (Alabadi *et al*., 2001; Schaffer *et al*., 1998; Strayer *et al*., 2000; Wang *et al*., 1998), that is responsible to sustain the rhythmicity of the clock (Ding *et al*., 2007). Moreover, *ELF4* has been shown to be a positive regulator of *CCA1* (Doyle *et al*., 2002; Kikis *et al*., 2005). Since the expression of *ELF4* is lower in *SPL3OX*, this could have dampened the effect of higher *TOC1* levels on *CCA1*. Nevertheless, the higher *ELF4* expression levels do not explain the lowered *CCA1* levels in comparison to *SPL3OX* in the *ful SPL3* mutant. Interestingly, the *elf4* mutant has been reported to be at least

partially a gating mutant that is hyposensitive to red-light repression of elongation of hypocotyl growth (McWatters *et al*., 2007). Also several other clock-associated genes have been shown to be involved in phytochrome-dependent red-light signaling. For example *TOC1* and *PRR7* are discussed to be positive regulators of the red-light signaling pathway acting downstream of *PHYB*, while *CCA1* and *LHY* are believed to be negative regulators in this pathway (Ito *et al*., 2007).

GI is known to mediate the output of the circadian clock by positively affecting the expression of *CO* and *FT* (Kardailsky *et al*., 1999; Kobayashi *et al*., 1999; Samach *et al*., 2000; Suarez-Lopez *et al*., 2001). Additionally, it has been more recently reported to be also involved in light input signalling (Huq *et al*., 2000) and blue light signalling (Martin-Tryon *et al*., 2007) and therefore might have separable roles in circadian clock regulation and flowering time regulation. Since the expression of *GI* is significantly enriched in both *SPL3OX* and *ful SPL3* plants under both LD and SD conditions, but the expression of the downstream genes of GI, that were tested (i.e. *CO*, *FT*, *SOC1*, *AGL24*), was only slightly affected in *SPL3OX* plants it seems unlikely that the altered *GI* expression in those plants is causing the early flowering phenotype of *SPL3OX*. It is more likely, that the effect of overexpression of *SPL3* on the circadian clock is secondary and of indirect nature.

5. Role for SPL3 during the floral transition

Taken together the data obtained in this study suggest a decisive role for *SPL3* during the transition from vegetative to reproductive growth. In the *SPL3OX* transgenic plants, *SPL3* is responsible for an early upregulation of *FUL*, primarily in the vasculature of the leaves. Moreover, *SPL3* seems to positively affect sugar availability in the leafs. It is interestingly to speculate that what is actually decisive is, that *FUL* might facilitate the loading of leaf produced substances into the phloem. Together with the leaf carbohydrates the FT protein, also produced in the leaves, could be earlier transported into the phloem and transported into the apex, which causes the early flowering phenotype.

Upon photoperiodic stimulus *SPL3* is among the fastest responding genes (Schmid *et al*., 2003). Since its expression in wild-type is detectable in the apex, in leaf primordia and early leaves it seems likely that SPL3 activates *FUL* in theses organs. The expression of *FUL* may then promote the floral transition through an enhanced and facilitated transport of leaf produced substances into the phloem, through which they are transported into the apex. In the apex then the role of SPL3 could not only be the activation of *FUL* to act as a floral meristem identity gene, but also as a factor that facilitates the transport of phloem substances into the

apex, thus creating a sink-like activity of the SAM. This role of *FUL* during the floral transition could correspond to the early function of FUL previously described (Mandel *et al*., 1992, Ferrandiz *et al*. 2000). Notably, the GUS analysis did not show an ectopic GUS staining in the apices of seedlings.

Nevertheless it will be interesting to further investigate how sugar metabolism, red-light signalling and the circadian clock are influenced by SPL3.

In the future it will be important to further elucidate the function of all microRNA regulated SPL-genes during the phase change from vegetative to reproductive growth to gain a better understanding of how the members of this plant-specific transcription factor family enable a plant to interpret environmental signals and to adjust their most crucial developmental processes to their environment in order to guarantee a maximized success of reproduction.

References

Abe M, Kobayashi Y, Yamamoto S, Daimon Y, Yamaguchi A, Ikeda Y, Ichinoki H, Notaguchi M, Goto K, Araki T. (2005) FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science. 2005 Aug 12;309(5737): 1052-6.

Achard P, Herr A, Baulcombe DC, Harberd NP. (2004) Modulation of floral development by a gibberellin-regulated microRNA. Development. 2004 Jul;131(14): 3357-65.

Alabadí D, Oyama T, Yanovsky MJ, Harmon FG, Más P, Kay SA. (2001) Reciprocal regulation between TOC1 and LHY/CCA1 within the Arabidopsis circadian clock. Science. 2001 Aug 3;293(5531): 880-3

Alexandre CM, Hennig L. (2008) FLC or not FLC: the other side of vernalization. J Exp Bot. 2008;59(6): 1127-35

Arabidopsis Genome Initiative, The (2000), Analysis of the genome sequence of the flowering plant Arabidopsis thaliana, Nature, 408: 796-815

Araki, T.,Kobayashi, Y.,Kaya, H.,Iwabuchi, M. (1998) The flowering-time gene FT and regulation of flowering in Arabidopsis. Journal of plant research, 111, 277-281

Aukerman, M.J. and Sakai, H. (2003) Regulation of Flowering Time and Floral Organ Identity by a MicroRNA and Its APETALA2-Like Target Genes. The Plant Cell, 15: 2730-2741.

Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, Zoran MJ. (2005) Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat Rev Genet. 2005 Jul;6(7): 544-56.

Bernier, G., Havelange, A., Houssa, C., Petitjean, A., Lejeune, P. (1993) Physiological Signals That lnduce Flowering, The Plant Cell, Vol. 5: 1147-1155

Benfey, P.N. and Chua, N.-H. (1990) The Cauliflower Mosaic Virus 35S Promoter: Combinatorial Regulation of Transcription in Plants. Science 16 November 1990: Vol. 250. no. 4983: 959 - 966

Birkenbihl, R.P., Jach, G., Saedler, H. and Huijser, P. (2005) Functional Dissection of the Plant-specific SBP-Domain: Overlap of the DNA-binding and Nuclear Localization Domains. Journal of Molecular Biology, 352, 585-596.

Blázquez MA, Weigel D. (2000) Integration of floral inductive signals in Arabidopsis. Nature. 2000 Apr 20;404(6780): 889-92.

Bläsing OE, Gibon Y, Günther M, Höhne M, Morcuende R, Osuna D, Thimm O, Usadel B, Scheible WR, Stitt M. (2005) Sugars and circadian regulation make major contributions to the global regulation of diurnal gene expression in Arabidopsis. Plant Cell. 2005 Dec;17(12): 3257-81.

Bodson and Outlaw (1985), Elevation in the Sucrose Content of the Shoot Apical Meristem of Sinapis alba at Floral Evocation. Plant Physiol. 1985 Oct;79(2):420-424

Cardon, G., Höhmann, S., Klein, J., Nettesheim, K., Saedler, H. and Huijser, P. (1999) Molecular characterisation of the Arabidopsis SBP-box genes. Gene, 237: 91-104.

