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Analysis of Two Transcriptional Regulators that Affect Meristem Function

Arabidopsis thaliana TERMINAL FLOWER2 and *Picea abies APETELA2*

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Abstract
Nilsson, L. 2007. Analysis of Two Transcriptional Regulators that Affect Meristem Function. *Arabidopsis thaliana TERMINAL FLOWER2* and *Picea abies APETELA2*. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 263. 50 pp. Uppsala. ISBN 978-91-554-6774-1.

The aerial plant body is derived from undifferentiated cells in the shoot apical meristem that in *Arabidopsis thaliana* is active throughout the plant life cycle. Upon transition to flowering the activity of the meristem is altered and the meristem starts to produce secondary inflorescences and floral meristems instead of leaves. Both the activity of the meristem and the decision of when to flower are processes strictly regulated by several mechanisms. In this thesis I describe the function of two genes that are active in the regulation of meristem function and in the regulation of when to shift to reproductive development.

First, the Arabidopsis gene encoding TERMINAL FLOWER2 (TFL2), homologous to HETEROCHROMATIN PROTEIN1, was isolated and characterised. Mutations in *TFL2* result in plants that are dwarfed, flowers early, have reduced sensitivity to day length and terminate the inflorescence in an apical flower. As homologues from other organisms *TFL2* is active in gene regulation by gene repression. I show that the gene affect flowering time by the autonomous and the photoperiod pathways, two of four floral inductive pathways. *TFL2* act to repress the activity of genes that are promoters of floral meristem identity and interacts genetically with factors known to alter the chromatin state. Further *tfl2* is shown to have altered levels of and response to auxin. All together this shows that *TFL2* is active as a regulator of several different processes during plant development.

Second, I have characterised and studied the function of three genes encoding *APETALA2 LIKE* proteins in Norway spruce (*Picea abies*). In spruce these genes are expressed in meristems and reproductive tissues. When constitutively expressed in Arabidopsis two of the genes delays flowering time and alter the function of shoot apical and floral meristems. Together this suggests a function similar to the Arabidopsis homologues.

Keywords: Arabidopsis thaliana, Picea abies, gene regulation, development, meristem

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List of papers

This thesis is based on the following papers that will be referred to in the text by their Roman numerals:

I Katarina Landberg, Lars Nilsson and Annika Sundås Larsson. ***TERMINAL FLOWER2* (*TFL2*) Regulates the Transition to Flowering Through the Autonomous Pathway.** (manuscript)

II Lars Nilsson, Katarina Landberg, Kristina Rizzardi, Alessia Para and Annika Sundås Larsson. **Homeotic gene expression is regulated by TERMINAL FLOWER2 and the CAF-1 complex.** (manuscript)

III Katarina Landberg*, Lars Nilsson*, Kristina Rizzardi, Karin Ljung and Annika Sundås Larsson. ***TERMINAL FLOWER2* regulates auxin levels and auxin response in Arabidopsis.** (manuscript)

IV Lars Nilsson, Annelie Carlsbecker, Annika Sundås Larsson and Tiina Vahala. ***APETALA2* like genes from *Picea abies* show functional similarities to their Arabidopsis homologues.** Planta. 2006 Sep 5; [Epub ahead of print]

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Contents

Abbreviations and Nomenclature

ABA	abscisic acid
CD	chromo domain
CSD	chromo shadow domain
CZ	central zone
FM	floral meristem
GA	gibberellic acid
IAA	indole 3-acetic acid
IFM	inflorescence meristem
L1	layer one
LD	long day
mRNA	messenger RNA
miRNA	microRNA
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
PZ	peripheral zone
RT-PCR	reverse transcriptase PCR
RZ	rib zone
SAM	shoot apical meristem
SD	short day
TF	transcription factor
Trp	tryptophan
VM	vegetative meristem

The following nomenclature is used in this thesis:

Gene names are written in upper case italic letters, e.g. *TERMINAL FLOWER2* (*TFL2*)
Mutant gene names are written in lower case italic letters, e.g. *terminal flower2* (*tfl2*)
Protein names are written in capital letters, e.g. TERMINAL FLOWER2 (TFL2)

Introduction

Mechanisms of gene regulation

Multicellular organisms as plants have cells specialised to carry out different functions and like all other living organisms plants carry their blueprints of life, their genes, in the form of DNA. Cells are specialised to carry out different functions and to be able to respond to different conditions depending on for example environmental factors the gene expression has to be strictly regulated at several different levels.

In the cell, DNA is wrapped around histones creating nucleosomes that are the small building blocks creating a higher order structure of DNA, the chromatin. Chromatin has historically been described as heterochromatin and euchromatin. Heterochromatin harbours few genes and is defined as highly condensed and densely stained throughout the cell cycle, while euchromatin contain most of the genes and is less condensed during the interphase of the cell cycle. Alteration of the chromatin condensation around a certain gene affects the accessibility for factors regulating transcription.

When DNA becomes accessible by relaxation of compact DNA structures, proteins known as transcription factors (TFs) can bind to short sequences, *cis*-elements, located at the gene promoter to regulate the transcription of the downstream gene. An estimation of the Arabidopsis genome predicts that 5% of the genes encode TFs (AGI, 2000). In addtition several classes of TFs have been shown to be unique to plants, regulate developmental processes and display redundancy (Riechmann and Ratcliffe, 2000). In plants as in other higher eukaryotes RNA polymerase II dependent transcription result in the mediator between DNA and protein, messenger RNA (mRNA).

During recent years several classes of mRNAs have been shown to interact with small RNAs to post transcriptionally down regulate the specific gene. One type of small RNAs, microRNA (miRNA) is non coding, 21-24 nt long and generated from longer transcripts (Chen, 2005). Regulation by miRNA is a mechanism conserved between animals and plants and in the latter group miRNA has been described from seed plants as well as from ferns and mosses (Axtell and Bartel, 2005). In plants several genes regulating developmental processes, such as genes in the auxin signalling pathway and at least one floral homeotic gene, have been shown to be directly regulated by miRNA (Chen, 2004; Mallory et al., 2005).