Cardon, G.H., Höhmann, S., Nettesheim, K., Saedler, H. and Huijser, P. (1997) Functional analysis of the Arabidopsis thaliana SBP-box gene SPL3: a novel gene involved in the floral transition. The Plant Journal, 12: 367-377.

Carré, I.A. and Kim, J.-Y. (2002) MYB transcription factors in the Arabidopsis circadian clock. Journal of Experimental Botany, 53: 1551-1557.

Carré IA. (2001) Day-length perception and the photoperiodic regulation of flowering in Arabidopsis. J Biol Rhythms. 2001 Aug;16(4): 415-23.

Caspar T, Huber SC, Somerville C. (1985) Alterations in Growth, Photosynthesis, and Respiration in a Starchless Mutant of Arabidopsis thaliana (L.) Deficient in Chloroplast Phosphoglucomutase Activity. Plant Physiol. 1985 Sep;79(1): 11-17.

Caspar T, Lin TP, Kakefuda G, Benbow L, Preiss J, Somerville C. (1991) Mutants of Arabidopsis with Altered Regulation of Starch Degradation. Plant Physiol. 1991 Apr;95(4): 1181-1188.

Chandler J, Wilson A, Dean C. (1996) Arabidopsis mutants showing an altered response to vernalization. Plant J. 1996 Oct;10(4): 637-44

Chen, K. and Rajewsky, N. (2007) The evolution of gene regulation by transcription factors and microRNAs. Nature Reviews Genetics, 8: 93-103.

Chen, X. (2008) MicroRNA Metabolism in Plants, Curr Top Microbiol Immunol. 320: 117-136

Chuck G, Meeley R, Hake S. (2007) Floral meristem initiation and meristem cell fate are regulated by the maize AP2 genes ids1 and sid1. Development. 2008 Sep;135(18): 3013-9.

Ciannamea S, Kaufmann K, Frau M, Tonaco IA, Petersen K, Nielsen KK, Angenent GC, Immink RG. (2006) Protein interactions of MADS box transcription factors involved in flowering in Lolium penne. J Exp Bot. 2006;57(13): 3419-31.

Corbesier L, Lejeune P, Bernier G. (1998) The role of carbohydrates in the induction of flowering in Arabidopsis thaliana: comparison between the wild type and a starchless mutant. Planta. 1998 Sep;206(1): 131-7.

Corbesier L, Bernier G, Périlleux C. (2002) C : N ratio increases in the phloem sap during floral transition of the long-day plants Sinapis alba and Arabidopsis thaliana. Plant Cell Physiol. 2002 Jun;43(6): 684-8.

Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, Coupland G. (2007) FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science. 2007 May 18;316(5827): 1030-3

David KM, Armbruster U, Tama N, Putterill J. (2006) Arabidopsis GIGANTEA protein is post-transcriptionally regulated by light and dark. FEBS Lett. 2006 Feb 20;580(5): 1193-7

Ding Z, Doyle MR, Amasino MR, Davis, SJ (2007), A Complex Genetic Interaction Between Arabidopsis thaliana TOC1 and CCA1/LHY in Driving the Circadian Clock and in Output Regulation, Genetics, Vol. 176:1501-1510

Doyle MR, Davis SJ, Bastow RM, McWatters HG, Kozma-Bognár L, Nagy F, Millar AJ, Amasino RM. (2002) The ELF4 gene controls circadian rhythms and flowering time in Arabidopsis thaliana. Nature. 2002 Sep 5;419(6902): 74-7.

Dunlap JC. (1996) Genetics and molecular analysis of circadian rhythms. Annu Rev Genet. 1996;30: 579-601

Edwards and Miller, (2007) Analysis of Circadian Leaf Movement Rhythms in Arabidopsis thaliana, in Circadian Rhythms: Methods and Protocols, Methods in Molecular Biology, 362, Feb 02 2007, 103-113, Edinburgh

Farré EM, Harmer SL, Harmon FG, Yanovsky MJ, Kay SA. (2005) Overlapping and distinct roles of PRR7 and PRR9 in the Arabidopsis circadian clock. Curr Biol. 2005 Jan 11;15(1): 47-54.

Ferrándiz C, Liljegren SJ, Yanofsky MF (2000) Negative regulation of the SHATTERPROOF genes by FRUITFULL during Arabidopsis fruit development. Science. 2000 Jul 21;289(5478): 436-8.

de Folter, S., Immink, R.G.H., Kieffer, M., Parenicová, L., Henz, S.R., Weigel, D., Busscher, M., Kooiker, M., Colombo, L., Kater, M.M., Davies, B. and Angenent, G.C. (2005) Comprehensive Interaction Map of the Arabidopsis MADS Box Transcription Factors. The Plant Cell, 17: 1424-1433.

Fowler S, Lee K, Onouchi H, Samach A, Richardson K, Morris B, Coupland G, Putterill J. (1999) GIGANTEA: a circadian clock-controlled gene that regulates photoperiodic

flowering in Arabidopsis and encodes a protein with several possible membrane-spanning domains. EMBO J. 1999 Sep 1;18(17): 4679-88.

Gandikota, M., Birkenbihl, R.P., Höhmann, S., Cardon, G.H., Saedler, H. and Huijser, P. (2007) The miRNA156/157 recognition element in the 3' UTR of the Arabidopsis SBP box gene SPL3 prevents early flowering by translational inhibition in seedlings. The Plant Journal. Feb;49(4): 683-93

Gendall AR, Levy YY, Wilson A, Dean C. (2001) The VERNALIZATION 2 gene mediates the epigenetic regulation of vernalization in Arabidopsis. Cell. 2001 Nov 16;107(4): 525-35.

Green RM, Tobin EM. (1999) Loss of the circadian clock-associated protein 1 in Arabidopsis results in altered clock-regulated gene expression. Proc Natl Acad Sci USA. 1999 Mar 30;96(7): 4176-9.

Gregis V, Sessa A, Colombo L, Kater MM. (2006) AGL24, SHORT VEGETATIVE PHASE, and APETALA1 redundantly control AGAMOUS during early stages of flower development in Arabidopsis. Plant Cell. 2006 Jun;18(6): 1373-82.

Gu, Q., Ferrandiz, C., Yanofsky, M.F. and Martienssen, R. (1998) The FRUITFULL MADS-box gene mediates cell differentiation during Arabidopsis fruit development. Development, 125: 1509-1517.

Guo, A., He, K., Liu, D., Bai, S., Gu, X., Wei, L. and Luo, J. (2005) DATF: a database of Arabidopsis transcription factors. Bioinformatics, 21: 2568-2569.

Hanano, S. and Davis, S (2005) Pseudo-response regulator genes "tell" the time of day: multiple feedbacks in the circadian system of higher plants; in: Endogenous plant rhythms; Annual Plant Reviews, Vol 21; Edited by Anthony J.W. Hall, Harriet G. McWatters, Blackwell Publishing, Oxford

Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA, Kay SA. (2000) Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science. 2000 Dec 15;290(5499): 2110-3.

Harmer SL, Panda S, Kay SA. (2001) Molecular bases of circadian rhythms. Annu Rev Cell Dev Biol. 2001;17: 215-53

Hartmann U, Höhmann S, Nettesheim K, Wisman E, Saedler H, Huijser P. (2000) Molecular cloning of SVP: a negative regulator of the floral transition in Arabidopsis. Plant J. 2000 Feb;21(4): 351-60.

Hazen SP, Schultz TF, Pruneda-Paz JL, Borevitz JO, Ecker JR, Kay SA. (2005) LUX ARRHYTHMO encodes a Myb domain protein essential for circadian rhythms. Proc Natl Acad Sci U S A. 2005 Jul 19;102(29): 10387-92.

Helliwell CA, Wood CC, Robertson M, James Peacock W, Dennis ES. (2006) The Arabidopsis FLC protein interacts directly in vivo with SOC1 and FT chromatin and is part of a high-molecular-weight protein complex. Plant J. 2006 Apr;46(2): 183-92.

Hempel FD, Weigel D, Mandel MA, Ditta G, Zambryski PC, Feldman LJ, Yanofsky MF. (1997) Floral determination and expression of floral regulatory genes in Arabidopsis. Development. 1997 Oct;124(19): 3845-53

Houssa C, Bomans J, Greimers R, Jacqmard A. (1991), High-yield isolation of protoplasts from microgram amounts of shoot meristematic tissues and rapid DNA content determination by flow cytometry. Exp Cell Res. 1991 Dec;197(2): 153-7

Huijser, P., Klein, J., Lönnig, W.-E., Meijer, H., Saedler, H. and Sommer, H. (1992) Bracteomania, an inlforescence anomaly, is caused by the loss of function of the MADS-box gene squamosa in Antirrhinum majus. EMBO J. 11, 1239–1249.

Huq E, Tepperman JM, Quail PH. (2000) GIGANTEA is a nuclear protein involved in phytochrome signaling in Arabidopsis. Proc Natl Acad Sci U S A. 2000 Aug 15;97(17): 9789-94.

Ito S, Nakamichi N, Nakamura Y, Niwa Y, Kato T,2007), Genetic linkages between circadian clock-associated components and phytochrome-dependent red light signal transduction in Arabidopsis thaliana. Plant Cell Physiol. 2007 Jul;48(7): 971-83

Jaeger KE, Wigge PA (2007) FT protein acts as a long-range signal in Arabidopsis. Curr Biol. 2007 Jun 19;17(12): 1050-4.

Jung JH, Seo YH, Seo PJ, Reyes JL, Yun J, Chua NH, Park CM. (2007) The GIGANTEA-regulated microRNA172 mediates photoperiodic flowering independent of CONSTANS in Arabidopsis. Plant Cell. 2007 Sep;19(9): 2736-48.

Kardailsky I, Shukla VK, Ahn JH, Dagenais N, Christensen SK, Nguyen JT, Chory J, Harrison MJ, Weigel D. (1999) Activation tagging of the floral inducer FT. Science. 1999 Dec 3;286(5446): 1962-5.

Kaufmann, K., Melzer, R. and Theißen, G. (2005) MIKC-type MADS-domain proteins: structural modularity, protein interactions and network evolution in land plants. Gene, 347: 183-198.

Kempin SA, Savidge B, Yanofsky MF. (1995) Molecular basis of the cauliflower phenotype in Arabidopsis. Science. 1995 Jan 27;267(5197): 522-5.

Kikis EA, Khanna R, Quail PH. (2005) ELF4 is a phytochrome-regulated component of a negative-feedback loop involving the central oscillator components CCA1 and LHY. Plant J. 2005 Oct;44(2): 300-13.

Klein, J., Saedler, H. and Huijser, P. (1996) A new family of DNA binding proteins incude putative transcriptional regulators of the Antirrhinum majus floral meristem identity gene SQUAMOSA. Molecular Genetics and Genomics, 250: 7-16.

Kobayashi Y, Kaya H, Goto K, Iwabuchi M, Araki T. (1999) A pair of related genes with antagonistic roles in mediating flowering signals. Science. 1999 Dec 3;286(5446): 1960-2.

Kropat, J., Tottey, S., Birkenbihl, R.P., Depège, N., Huijser, P. and Merchant, S. (2005) A regulator of nutritional copper signaling in Chlamydomonas is an SBP domain protein that recognizes the GTAC core of copper response element. PNAS, 102: 18730-18735.

Larsson AS, Landberg K, Meeks-Wagner DR. (1998) 1998The TERMINAL FLOWER2 (TFL2) gene controls the reproductive transition and meristem identity in Arabidopsis thaliana. Genetics. 1998 Jun;149(2): 597-605

Lauri, A (2005) Doktorarbeit Molecular analysis of petal development by X-ChIP and two-hybrid technology, MPIZ Köln

Lee JH, Yoo SJ, Park SH, Hwang I, Lee JS, Ahn JH. (2007) Role of SVP in the control of flowering time by ambient temperature in Arabidopsis. Genes Dev. 2007 Feb 15;21(4): 397-402.

Lee, I.,Bleecker, A.,Amasino, R. (1993) Analysis of naturally occurring late flowering in Arabidopsis thaliana. Mol Gen Genet. 1993 Feb;237(1-2): 171-6.

Lee H, Suh SS, Park E, Cho E, Ahn JH, Kim SG, Lee JS, Kwon YM, Lee I. (2002) Genes Dev. 2000 Sep 15;14(18): 2366-76.

Lee J, Oh M, Park H, Lee I. (2008) SOC1 translocated to the nucleus by interaction with AGL24 directly regulates leafy. Plant J. 2008 Sep;55(5): 832-43.

Liang, X., Nazarenus, T.J. and Stone, J.M. (2008) Identification of a Consensus DNA-Binding Site for the Arabidopsis thaliana SBP domain Transcription Factor, AtSPL14, and Binding Kinetics by Surface Plasmon Resonance. Biochemistry, 47, 3645-3653.

Litt A, Irish VF.(2003) Duplication and diversification in the APETALA1/FRUITFULL floral homeotic gene lineage: implications for the evolution of floral development. Genetics. 2003 Oct;165(2): 821-33.

Liu, C., Chen, H., Hong, L.E., Soo, H.M., Kumar, P.P., Han, J.-H., Liou, Y.C. and Yu, H. (2008) Direct interaction of AGL24 and SOC1 integrates flowering signals in Arabidopsis. Development, 135: 1481-1491.

Makino S, Kiba T, Imamura A, Hanaki N, Nakamura A, Suzuki T, Taniguchi M, Ueguchi C, Sugiyama T, Mizuno T. (2000) Genes encoding pseudo-response regulators: insight into His-to-Asp phosphorelay and circadian rhythm in Arabidopsis thaliana. Plant Cell Physiol. 2000 Jun;41(6): 791-803.

Mandel MA, Gustafson-Brown C, Savidge B, Yanofsky MF. (1992) Molecular characterization of the Arabidopsis floral homeotic gene APETALA1. Nature. 1992 Nov 19;360(6401): 273-7.