Regulation at the level of chromatin: HETEROCHROMATIN PROTEIN1

One of the characterised genes encoding a chromatin regulating factor in plants, *TERMINAL FLOWER2* (*TFL2*) has also been named *LIKE HETEROCHROMATIN PROTEIN1* (*LHP1*) and *TU8* (Gaudin et al., 2001; Kim et al., 2004). *TFL2* is the Arabidopsis homologue to *Su(var)2-5,* the gene encoding HETEROCHROMATIN PROTEIN1 (HP1), first described in *Drosophila melanogaster* (Eissenberg et al., 1990). HP1 proteins are characterised by their N-terminal chromo domain (chromosome organisation modifier; CD; Paro and Hogness, 1991) and the highly similar C-terminal chromo shadow domain (CSD) which are separated by an in length variable and in amino acid sequence less conserved hinge region (Eissenberg and Elgin, 2000). The CD is found in several gene families among different organisms such as yeasts, insects, mammals and plants (Lorentz et al., 1994; Kiyosue et al., 1998; Eissenberg, 2001; Zemach et al., 2006). The originally proposed function of HP1 proteins as solely components of heterochromatin has during the years been revised (Minc et al., 2000; Fanti et al., 2004; Libault et al., 2005). In mammals such as in human three isoforms of HP1 have been described; HP1α, HP1β and HP1γ which have been shown to have different localisation patterns on the chromosomes and to display non redundant functions (Jones et al., 2000). HP1α and HP1β are mainly found in the heterochromatic regions while HP1γ is localised in euchromatic regions (Minc et al., 2000). The localisation of TFL2 has been investigated and several reports suggest a function for TFL2 mainly in euchromatic regions and not as a primary component of constitutive heterochromatin (Libault et al., 2005; Zemach et al., 2006). In addition HP1/TFL2 has been described to repress genes outside true heterochromatin regions in different species including plants by formation of facultative heterochromatin (Li et al., 2002; Kotake et al., 2003; Libault et al., 2005).

The *tfl2* mutant plant has a pleiotropic phenotype displaying dwarfness, early flowering, termination of the inflorescence and reduced photoperiod sensitivity that suggests a function of the gene in the regulation of several different processes during plant development (Sundås Larsson et al., 1998). Indeed *TFL2* has been shown to function in the regulation of flowering time by repression of *FLOWERING LOCUS T* (*FT*), as a regulator of floral homeotic gene expression by repressing e.g. *AGAMOUS* (*AG*) and *PISTILLATA* (*PI*), repressor of *FLOWERING LOCUS C* (*FLC*) in response to low temperatures and a component in the regulation of indole glucosinolate levels (Ludwig-Müller et al., 1999; Kotake et al., 2003; Sung et al., 2006).

Regulation by transcription factors: APETALA2 (AP2)

Until recently genes encoding an AP2 domain had only been described from plants, but in the reports by Magnani et al. (2004) and Wuitschick et al., (2004), genes encoding an AP2 domain were described from cyanobacteria, ciliates and viruses. The AP2 domain is a DNA binding domain consisting of approximately 70 amino acids (Ohme-Takagi and Shinshi, 1995). Genes encoding AP2 domains are divided in two sub families depending on the number of AP2 domains. The EREBP (ethylene responsive element binding protein) subfamily contain one AP2 domain and most members of this subfamily display activity in the response to biotic and environmental stress (Riechmann and Meyerowitz, 1998). The AP2 subfamily harbours two AP2 domains separated by a linker region. The sequence similarity among proteins in the AP2 superfamily is limited to the AP2 domain but in the AP2 subfamily the linker region between the AP2 domains is conserved among species and proteins (Riechmann and Meyerowitz, 1998). In Arabidopsis the *AP2* subfamily is a large gene family consisting of over 140 members that has been shown be active in several processes during plant development (Riechmann and Meyerowitz, 1998; Sakuma et al., 2002).

The *AP2* gene from Arabidopsis was the first isolated member of the *AP2* superfamily and has been shown to regulate floral organ identity as one of the two A-class genes in the ABCE model (Jofuku et al., 1994; Jack, 2001), described below. The gene also affects ovule and seed development and is an important factor in the maintenance of the shoot apical meristem (SAM; Jofuku et al. 2005; Würschum et al., 2006). The *AP2* class gene *AINTEGUMENTA* (*ANT*) from Arabidopsis has been shown to function in the regulation of floral organ growth in addition to ovule development (Elliott et al., 1996). Certain genes in the *AP2* subgroup such as the *TARGET OF EAT* genes (*TOE1*) and (*TOE2*) from Arabidopsis and *Glossy15* from maize are active players in the regulation of transition between different development stages (Moose and Sisco, 1994; Aukerman and Sakai, 2003). Together this shows that genes from the AP2 family function as regulators of several processes throughout plant development

Several *AP2* genes have been shown to be regulated by and/or show sequence complementarity to *miRNA172* (Aukerman and Sakai, 2003; Chen, 2004; Shigyo and Ito, 2004; Lauter et al., 2005). By constitutive expression of *miRNA172* in Arabidopsis phenotypical traits similar to mutant *AP2* plants were displayed, suggesting a function of miRNA in *AP2* gene repression (Chen, 2004).

Regulation of the shoot apical meristems

Organisation of the shoot apical meristem

In plants organs are initiated and develop throughout the plant life cycle. All of the organs formed during postembryonic growth are derived from the pool of undifferentiated stem cells in the meristems. The two apical meristems in plants, the SAM and the root apical meristem, are established and can easily be distinguished already during embryogenesis. During seed dormancy the meristems are inactive but when the seed starts to germinate the meristems are activated and cell division resumes.

The dome shaped SAM of Arabidopsis display three discrete layers (L1 – L3) based on cell division patterns. L1 and L2, in order the two outer layers, are both one cell layer thick and division in these layers are anticlinal. L1 give rise to the epidermis and L2 to the layer of sub epidermal cells. In the innermost layers, L3, also referred to as corpus, cell division occurs in all three planes, generating the internal parts of the plant.

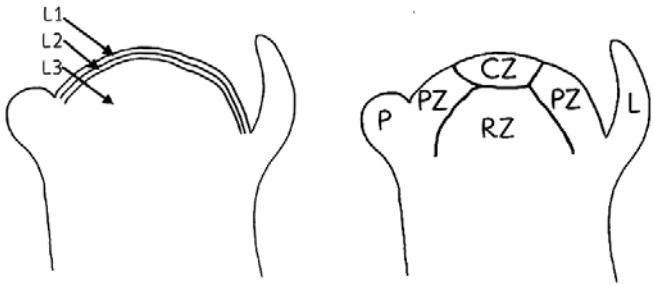

Figure 1. Zonation of the apical meristem. L1 - layer one, L2 - layer two, L3 - layer three, CZ - central zone, PZ - peripheral zone, RZ - rib zone, L – young leaf and P - leaf primordia

The SAM also displays distinct zones where cell division rates characterise the different zones. The central zone (CZ) contains large vacuolated cells that are slowly dividing (Laufs et al., 1998). As cells divide older cells are displaced to zones surrounding the CZ, the underlying rib zone (RZ) and the peripheral zone (PZ). In the RZ cell division is faster and in this part of the meristem the cells that will give rise to the interior part of the plant stem proliferate. Surrounding the CZ, the doughnut shaped PZ harbouring cytoplasmically dense cells, display an enhanced cell division rate compared to the CZ. Cells proliferating in this zone will build up the aerial organs of the plant body.