Mandel MA, Yanofsky MF. (1995) The Arabidopsis AGL8 MADS box gene is expressed in inflorescence meristems and is negatively regulated by APETALA1. Plant Cell. 1995 Nov;7(11): 1763-71

Mandel MA, Yanofsky MF. (1995) A gene tri ggering flower formation in Arabidopsis. Nature. 1995 Oct 12;377(6549): 522-4.

Mandel MA, Yanofsky MF. (1998) The Arabidopsis AGL9 MADS box gene is expressed in young flower primordia. Sexual plant reproduction, 11,1: 22

Martin-Tryon EL, Kreps JA, Harmer SL. (2007) GIGANTEA acts in blue light signaling and has biochemically separable roles in circadian clock and flowering time regulation. Plant Physiol. 2007 Jan;143(1): 473-86.

Más, P. (2008) Circadian clock function in Arabidopsis thaliana: time beyond transcription. Trends Cell Biol. 2008 Jun;18(6): 273-81.

Matsushika A, Makino S, Kojima M, Mizuno T. (2000) Circadian waves of expression of the APRR1/TOC1 family of pseudo-response regulators in Arabidopsis thaliana: insight into the plant circadian clock. Plant Cell Physiol. 2000 Sep;41(9): 1002-12.

McWatters HG, Kolmos E, Hall A, Doyle MR, Amasino RM, Gyula P, Nagy F, Millar AJ, Davis SJ. (2007) ELF4 is required for oscillatory properties of the circadian clock. Plant Physiol. 2007 May;144(1): 391-401.

Melzer, S., Lens, F., Gennen, J., Vanneste, S., Rhode, A. and Beeckman, T. (2008) Flowering-time genes modulate meristem determinancy and growth form in Arabdopsis thaliana. Nature Genetics, 40: 1489-1492.

Michales, S.D. and Amasino, R.M. (1999) FLOWERING LOCUS C encodes a novel MADS Domain Protein That Acts as a Repressor of Flowering. The Plant Cell, 11, 949-956.

Michaels SD, Bezerra IC, Amasino RM. (2004) FRIGIDA-related genes are required for the winter-annual habit in Arabidopsis. Proc Natl Acad Sci U S A. 2004 Mar 2;101(9): 3281-5.

Michaels, TP, Breton, G, Hazen SP, Priest, H, Mokkler, TC, Kay, SA, Chory, J (2008) A Morning-Specific Phytohormone Gene Expression Program underlying Rhythmic Plant Growth, PLoS Biol. 2008 September; 6(9): e225

Mizoguchi T, Wheatley K, Hanzawa Y, Wright L, Mizoguchi M, Song HR, Carré IA, Coupland G. (2002) LHY and CCA1 are partially redundant genes required to maintain circadian rhythms in Arabidopsis. Dev Cell. 2002 May;2(5): 629-41.

Mizoguchi, T., Wright, L., Fujiwara, S., Cremer, F., Lee, K., Onouchi, H., Mouradov, A., Fowler, S., Kamada, H., Putterill, J. and Coupland, G. (2005) Distinct Roles of GIGANTEA in Promoting Flowering and Regulating Circadian Rhythms in Arabidopsis. The Plant Cell, 17: 2255-2270.

Moreno, M.A., Harper, L.C., Krueger, R.W., L., D.S. and Michael, F. (1997) liguleless1 encodes a nuclear-localized protein required for induction of ligules and auricles during maize leaf organogenesis. Genes & Development, 11: 616-628.

Murakami M, Matsushika A, Ashikari M, Yamashino T, Mizuno T. (2005) Circadian-associated rice pseudo response regulators (OsPRRs): insight into the control of flowering time. Biosci Biotechnol Biochem. 2005 Feb;69(2): 410-4.

Mylne JS, Barrett L, Tessadori F, Mesnage S, Johnson L, Bernatavichute YV, Jacobsen SE, Fransz P, Dean C. (2006) LHP1, the Arabidopsis homologue of HETEROCHROMATIN PROTEIN1, is required for epigenetic silencing of FLC. Proc Natl Acad Sci U S A. 2006 Mar 28;103(13): 5012-7.

Nagae, M., Nakata, M. and Takahashi, Y. (2008) Identification of Negative cis-Acting Elements in Response to Copper in the Chloroplastic Iron Superoxide Dismutase Gene of the Moss Barbula unguiculata. Plant Physiology, 146: 1687-1696.

Nelson DC, Lasswell J, Rogg LE, Cohen MA, Bartel B. (2000) FKF1, a clock-controlled gene that regulates the transition to flowering in Arabidopsis. Cell. 2000 Apr 28;101(3): 331-40.

Orlando, V. (2000) Mapping chromosomal proteins in vivo by formaldehyde-crosslinked-chromatin immunoprecipitation; Trends Biochem Sci. 2000 Mar;25(3): 99-104.

Park DH, Somers DE, Kim YS, Choy YH, Lim HK, Soh MS, Kim HJ, Kay SA, Nam HG. (1999) Control of circadian rhythms and photoperiodic flowering by the Arabidopsis GIGANTEA gene. Science. 1999 Sep 3;285(5433): 1579-82.

Pelaz, S., Tapia-Lopez, R., Alvarez-Buylla, E.R. and Yanofsky, M.F. (2001) Conversion of leaves into petals in Arabidopsis. Current Biology, 11: 182-184.

Purugganan MD. (1997) The MADS-box floral homeotic gene lineages predate the origin of seed plants: phylogenetic and molecular clock estimates. J Mol Evol. 1997 Oct;45(4): 392-6.

Putterill J, Robson F, Lee K, Simon R, Coupland G. (1995) The CONSTANS gene of Arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell. 1995 Mar 24;80(6): 847-57.

Quinn, J.M., Kropat, J. and Merchant, S. (2003) Copper Response Element and Crr1-Dependent Ni 2+ -Responsive Promoter for Induced, Reversible Gene Expression in Chlamydomonas reinhardtii. Eukaryotic Cell, 2: 995-1002.

Ratcliff, O.J. and Riechmann, J.L. (2002) Arabidopsis Transcription Factors and the Regulation of Flowering Time: A genomic Perspective. Curr. Issues Mol. Biol., 4: 77-91.

Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B. and Bartel, D.P. (2002) Prediction of Plant MicroRNA Targets. The Cell, 110: 513-520.

Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, Z.C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K., Yu, G.L. (2000) Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. Science 290: 2105-2110.

Riese, M., Höhmann, S., Saedler, H., Münster, T. and Huijser, P. (2007) Comparative analysis of the SBP-box gene families in P. patens and seed plants. Gene, 401, 28-37.

Rosea, A.B., Elfersib, T., Parrab, G. and Korf, I. (2008) Promoter-Proximal Introns in Arabidopsis thaliana Are Enriched in Dispersed Signals that Elevate Gene Expression. The Plant Cell 20:543-551

Samach A, Onouchi H, Gold SE, Ditta GS, Schwarz-Sommer Z, Yanofsky MF, Coupland G. (2000) Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science. 2000 Jun 2;288(5471): 1613-6.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Salomé, P.A. and McClung, C.R. (2004) The Arabidopsis thaliana Clock. Journal of Biological Rhythms, 19: 425-435.

Sawa M, Nusinow DA, Kay SA, Imaizumi T. (2007). FKF1 and GIGANTEA complex formation is required for day-length measurement in Arabidopsis. Science. 2007 Oct 12;318(5848): 261-5.

Schaffer R, Ramsay N, Samach A, Corden S, Putterill J, Carré IA, Coupland G. (1998) The late elongated hypocotyl mutation of Arabidopsis disrupts circadian rhythms and the photoperiodic control of flowering. Cell. 1998 Jun 26;93(7): 1219-29.

Schauer, S.E., Baskar, R., Schlüter, P., Gheyselinck, J., Bolaños, A., Curtis, M.D. and Grossniklaus, U. (2008) Regulatory elements encoded in the first intron are necessary for proper expression of the MADS-box transcription factors AGL6 and AGL13 in Arabidopsis thaliana. Dev. Biol. 319: 587 Meeting abstract

Schmid, M., Uhlenhaut, N.H., Godard, F., Demar, M., Bressan, R., Weigel, D. and Lohmann, J.U. (2003) Dissection of floral induction pathways using global expression analysis. Development, 130: 6001-3012.

Schmitz RJ, Sung S, Amasino RM (2008) Histone arginine methylation is required for vernalization-induced epigenetic silencing of FLC in winter-annual Arabidopsis thaliana. Proc Natl Acad Sci U S A. 2008 Jan 15;105(2): 411-6

Schultz, E. A.,Haughn, G. W. (1991) LEAFY, a Homeotic Gene That Regulates Inflorescence Development in Arabidopsis. The Plant Cell, 1991, 3: 771-781

Schwab, R., Palatnik, J.F., Riester, M., Schommer, C., Schmid, M. and Weigel, D. (2005). Specific Effects of MicroRNAs on the Plant Transcriptome. Developmental Cell,Vol 8: 517-527

Schwab, R. (2006) Doktorarbeit Functions and target selection of Arabidopsis microRNAs, Universität Tübingen

Schwarz S, Grande AV, Bujdoso N, Saedler H, Huijser P. The microRNA regulated SBP-box genes SPL9 and SPL15 control shoot maturation in Arabidopsis. Plant Mol Biol. 2008 May;67(1-2): 183-95.

Sheldon, C.C., Finnegan, E.J., Dennis, E.S. and Peacock, W.J. (2006) Quantitative effects of vernalization in FLC and SOC1 expression. The Plant Journal, 45: 871-883.

Sheldon CC, Burn JE, Perez PP, Metzger J, Edwards JA, Peacock WJ, Dennis ES. (1999) The FLF MADS box gene: a repressor of flowering in Arabidopsis regulated by vernalization and methylation. Plant Cell. 1999 Mar;11(3): 445-58.

Stone, J.M., Liang, X., Nekl, E., R. and Stiers, J.J. (2005) Arabidopsis AtSPL14, a plant-specific SBP-domain transcription factor, participates in plant development and sensitivity to fumonisin B1. The Plant Journal, 41: 744-754.

Strayer C, Oyama T, Schultz TF, Raman R, Somers DE, Más P, Panda S, Kreps JA, Kay SA. (2000) Cloning of the Arabidopsis clock gene TOC1, an autoregulatory response regulator homolog. Science. 2000 Aug 4;289(5480): 768-71.

Suárez-López P, Wheatley K, Robson F, Onouchi H, Valverde F, Coupland G. (2001) CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. Nature. 2001 Apr 26;410(6832): 1116-20.

Sung S, Amasino RM (2006) Molecular genetic studies of the memory of winter. J Exp Bot. 2006;57(13): 3369-77

Takada S, Goto K. (2003) Terminal flower2, an Arabidopsis homolog of heterochromatin protein1, counteracts the activation of flowering locus T by constans in the vascular tissues of leaves to regulate flowering time. Plant Cell. 2003 Dec;15(12): 2856-65.

Teper-Bamnolker, P. and Samach, A. (2005) The Flowering Integrator FT Regulates SEPALLATA3 and FRUITFULL Accumulation in Arabdiopsis Leaves. the Plant Cell, 17: 2661-2675.

Unte, U.S., Sorensen, A.-M., Pesaresi, P., Madhuiri, G., Leister, D., Seadler, H. and Huijser, P. (2003) SPL8, an SBP-Box Gene That Affects Pollen Sac Development in Arabidopsis. The Plant Cell, 15: 1009-1019.

Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, Coupland G. (2004) Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science. 2004 Feb 13;303(5660): 1003-6

Wang ZY, Tobin EM. (1998) Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. Cell. 1998 Jun 26;93(7): 1207-17.

Wang, H., Nussbaum-Wagler, T., Li, B., Zhao, Q., Vigouroux, Y., Faller, M., Bomblies, K., Liukens, L. and Doebley, J.F. (2005) The origin of the naked grains of maize. Nature, 463: 714-719.

Wang JW, Schwab R, Czech B, Mica E, Weigel D. (2008) Dual effects of miR156-targeted SPL genes and CYP78A5/KLUH on plastochron length and organ size in Arabidopsis thaliana. Plant Cell. 2008 May;20(5): 1231-43.

Wijnen H, Young MW. (2006) Interplay of circadian clocks and metabolic rhythms. Annu Rev Genet. 2006;40: 409-48

Wilson RN, Heckman JW, Somerville CR. (1992) Gibberellin Is Required for Flowering in Arabidopsis thaliana under Short Days. Plant Physiol. 1992 Sep;100(1): 403-408

Wu, G. and Poething, R.S. (2006) Temporal regulation of shoot development in Arabidopsis thaliana by miR156 and its target SPL3. Development, 133: 3539-3547.

Yamaguchi A, Kobayashi Y, Goto K, Abe M, Araki T. (2005) TWIN SISTER OF FT (TSF) acts as a floral pathway integrator redundantly with FT. Plant Cell Physiol. 2005 Aug;46(8): 1175-89

Yamasaki, K., Kigawa, T., Inoue, M., Tateno, M., Yamasaki, T., Yabuki, T., Aoki, Y., Seki, E., Matsuda, T., Nunokawa, E., Ishizuka, Y., Terada, T., Shirouzu, M., Osanai, T., Tanaka, A., Seki, M., Shinozaki, K. and Yokoyama, S. (2004) A Novel Zinc-binding Motif Revealed by Solution Structures of DNA-binding Domains of Arabidopsis SBP-family Transcription Factors. Journal of Molecular Biology, 337: 49-63.

Yamasaki K, Kigawa T, Inoue M, Yamasaki T, Yabuki T, Aoki M, Seki E, Matsuda T, Tomo Y, Terada T, Shirouzu M, Tanaka A, Seki M, Shinozaki K, Yokoyama S. (2006) An Arabidopsis SBP-domain fragment with a disrupted C-terminal zinc-binding site retains its tertiary structure FEBS Lett. 2006 Apr 3;580(8): 2109-16.