Genetic regulation of the SAM

Since plants are sessile and have to adjust their growth according to existing environmental conditions the SAM has to be highly flexible and respond to a wide range of internal and external signals. Several genes in a complex network regulate meristem size and function.

The main actor in initiation and maintenance of an undifferentiated pool of actively dividing cells is *WUSCHEL* (*WUS*). *WUS* expression is first detected during early embryo development with a continued expression throughout plant development in cells underlying the stem cells in the CZ of the meristems (Mayer et al., 1998). *WUS* is a member of the *WUSCHEL RELATED HOMEOBOX* (*WOX*) family of TFs (Haecker et al., 2004) and when mutated no cell division occurs in the area where the functional SAM should have been located resulting in arrested growth of the plant. Since *WUS* is expressed in the cells underneath the stem cells, were the gene promotes cell division, it must work by cell to cell signalling (Mayer et al., 1998). The signal being its gene product or another signalling molecule moving from cell to cell.

WUS has been shown to be regulated by several different genes both transcriptionally and at the level of chromatin (see Williams and Fletcher, 2005 and references therein). One of the mechanisms regulating *WUS* includes the *CLAVATA* (*CLV*) ligand-receptor complex. *CLV3* is expressed in the CZ and is positively regulated by *WUS, CLV* in turn creates a negative feedback loop downregulating *WUS* and thereby preventing the mersitematic domain to expand (Schoof et al., 2000). The *CLV* pathway contains three components: CLV3, a small protein exported from the mid apical cells in the CZ to the apoplast were it moves down to the underlying cells, binds to and activates the CLV1/CLV2 complex (Clark et al., 1997; Fletcher et al., 1999; Jeong et al., 1999). When activated the CLV1/CLV2 complex triggers a signalling cascade to inhibit the expression of *WUS*. *CLV1* encodes a leucine rich repeat kinase with an extra cellular ligand binding domain that forms a heterodimer with CLV2. CLV2 is similar to CLV1 but lacks the kinase domain. Even though the mechanism including *CLV* and *WUS* creates an excellent self regulating loop to define meristem size several other genes also participates in the regulation and organisation of the apical meristem reviewed by Williams and Fletcher (2005) and Bhalla and Singh (2006).

In addition to the regulation of meristem size the cells in this area has to be kept in an undifferentiated state until they are designated to form an organ. Differentiation is prevented by *SHOOT MERISTEMLESS* (*STM*) and the *KNOTTED-like in Arabidopsis* (*KNAT*) genes *KNAT1* and *KNAT6* that function independently of *WUS* (Lincoln et al., 1994; Endrizzi et al., 1996; Lenhard et al., 2002; Belles-Boix et al., 2006). *STM* is expressed throughout the meristem and when mutated plants fail to establish and maintain a functional SAM. (Long et al., 1996)

Regulation of meristem function by factors that alter the accessibility of DNA

Genes involved in the regulation of chromatin structure has during recent years been shown to take part in the regulation of plant development by altering the expression of meristem identity genes and thereby also the function of the SAM, (reviewed by Wagner, 2003 and Reyes, 2006). The Arabidopsis *FASCIATA* (*FAS*) genes, *FAS1* and *FAS2* together with *MULTICOPY SUPRESSOR OF IRA1* (*MSI1*) encodes the three subunits of the chromatin assembly complex homologous to the animal CHROMATIN ASSEMBLY FACTOR (CAF-1) complex (Leyser and Furner, 1992; Kaya et al., 2001). In *fas* mutants the regulation of the meristem is altered resulting in a laterally expanded *WUS* expression domain causing meristem enlargement (Kaya et al., 2001). In addition to the broadened *WUS* expression domain expression is occasionally detected in L2 and in extreme cases in L1. This indicates a role for *FAS* genes as spatial repressors of *WUS*, probably as components in the rapid chromatin assembly after replication.

The *SNF2* ATPase *SPLAYED* (*SYD*) functions as a chromatin remodelling factor and has been shown to bind to the promoter of *WUS* and positively regulate its expression (Kwon et al., 2005). In *syd* mutants the expression of *WUS* is down regulated resulting in loss of the SAM maintenance. Regulation of *STM* and *KNAT2* has been shown to involve *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*) and *CURLY LEAF* (*CLF*), members of a suggested polycomb group complex similar to PRC2 in *Drosophila* (Lund and van Lohuizen, 2004). In plants with a suppressed *FIE* activity and in *clf* mutant plants *STM* and *KNAT2* have been shown to be ectopically expressed (Katz et al., 2004).

Involvement of *AP2* genes in SAM regulation

In addition to being a regulator of flower development and seed size, *AP2* has recently been shown to control meristem size as the *ap2* allele *I28* terminates the inflorescence (Würschum et al., 2006). Data suggest a mechanism where *AP2* regulates *WUS* transcription either by regulating the *CLV* signalling pathway or by acting antagonistically with *CLV3* to regulate *WUS*. In addition to *AP2,* mutants of a second member of the *AP2* superfamily *ENHANCER OF SHOOT REGENERATION1*/*DORNRÖSHEN* (*DRN*), show arrested cell division in the SAM which indicates that the gene is active in the regulation of meristem function (Kirch et al., 2003). The *ANT* gene has a suggested function in maintenance of cell division in developing organs, giving rise to an over expressor phenotype displaying enlarged organs (Mizukami and Fischer, 2000). These examples suggest a role of *AP2* genes in the positive regulation of cell division not only in the meristems but also in organs initiated by the meristem.

Flowering

The decision to flower

The time point when to initiate reproductive development differs between plant species. Certain species like spruce commit to reproductive development after 20 years or more while other species flower within a month from germination. The mechanisms underlying the late developmental switch in some species are poorly understood. In Arabidopsis, as in several other plant species that flowers early, two different strategies have evolved to regulate the change to reproductive development, thus giving rise to ecotype differences. Some ecotypes are summer annuals and set seeds in the same growth season as they germinate. Winter annual ecotypes use an alternative strategy; after germination the plant need a prolonged exposure to cold during the winter before they flower and set seeds during the following growth season.

The decision to flower depends on several factors and for Arabidopsis four major genetic pathways in control of the induction of reproductive development have been described. In addition to the photoperiod and vernalisation pathways that depend on environmental signals, the autonomous and gibberellic acid (GA) signalling pathways which depend on endogenous factors have been described. Even though described as pathways they can not be seen as straight highways but rather as parts of a network since several interactions between the pathways have been identified (Parcy, 2005).

As final regulators, connecting the signals from these pathways, genes known as floral pathway integrators, *FT* and *SUPPRESSOR OF CONSTANS1*/*AGAMOUS LIKE20* (*SOC1/AGL20*) function to activate the floral identity genes *AP1* and *LEAFY* (*LFY*) (Kardailsky et al., 1999; Lee et al., 2000; Simpson and Dean, 2002). Beside the role as a meristem identity gene *LFY* functions as a floral pathway integrator since both *CONSTANS* (*CO*) and signals from the GA pathway activate its transcription.

Arabidopsis is a facultative long day (LD) plant, i.e. the flowering is promoted by LD conditions and delayed by short day (SD) conditions. The regulation of flowering by photoperiod depends on the detection of light by photoreceptors. In Arabidopsis the photoreceptors are represented by the PHYTOCHROMES (PHY), encoded by *PHYA* to *PHYE* (Clack et al., 1994), the CRYPTOCROMES (CRY), encoded by *CRY1* and *CRY2* (Ahmad and Cashmore, 1993; Lin et al., 1998), and the PHOTOTROPINS (PHOT) encoded by *PHOT1* and *PHOT2* (Liscum and Briggs, 1995; Jarillo et al., 1998). Phytochromes perceive red and far red light while cryptochromes and phototropins perceive blue and UV qualities of light. Photoperiod is sensed in the leaves where photoreceptors act both to regulate the circadian clock and *CO*. The levels of CO have been shown to be dependent on the activity of both phytochromes and cryptochromes (Valverde et al., 2004) but CO is also activated by *GIGANTEA* (*GI*) that is an output from the circadian clock

(Mizoguchi et al., 2005). *CO* encodes a TF that is a positive regulator of the floral pathway integrators *FT* and *SOC1* (Putterill et al., 1995; Samach et al., 2000). FT in turn interacts with FLOWERING LOCUS D (FD) to upregulate the floral identity gene *AP1* (Abe et al., 2005). Repression of *FT* has been shown to be carried out by *TFL2* and *FLC*, (Michaels and Amasino, 1999; Samach et al., 2000; Kotake et al., 2003). In *tfl2* mutants *FT* expression is elevated from seedling stages and onward which results in an early transition to reproductive phase in both LD and SD conditions.

In addition to being a repressor of *FT*, *FLC* also repress *SOC1* (Hepworth et al., 2002). *FLC* is a convergence point between the vernalisation and autonomous pathways, that both repress *FLC* expression. Vernalisation is achieved by a prolonged exposure to low temperatures and ensures that the plant flowers during the following growth season. Vernalisation is a non reversible commitment to flowering and should be seen as a pathway that makes the plant competent to flower rather than a flower promoting pathway. Arabidopsis summer annuals have been shown to be mutant alleles of *FLC* and/or *FRIGIDA* (*FRI*) (Gazzani et al., 2003; Michaels et al., 2003). *FRI* encodes a protein with unknown cellular function that is a positive regulator of *FLC* (Johanson et al., 2000).

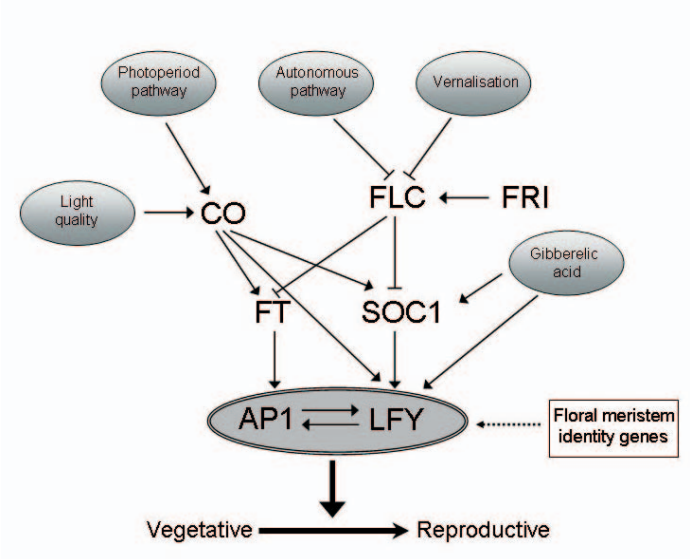

Figure 2. Regulation of flowering by flowering promoting pathways

The vernalisation pathway is active through epigenetic repression of *FLC*. This function is in Arabidopsis carried out by the *VERNALISATION* (*VRN*) genes *VRN1* and *VRN2* and *VERNALISATION INSENSITIVE3* (*VIN3*), the second gene homologous to *SU(Z)12* in *Drosophila* that encodes members of Polycomb group complexes that are factors known to modify chromatin structure (Gendall et al., 2001; Levy et al., 2002; Sung and Amasino, 2004).

Upon vernalisation these genes have been shown to modify the histone code, that result in a compact chromatin structure in the region covering the *FLC* locus (Bastow et al., 2004). *TFL2* has been shown to be crucial for the maintenance of *FLC* repression after cold exposure as the expression of *FLC* is increased with time post vernalisation in *tfl2* (Mylne et al., 2006; Sung et al., 2006).