Yamasaki H, Hayashi M, Fukazawa M, Kobayashi Y, Shikanai T. (2009) SQUAMOSA Promoter Binding Protein-Like7 Is a Central Regulator for Copper Homeostasis in Arabidopsis. Plant Cell. 2009 Jan;21(1): 347-61.

Yoo, B.C., Kragler, F., Varkonyi-Gasic, E., Haywood, V., Archer-Evans, S., Lee, Y.M., Lough, T. J., Lucas, W.J. (2005) A systemic small RNA signalling system in plants. The Plant Cell, Aug., (16): 1979-2000

Yu H, Xu Y, Tan EL, Kumar PP. (2002) AGAMOUS-LIKE 24, a dosage-dependent mediator of the flowering signals. Proc Natl Acad Sci U S A. 2002 Dec 10;99(25): 16336-41.

Zeilinger MN, Farré EM, Taylor SR, Kay SA, Doyle FJ 3rd. (2006) A novel computational model of the circadian clock in Arabidopsis that incorporates PRR7 and PRR9. Mol Syst Biol. 2006;2:58.

Zhang Y, Schwarz S, Saedler H, Huijser P. (2007) SPL8, a local regulator in a subset of gibberellin-mediated developmental processes in Arabidopsis. Plant Mol Biol. 2007 Feb;63(3): 429-39.

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W. (2004) GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol. 2004 Dec;136(4): 4335.

Appendix

Appendix A

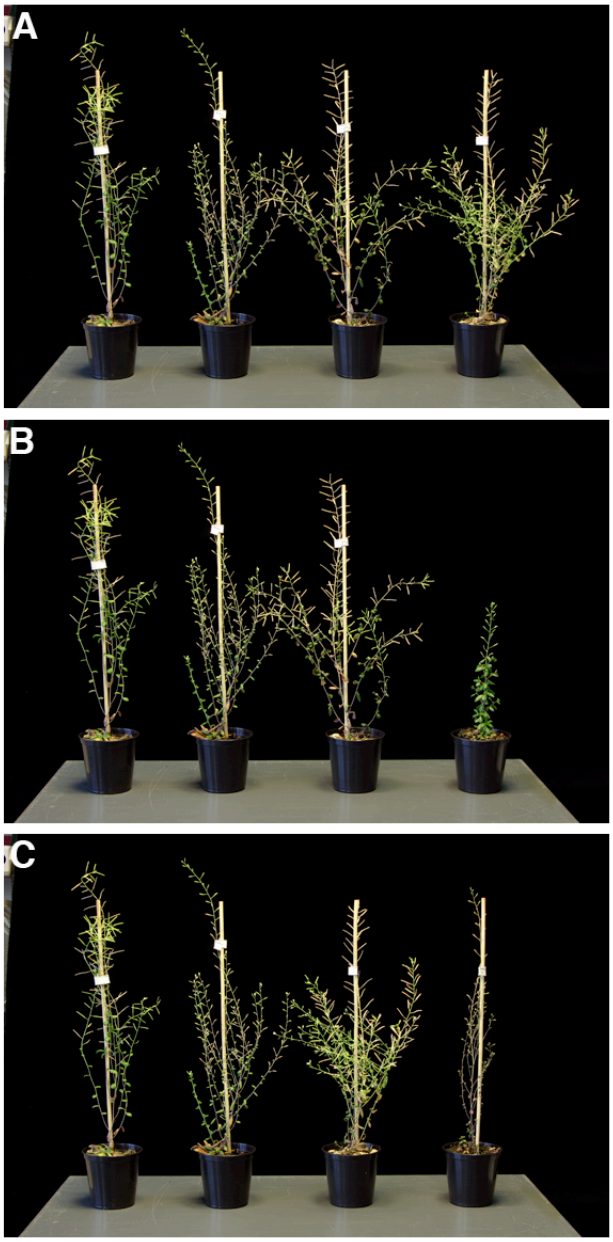
Table Appendix A
List of primers used in this study; Efficiency for qRT-PCR was tested for an annealing temperature of 58°C.

Primer No.	Gene	Sequence 5' -3'	Orientation	Efficiency at 58°C [%]
NB1	FUL	ATATCACATCGATCATATCTATC	forward	89.7
NB2	FUL	GGTGAGTCCACAAAACATCATAG	reverse	80.7
NB3	FUL	TGCACGTGCTTCCATACATCAAAAGG	reverse	89.7
NB4	FUL	AGCACGTGCACTCAGACACGTAC	forward	80.7
NB7	FUL	TAGTCGTTATAGTGTTACTGTAG	forward	84.8
NB8	FUL	CATAGACGATGATATTTGTGG	reverse	84.8
NB9	FUL	CAATGCTCCAACTCTTCTTCAG	forward	89.2
NB10	FUL	TCGTTCGTAGTGGTAGGACG	reverse	89.2
NB11	FUL	TGGACTTCAATTCAACTAGGCCCAG	forward	94.7
NB12	FUL	TTGGGTGAGATTCTCTGCCACAC	reverse	94.7
NB25	FUL	TGTATTCACGTCACATACCG	forward	99.7
NB26	FUL	GAGAAAGTAACTTGCCTATTGA	reverse	99.7
NB27	FUL	CGAGTCAGGAGGGAAACTCGAATC	forward	93.7
NB28	FUL	TTGTGAAACGTCTCGGCCAAC	reverse	93.7
NB29	FUL	GTTGTAGTAACTAATGTTTATCATACA	forward	84.1
NB30	FUL	CACGATCAATACACATTCATTC	reverse	84.1
NB31	FUL	CCTTTACTTAAGAGGGAATGAATG	forward	78.7
NB32	FUL	GTATGTAACTATGTATCCATGTTGTCC	reverse	78.7
NB33	FUL	GGACAACATGGATACATAGTTACA	forward	75.4
NB34	FUL	GCAAGGCTTTATCCTGATGA	reverse	75.4
NB35	FUL	CATCAGGATAAAGCCTTGCAAG	forward	88.1
NB36	FUL	TGGTCTCCATTATATATTAGTACCGG	reverse	88.1
NB37	FUL	CGGTACTAATATATAATGGAGACC	forward	70.8
NB38	FUL	TTACCACTTTGAGTCTTACGAAG	reverse	70.8
NB39	FUL	TTGCAACTGTAGATTTATGCTGG	forward	88.6
NB40	FUL	GATGCACCACCGTTCTCTCC	reverse	88.6
NB45	AP1 cDNA	GCACCAAATCCAGCATCCTT	forward	97.1
NB46	AP1 cDNA	CAGACCACCCATGTTGAGAAAA	reverse	97.1
NB47	CAL cDNA	TCTCACGTTAATGCACAGACGA	forward	108.9
NB48	CAL cDNA	TCAATCTTGGCCTTAAGCCTG	reverse	108.9
NB49	AG cDNA	GCGTACCAATCGGAGCTAGGAGG	forward	92.5
NB50	AG cDNA	GACGCAATTTGGCTGATTCTTGTTG	reverse	92.5
NB51	FUL	CACTAGTGAAGGGAAACAATG	forward	
NB52	FUL	ACTAGTGACTTTTGTTATTG	reverse	
NB55	FT cDNA	GAGACCCTCTTATAGTAAGCAGAG	forward	96.5
NB56	FT cDNA	CGTAACACACAATCTCATTGCC	reverse	96.5
NB59	SOC1 cDNA	GGGGCAAAACTCAGATGAAG	forward	96.5
NB60	SOC1 cDNA	TCCTATGCCTTCTCCCAAGA	reverse	96.5
NB61	FD cDNA	ACCACCTAAACCGACACAGC	forward	89.5
NB62	FD cDNA	CATGAGCGTTTGAGAGGTGA	reverse	89.5
NB63	CO cDNA	CATGGAAACTGGTGTTGTGC	forward	92.9
NB64	CO cDNA	ATCGTGTTGAACCCTTGCTC	reverse	92.9
NB65	SEP3 cDNA	ATGCTTCGGACACTGGAGAG	forward	89.6
NB66	SEP3 cDNA	CAGTCAGCATGCGTTCCTTA	reverse	89.6
NB67	JH2295	TAAGCTCAATGATATTCCCGTACA		
NB68	JH2295	CAGGTTCAAAACAAGCCAAGA		
NB69	JH2295	CCCATTTGACGTGAATGTAGACAC		