As previously mentioned both the vernalisation and the autonomous pathways regulate *FLC* expression. Mutants of genes in the autonomous pathway display a late flowering phenotype during both LD and SD conditions, not detected in combination with the *flc* mutant (Michaels and Amasino, 2001). The late flowering phenotype of mutants in the autonomous pathway can also be restored by vernalisation which shows the strength and robustness in epigenetic silencing of *FLC* (Michaels and Amasino, 2001). The genes of the autonomous pathway regulate *FLC* mainly through two mechanisms. The first mechanism involve *FCA*, *FLOWERING LOCUS K* (*FLK*) and *FPA* that all encode RNA binding proteins (Macknight et al., 1997; Schomburg et al., 2001; Lim et al., 2004). FCA interacts with another gene product in this pathway, FY, to downregulate *FLC* by binding to its mRNA resulting in altered polyadenylation sites (Simpson et al., 2003; Henderson et al., 2005). FCA-FY also creates a negative feedback loop to regulate *FCA* transcript by altered processing of its pre-mRNA (Macknight et al., 2002; Simpson et al., 2003). *FCA* has been shown to be a receptor for abscisic acid (ABA), one of several hormones described from the plant kingdom. The binding to ABA blocks the interaction between FCA and FY resulting in abolished activity of this complex (Razem et al., 2006). *LUMINIDEPENDENS* (*LD*) is a homeodomain protein with unknown function but genes in the same class have been shown to bind RNA (Lee et al., 1994). The second mechanism by which the autonomous pathway regulates *FLC* is epigenetic and independent of the RNA modification proteins. Here *FLD* and *FVE* has been shown to reduce the histone acetylation level which creates a downregulation of FLC transcript (He et al., 2003; Ausín et al., 2004).

In the last of the four described genetic pathways flowering is promoted by GA. In mutants displaying constitutive activation of GA signalling flowering is promoted and in line with this, mutants defective in GA synthesis or insufficient in GA signalling are late flowering predominantly during SD conditions. This suggests GA to be an important factor during non inductive photoperiods by activating the downstream targets *LFY* and *SOC1* (Blazquez and Weigel, 1999; Moon et al., 2003).

In addition to the genes of the four defined signalling pathways other factors not yet designated to a certain pathway have been shown to alter the transition to flowering. Several genes from the *AP2* family of TFs have been shown to change the time when to adopt reproductive development. *SCHLAFMÜTZE* (*SMZ*), *SCHNARCHZAPFEN* (*SNZ*), *TOE1* and *TOE2* are down regulated upon floral induction in LD conditions. The genes have been

described as floral repressors acting downstream of both *CO* and *FT* but upstream of *LFY* (Aukerman and Sakai, 2003; Schmid et al., 2003).

Meristem identity upon flowering

The transition to flowering in Arabidopsis alters the morphology of the dome shaped vegetative meristem (VM) to a higher dome caused by an enhanced rate of cell division. The phyllotaxy of which the anlagen are initiated is maintained but instead of producing leaves on the flanks of the meristem the plant starts to produce flowers. During this phase the internodes elongate which results in an inflorescence with spirally arranged flowers.

When the developmental program of flowering is initiated two groups of meristem identity genes work in opposite directions to keep an active inflorescence meristem (IFM) or to promote flowers on the meristem flank. In Arabidopsis plants defective in *LFY*, flowers are replaced by vegetative shoots producing leaves, which suggest a function of *LFY* in the floral identity of a meristem (Schultz and Haughn, 1991). *LFY* encodes a TF and is expressed from the early development of floral buds (Weigel et al., 1992). In addition to being an integrator of signals from flowering pathways, as discussed in the previous section, *LFY* has been shown to activate the floral homeotic genes *AP1*, *AP3* and *AG* (Parcy et al., 1998; Busch et al., 1999).

When mutated the flowers of *ap1* produces adventious flowers in the axils of the first whorl organs indicating a role of *AP1* in the identity of a determinate floral meristem (FM; Irish and Sussex, 1990). The to *AP1* closely related gene *CAULIFLOWER* (*CAL*) show an expression pattern similar to that of *AP1* throughout the FM (Kempin et al., 1995). During early floral development *AP1* and *CAL* are globally expressed in the flower bud, later the expression of *CAL* declines and *AP1* expression is restricted to the two outer whorls (Mandel et al., 1992; Kempin et al., 1995), specifying sepals and petals described in detail in the next section. It has been shown that *AP1* and *CAL* act redundantly to specify the identity of the FM since *ap1* and *cal* single mutants produce flowers but FMs of *ap1cal* double mutants behave like IFMs (Bowman et al., 1993). As mentioned above *AP1* is activated by *LFY* during early flower development but *AP1* is also an important factor in maintaining the expression of *LFY* in floral buds (Bowman et al., 1993). In addition to being activated by *LFY, AP1* is activated by the flowering pathway gene *FT* (Abe et al., 2005; Wigge et al., 2005).

With opposing effect to *AP1* and *LFY*, *TFL1* functions to keep the identity of the IFM. *TFL1* is not a TF but encodes a protein related to phosphatidyl ethanolamine binding proteins described from different kingdoms, sharing over 50% amino acids identity with FT (Ohshima et al., 1997; Kardailsky et al., 1999). *tfl1* mutants display an early transition to flowering, the inflorescence terminates in a floral structure and occasionally a flower is produced from the axil of a cauline leaf instead of a secondary inflorescence (Shannon

and Meeks-Wagner, 1991). This indicates that *TFL1* has a function as a regulator of the phase transition from vegetative to reproductive development and a function to maintain the identity of the IFM. An alternative explanation could also be that *TFL1* have a function influencing the rate of progression through all phases of the SAM. *TFL1* is expressed already in the VM and is upregulated upon the transition to flowering (Ratcliffe et al., 1999). In the IFM *TFL1* represses both the FM identity genes *AP1* and *LFY,* and inversely in FMs *TFL1* expression is prevented by *AP1* and *LFY* (Gustafson-Brown et al., 1994; Liljegren et al., 1999; Ratcliffe et al., 1999)

The patterning of the angiosperm flower is based on the ABCE model

LFY and *AP1* are factors important for the initiation and identity of a flower meristem. After the bud has appeared on the flank of the IFM several genes are activated in a specific pattern and time frame to specify the formation of the multiplex reproductive structure, the flower. In 1991, Coen and Meyerowitz suggested a model for the patterning of a flower based on observations from Arabidopsis and *Antirrhinum* mutants. In the proposed ABC model they described how the identity of the floral organs, located in four concentric whorls of the flower, are based on the combinatorial activity of the three organ identity factors A, B and C. The A factor alone specify sepals, the combination of A and B specify petals, B and C specify stamens and the C factor solely specify carpels. Later Pelaz et al. (2000; 2001) revised this model including an other set of genes important for the formation of organs in the three inner whorls, described as E factors.

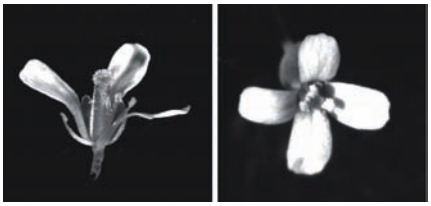

Figure 3. Arabidopsis flower with one sepal and petal removed (left) and a complete flower (right)

In Arabidopsis A function is determined by *AP1* and *AP2*. Interesting to note is that *AP2* is the only homeotic gene outside the large MADS box family of genes reviewed by Theissen (2001). A class activity restrict the expression of C class genes in the two outer whorls of the flower with the opposing effect in the two inner whorls where C function restrict A class genes. *AP2* creates an exception and is widely expressed in the flower bud during all stages of development, but is on the other hand post transcriptionally

repressed by miRNA (Chen, 2004). In flowers of strong A class mutants sepals are converted into carpelloid or leaf like organs and petals are absent (Bowman et al., 1991). Mutants of *AP3* and *PI*, the B factors, show homeotic changes of petals into sepalloid organs and stamens into carpelloid structures (Bowman et al., 1991). In mutant plants of the C factor, *AG*, stamens are replaced by petals and instead of carpels repeated whorls of sepals, petals and petals are produced, indefinitely (Yanofsky et al., 1990). E function is specified by the *SEPALLATA* (SEP) genes *SEP1*, *SEP2* and *SEP3* that has been shown to function redundantly (Pelaz et al., 2000). As a consequence of the redundancy the *sep* single mutants and double mutant combinations show no alteration of the floral organs, however the triple mutant flowers are indeterminate and only sepals are specified in all the four whorls. This indicates a function of the *SEP* genes in the specificity of the three inner whorls during flower development adding an E function to the earlier described ABC model.

Conifer reproductive structures

Present day seed plants are phylogenetically divided in gymnosperms and angiosperms that most probably shared a common ancestor 300 million years ago (Savard et al., 1994). Gymnosperms are today represented by four phyla: *Cycadophyta*, *Ginkgophyta*, *Coniferophyta* and *Gnetophyta* with a total of ca. 800 species, while angiosperms make up one phylum *Anthophyta* with over 200 000 species. Gymno- and angiosperm reproductive organs differ in morphology as a consequence of evolution after the split between the two plant groups. Compared to most angiosperms that possess bisexual flowers gymnosperms have separate male and female reproductive structures.

Figure 4. Pollen cone (photo by A. Carlsbecker)

In the conifer Norway spruce (*Picea abies*) two structurally different cones are produced upon reproductive development; the seed producing megasporangia and the pollen producing microsporangia. Seed cones are determinate organs that produce the ovule bearing ovuliferous scales with subtending sterile bracts. The scales are produced with a spiral phyllotaxy.

Fully developed pollen cones are smaller compared to seed cones. The pollen producing organs, the microsporophylls, are arranged in a spiral pattern along the cone axis without a subtending bract.

Even though clear differences in morphology of reproductive organs exist, several reports have showed that homologues of genes regulating A, B and C function of Arabidopsis flower development are expressed in developing reproductive organs of gymnosperms read e.g. Tandre et al. (1995); Sundström et al. (1999) and Shigyo and Ito (2004). This suggests a functional conservation of the genes between gymno- and angiosperms. It also suggests a function of the conserved genes in reproductive development before the split between the two plant groups and that the specialised function of the different reproductive structures is a result from their separate evolution.

Regulation of plant development by the growth hormone auxin

Hormones have been shown to regulate development throughout the life cycle of plants. All plant hormones are structurally simple compounds divided into different groups depending on their chemical structure and affects on plant development. In addition to the previously mentioned GA and ABA, cytokinins, auxin, ethylene and recently brassinosteroids has also been identified as plant hormones.

The in plant produced auxin molecule, indole 3-acetic acid (IAA), is structurally related to tryptophan (Trp) and one of the two main pathways in IAA synthesis has been shown be dependent on a Trp precursor molecule. The Trp independent pathway depends on indole that also is structurally related to IAA or derivates of indole as precursor molecules (Bartel, 1997). Auxin has been shown to be active in the regulation of numerous processes during plant development such as: lateral root formation, phyllotaxy of the shoot, vascular patterning and different tropisms (Reed et al., 1998; Mattsson et al., 1999; Friml et al., 2002; Reinhardt et al., 2003). Hormones are often produced distant from their sites of action and might therefore require transport. In Arabidopsis seedlings, young leaves display the highest production rate of IAA but production has also been seen in the cotyledons and in the root tip (Ljung et al., 2001; Ljung et al., 2005).

Rapid responses with the accumulation of gene transcripts within minutes, when exposed to auxin include three well characterised gene families. First the *SMALL AUXIN UP-REGULATED RNAs* (*SAUR*) encode short lived proteins with unknown function (Gil et al., 1994; Hagen and Guilfoyle, 2002). Members of a second family, the *GH3* family encodes proteins with amino acid conjugating activity proposed to function in the down regulation

of free auxin levels and thereby reduce the auxin signal (Staswick et al., 2005). The third gene family, the *AUX/IAA* family has 29 members in Arabidopsis that are found to alter the transcription of genes in response to auxin (Reed, 2001; Remington et al., 2004).

AUX/IAA proteins has been shown to homo and hetero dimerise with proteins from the same family and interact with AUXIN RESPONSE FACTOR (ARF) proteins (Kim et al., 1997; Tiwari et al., 2003, 2004). There are 23 *ARF* genes in the Arabidopsis genome (Guilfoyle and Hagen, 2001) but in contrast to *SAUR, GH3* and *AUX/IAA* family genes no evidence exist today that *ARFs* are regulated by auxin. *ARFs* encode small TFs that show amino acid sequence homology with AUX/IAA proteins. Depending on the properties of certain amino acids ARFs can function as either positive or negative regulators of transcription (Guilfoyle and Hagen, 2001). To alter transcription ARFs have been shown to bind AuxRE either as homodimers, heterodimers or in complexes with AUX/IAA proteins (Ulmasov et al., 1997; Ulmasov et al., 1999). Many different possibilities to alter the combination of the different AUX/IAA and ARF proteins together with redundancy among the different genes in the same family results in a complex regulation of auxin action by the ARF-AUX/IAA complexes.