Table Appendix A condtinued

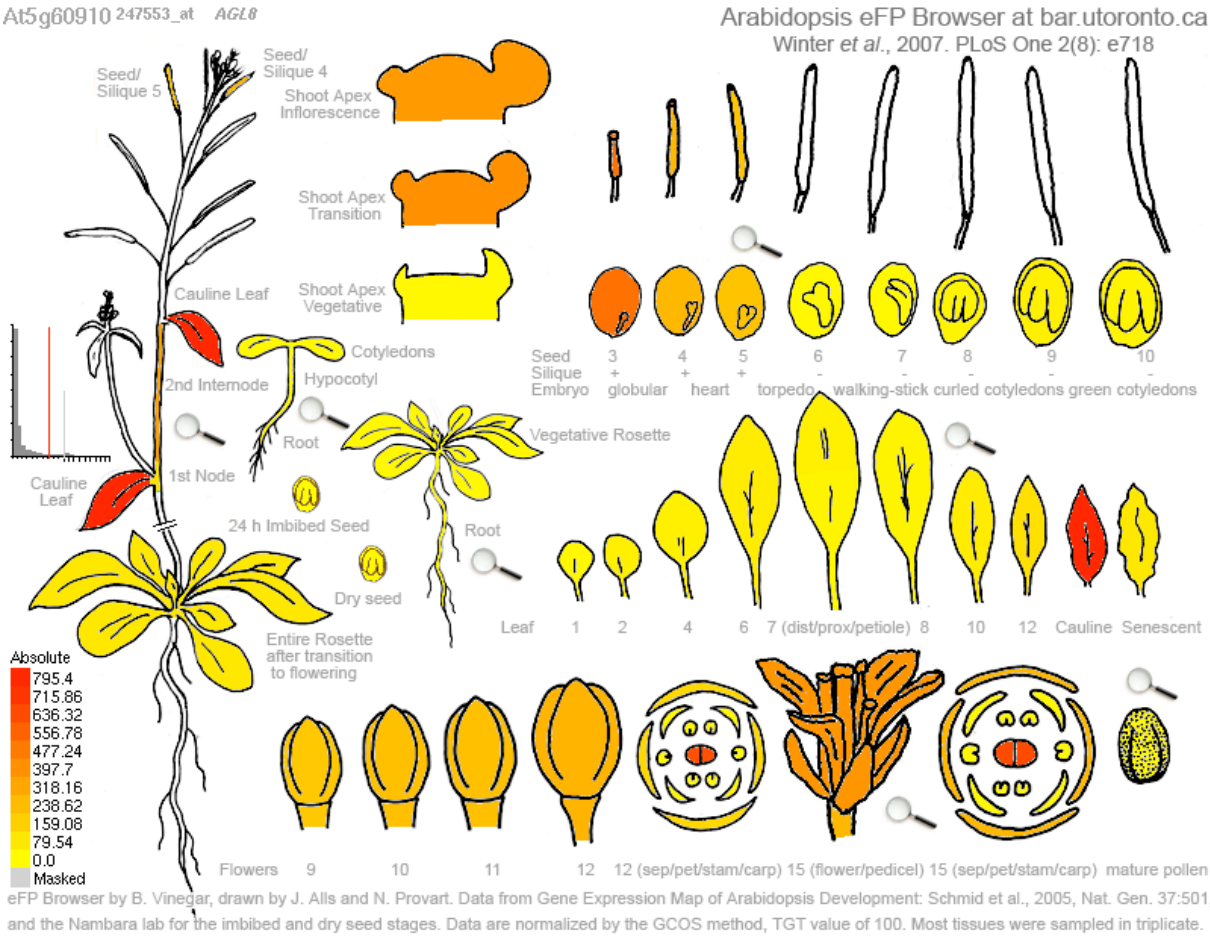
NB74	FPF1 cDNA	TCAGGATCCGACACACAGAA	forward	93.1
NB75	FPF1 cDNA	TCAATGGGAGTCTCGGACAT	reverse	93.1
NB76	LFY cDNA	CAACGAGAGCATTGGTTCAA	forward	102.2
NB77	LFY cDNA	CAAGAAGCTCCCAACGAAAG	reverse	102.2
NB86	AGL24 cDNA	GCGGCTGGAGAAACTACTTG	forward	91.9
NB87	AGL24 cDNA	CAGGGAAGTGTCGGAGTCAT	reverse	91.9
NB98	ELF4 cDNA	CGACAATCACCAATCGAGAATG	forward	90.3
NB99	ELF4 cDNA	AATGTTTCCGTTGAGTTCTTGAATC	reverse	90.3
N100	TOC1 cDNA	ATCTTCGCAGAATCCCTGTGATA	forward	82.6
N101	TOC1 cDNA	GCACCTAGCTTCAAGCACTTTACA	reverse	82.6
N102	CCA1 cDNA	TCTGTGTCTGACGAGGGTCGAATT	forward	92.2
N103	CCA1 cDNA	ACTTTGCGGCAATACCTCTCTGG	reverse	92.2
N104	LHY cDNA	CAACAGCAACAACAATGCAACTAC	forward	88.8
N105	LHY cDNA	AGAGAGCCTGAAACGCTATACGA	reverse	88.8
N106	ELF3 cDNA	GATGCCCACCATAATGAACC	forward	92.6
N107	ELF3 cDNA	TTGCTCGCGGATAAGACTTT	reverse	92.6
N108	GI cDNA	CTGTCTTTCTCCGTTGTTTCACTGT	forward	100.2
N109	GI cDNA	TCATTCCGTTCTTCTCTGTTGTTGG	reverse	100.2
N110	LUX cDNA	AGATGATGCAGATGCCAGTT	forward	87.6
N111	LUX cDNA	TAATTCTCATTTGCGCTTCC	reverse	87.6
N112	PRR9 cDNA	GCACAGAGAAACCAAAGGAA	forward	90.4
N113	PRR9 cDNA	CTTTCACTCGAGGACGTTGT	reverse	90.4
N114	PRR7 cDNA	TGAAAGTTGGAAAAGGACCA	forward	89.0
N115	PRR7 cDNA	GTTCCACGTGCATTAGCTCT	reverse	89.0
SH 258	SPL3	CAAGTAGTAGTGGAGTTTGTCAGGTCG	forward	97.9
SH 259	SPL3	TTTCCGCCTTCTCTCGTTGTGTCC	reverse	97.9
SH 286	PP2A	TAACGTGGCCAAAATGATGC	forward	97.0
SH 287	PP2A	GTTCTCCACAACCGCTTGGT	reverse	97.0