A model proposed is that under low auxin concentrations AUX/IAA bind to ARF and represses their function as transcriptional activators. With increased intracellular auxin concentration AUX/IAA proteins are degraded and the repression of ARFs is released (Ulmasov et al., 1997; Ulmasov et al., 1999; Tiwari et al., 2001; Tiwari et al., 2004). However, this is a simplified model since not all ARFs are positive regulators of transcription. The enhanced degradation of AUX/IAA by auxin has been shown to involve the SKP-Cullin/CDC53-F-Box (SCF) ubiquitin ligase SCF^{TIR} that ubiquitinate AUX/IAA proteins to target them for degradation (Gray et al., 2001). The F-box protein TRANSPORT INHIBITOR RESPONSE 1 (TIR1) has shown to be an auxin receptor and belongs to the family of AUXIN RECEPTOR F-BOX proteins (AFB; Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Since most of the AUX/IAA proteins are rapidly upregulated by auxin new AUX/IAA proteins are synthesised to dampen the general transcription activation, creating a regulatory feedback loop.

Results and Discussion

TFL2 cloning and expression (I)

The Arabidopsis *tfl2* mutant has already been described. The mutation that among other traits result in a plant that is dwarfed, display early flowering with a reduced photoperiod sensitivity and terminates with a flowerlike structure has been mapped to the upper arm of chromosome 5 (Sundås Larsson et al., 1998).

Figure 5. tfl2-1 (left) and Columbia, wild type, (right)

Using a positional cloning strategy the region harbouring the mutant alleles of *TFL2* was narrowed down to nine putative open reading frames (ORFs). Comparing the DNA sequence of *tfl2-1* and *tfl2-2* to wild type in the region spanning the ORFs a deletion covering two ORFs was found in *tfl2-2*. After DNA sequencing a base pair substitution was found in the *tfl2-1* allele resulting in a premature in frame stop codon. Complementation analy-

ses were performed by introducing the wild type allele of *TFL2* to the two mutant *tfl2* alleles. In both cases the *Agrobacterium* transformed plants showed a wild type phenotype. Since no differences were detected between the two mutant alleles and both were rescued by *TFL2*, we suggest that the deletion of the second gene in *tfl2-2* has no effect on the phenotype in our growth conditions and experimental setups. Since the gene is deleted in *tfl2-2* and the mutation in *tfl2-1* creates a stop codon leaving out an, for the function of the protein, essential domain both the mutations are likely to be null mutations.

Analyses of the *TFL2* sequence revealed that it is a single copy gene in the Arabidopsis genome. By comparing the cDNA and the genomic DNA encoding *TFL2* it was found that the gene contains six exons. *TFL2* encodes a putative protein of 445 amino acids that show homology to HP1 family proteins described from a wide range of organisms such as other plant species, yeast, mammals and *Drosophila* (Kiyosue et al., 1998; Eissenberg, 2001; Zemach et al., 2006). Analysing the protein we found a CD located at amino acids 107-156 and a CSD positioned at amino acids 382-442. CDs has been shown to bind to histone H3 methylated at lysine9 in Arabidopsis, yeast and mammalian systems (Bannister et al., 2001; Lachner et al., 2001; Jackson et al., 2002) while the CSD is involved in interactions with other proteins as well as in homodimersisation (see Singh and Georgatos, 2002 and references therein).

Comparing the TFL2 protein with homologues in e.g. mammals shows that the hinge region separating the CD and the CSD is longer in Arabidopsis. In addition to one nuclear localisation signal positioned in the CD, three other nuclear localisation signals are located along the protein sequence. Homology to HP1 proteins from other organisms in addition to nuclear localisation signals suggest a function of TFL2 in nuclear processes, probably as a regulator of gene transcription by the alteration of chromatin structure.

The expression pattern of *TFL2* in wild type plants was investigated by RNA *in situ* hybridisation and reverse transcriptase PCR (RT-PCR). Aerial tissues from one, two or three weeks old plants were analysed by *in situ* hybridisation. The *TFL2* gene showed a general low expression pattern in all tissues analysed and a high expression pattern in VM, IFMs, FMs and young developing organs. The results were confirmed and expression was also detected in the root by RT-PCR. The areas where *TFL2* transcription is high corresponds to zones of actively dividing cells and thereby also a high rate of gene replication. The expression pattern in this area is in line with a suggested function of *TFL2* in gene repression of newly replicated DNA. This is most likely through formation of dense chromatin structures, a function described for HP1 proteins in other organisms. Both the expression pattern and the pleiotropic phenotype of the *tfl2* mutants is consistent with a function in the regulation of several processes throughout plant development.

Characteristics of the TFL2 protein (I+II)

A wide variety of genes encoding CDs has been described in addition to genes encoding HP1 like proteins and proteins from the Polycomb group (Paro and Hogness, 1991; Koonin et al., 1995; Eissenberg, 2001). *TFL2* clearly belong to the group of *HP1*-like genes that encodes a CD and a CSD separated by an in length and sequence variable hinge region.

HP1 proteins from mammals have been shown to be biologically active as dimers (Brasher et al., 2000; Cowieson et al., 2000). We used the yeast two hybrid system to evaluate if TFL2 like its mammalian homologues has the ability to dimerise and if so which parts that interact. The CD, the hinge region, the CSD and the full length protein was expressed as AD and BD fusion proteins, respectively. In yeast, the full length fusion proteins and the CSD fusion proteins had the ability to form homodimers and thereby activate the reporter genes. An *in vitro* pull down assay was conducted to confirm the results from the yeast two hybrid system. TFL2-AD and TFL2 proteins were *in vitro* translated and by using an antibody recognising the AD domain of the TFL2-AD fusion protein, the dimeristation between full length TFL2 proteins was confirmed. Similar results have also been shown by Gaudin et al. (2001) and Yu et al. (2004). Gaudin also describe that TFL2 proteins lacking the last 11 amino acids is not able to rescue the *tfl2* mutant phenotype. In the CSD of HP1 proteins several amino acids creating a hydrophobic interphase have been shown to be important for the dimerisation (Brasher et al., 2000; Cowieson et al., 2000). The sequence homology among HP1 proteins in the CSD and the ability to form a dimer in yeast and *in vitro* strongly suggest that TFL2 dimerises through interactions of the CSDs *in planta*.

Comparing the amino acid sequences of the TFL2 protein with mammalian HP1 proteins several amino acids known to create the hydrophobic interphase important for homo dimerisation are conserved while others have changed during evolution (Thiru et al., 2004). To evaluate how critical certain of these amino acids are for the formation of a dimer by TFL2 several of the them were mutated. Our results show that all amino acids analysed affected the stability of the dimer. Even though the amino acid sequence of the TFL2 protein in this region is altered in comparison to other organisms, dimerisation occurs by the in Arabidopsis predicted hydrophobic interphase and the dimerisation is sensitive to additional changes of amino acid properties.