Appendix B



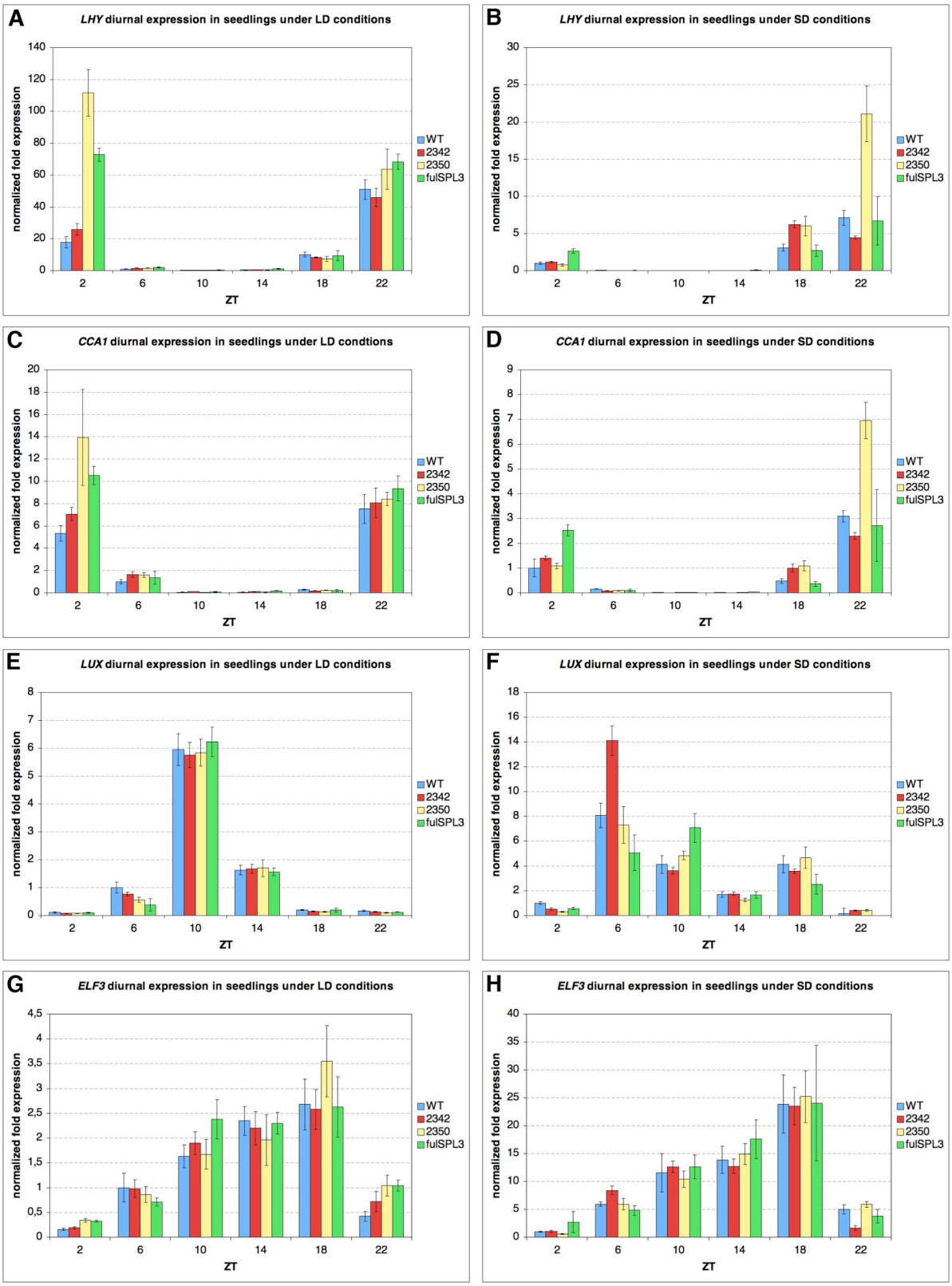
Phenotypes of wild-type, *SPL3OXs*, *ful*-2 and *ful SPL3OX* plants grown under SD conditions.
A) Phenoytpes of wild-type, *ful*-2 and *SPL3OX-2342*, *SPL3OX-2350* plants at the same age of about 12-14 weeks after sowing grown under SD conditions.
B) Phenotype of wild-type, *ful*-2 and *SPL3OX-2342*, and *ful SPL3OX-2342* plants at the same age of about 12-14 weeks after sowing grown under LD conditions.
C) Phenotype of wild-type, *ful*-2 and *SPL3OX-2350*, and *ful SPL3OX-2350* plants at the same age of about 12-14 weeks after sowing grown under LD conditions.

Appendix C



Overview on FUL expression in Arabidopsis organs. Figure taken from the TAIR homepage http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi?primaryGene=AT5G60910&modeInput=Absolute

Appendix D



Diural expression of several clock associated genes in 7 d old seedlings under LD and SD conditions. Diurnal Expression of *LHY* (A and B), *CCA1* (C and D), *LUX* (E and F) and *ELF3* (G and H) under LD and SD conditions. All genes were normalized against *PP2A* and ZT2 of WT was set arbitrarily set as one. blue = WT, red =*SPL3OX* line 2342, yellow = *SPL3OX* line 2350, green = *SPL3OX* line 2350 *ful*-2.

Nomenclature and Abbreviations

cDNA	Complementary Deoxyribonucleic Acid
Col-0	Columbia-0
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside Triphosphate
EtOH	Ethanol
g	Gram
GUS	β-Glucoronidase
kb	Kilo Base Pair
L	Liter
LD	Long Day
L. *er*	*Landsberg errecta*
min	Minute
mm	Millimeter
mRNA	Messenger Ribonucleic Acid
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RPM	Rounds per Minute
RT	Room Temperature
qRT-PCR	quantitative Reverse Transcription Polymerase Chain Reaction
SAM	Shoot Apical Meristem
SBP	Squamosa Promoter Binding Protein
SD	Short Day
Sec	Second
SPL	Squamosa Promoter Binding Protein Like
SPL3OX	SPL3 overexpressor
WT	Wild Type
µl	Microliter
M	Molar
ZT	Zeitgeber, here ZT0 = beginning of light period

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Erklärung

Köln, 2009

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzen Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken mit 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 noch nicht veröffentlicht worden ist, sowie dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Herrn Prof. Dr. Saedler und Prof. Dr. Werr sowie Herrn Dr. Huijser betreut worden.

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