TFL2 is active in the regulation of flowering time as a component of the photoperiod and the autonomous pathway (I)

Analyses of *tfl2* plants have shown that the transition to flowering occurs earlier than in wild type plants. This trait is more pronounced in SD than in LD conditions (Sundås Larsson et al., 1998). The hyposensitivity to photoperiod in *tfl2* mutants indicates an activity in the photoperiod pathway of flowering.

To evaluate the involvement of *TFL2* in the response to daylength *tfl2-1* was crossed to *early flowering3-1* (*elf3-1*) and *gi-1* (Zagotta et al., 1996; Fowler et al., 1999). Both these genes are active in sensing the day length as components of the circadian clock (Fowler et al., 1999; Hicks et al., 2001). Compared to wild type *elf3-1* flower early in both LD and SD conditions and in both these conditions *tfl2-1elf3-1* plants displayed an additive flowering time. In the double mutant the termination of the IFM was enhanced compared to *tfl2-1* single mutants, a trait not seen in *elf3* single mutants indicating a function of *ELF3* in the maintenance of an active IFM. Combining *tfl2-1* with the late flowering *gi-1* resulted in an intermediate flowering time. The double mutants flowered significantly later in LD conditions than in SD conditions compared to *tfl2-1* single mutants giving support to the described function of *GI* in promotion of flowering mainly during LD conditions (Fowler et al., 1999). No genetic interactions could be seen in *tfl2-1* mutant combinations with either *elf3-1* or *gi-1* concerning flowering time. However, the reduced sensitivity to daylength in *tfl2* mutants strongly indicates a function of *TFL2* as a factor in the response to daylength. Both *ELF3* and *GI* are functional early in the photoperiod pathway, as regulators of the circadian clock and our results does not rule out an involvement of *TFL2* further downstream in this pathway.

Since *tfl2* flowers early in both LD and SD conditions double mutants with genes known to be active in the autonomous pathway were created. Mutations in genes known to regulate this pathway result in late flowering plants showing that they are strong promoters of flowering (Koornneef et al., 1991). The *tfl2-1fca-1* double mutant flowered at the same time as *fca-1* single mutants indicating that *TFL2* and *FCA* are active in the same pathway in the regulation of flowering. *tfl2-1* was also combined with *fve-1* and *ld-1*. Both double mutant combinations displayed an intermediate flowering time indicating no interaction between them and *tfl2-1*. Since no interaction other than the epistatic relationship between *tfl2* and *fca-1* were seen, this indicates that *TFL2* functions as a specific repressor of *FCA* in the autonomous pathway.

Both the autonomous and the photoperiod pathways promote flowering by activation of *FT*. *FT* is upregulated in the *tfl2* background suggesting a

function of *TFL2* as a repressor of *FT* (Kotake et al., 2003). By non quantitative RT-PCR Gaudin et al. (2001) showed that *CO* is ectopically expressed in *tfl2* at the cotyledon stage. However our unpublished data and results by Kotake et al. (2003) show that the expression of *CO* is unaltered in *tfl2* compared to wild type. This indicates that *TFL2* act upstream of *FT* as a repressor of *FT* transcription in an opposite manner compared to *CO* in the regulation of flowering.

In addition to being a regulator of flowering time by influencing the photoperiod and autonomous pathways, *TFL2* was recently shown to be involved in the regulation of flowering time through the vernalisation pathway (Mylne et al., 2006; Sung et al., 2006). *TFL2* is specifically active in maintaining the repressed state of *FLC* after vernalisation. Our results and the results from others thus suggest a function of *TFL2* not as a general repressor of flowering but rather as a regulator of specific genes during the determination of flowering time in at least three of the four described pathways regulating flowering time.

TFL2 function to maintain an active inflorescence meristem by repression of *AP1* and *AG* (II)

Ectopic expression of *AP1*, *LFY* and *AG* result in termination of the IFM in a floral structure (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995; Mizukami and Ma, 1997). As previously reported no genetic interaction between *TFL2* and *LFY* could be detected (Sundås Larsson et al., 1998). In the same report a genetic interaction between *tfl2* and *ap1* was described with a suggested function of *TFL2* as a repressor of *AP1*. *AP1* is active in promotion and maintenance of the FM initiated on the flank of the IFM (Mandel et al., 1992). By introducing *tfl2-1* to a weak 35S::*AP1* line both flowering time and termination of the inflorescence were enhanced compared to the parental lines. Thus, *tfl2-1* causes an enhancement of the 35S::*AP1* phenotype. Analysing the expression pattern of *AP1* by RT-PCR, GUS staining and *in situ* hybridisation in the *tfl2-1* background *AP1* was found to be ectopically expressed. In addition to the wild type expression pattern, a low general expression was found in all the aerial parts of the plant. In the root expression was found in the quiescent centre and in the cell division zone. Our results suggest a function of *TFL2* as a repressor of *AP1* in different tissues and during several developmental stages. We suggest that the ectopic expression of *AP1* is a factor contributing to the early phase transition and early termination of the IFM of *tfl2* plants.

When combining *ag-1* and *tfl2-1* the double mutant plants showed *ag* like flowers and traits from the *tfl2* phenotype were partly rescued as seen by larger plant size and later termination of the IFM. Analyses of the *AG* ex-

pression pattern revealed similar results as for *AP1* in the *tfl2-1* background. In addition to a strong wild type expression pattern a general low level of expression could be detected in the aerial tissues examined. Together these results show that *TFL2* repress the meristem regulating and floral identity genes *AP1* and *AG*. This is seen by ectopic expression in different tissues and developmental stages where these genes should be at a repressed state.

In mouse HP1 has been shown to interact with p150, the homologue to Arabidopsis FAS1, which is the large subunit of the CAF-1 complex (Murzina et al., 1999; Kaya et al., 2001). To test if *TFL2* is active in the same mechanisms of gene repression as genes encoding proteins of the Arabidopsis CAF-1 complex, double mutants were created and analyses of protein interactions were conducted. In the double mutants of *tfl2-1fas1-1* and *tfl2-1fas2-1* nearly identical phenotypes were scored. The plant organs were reduced in size, flowers were produced in an altered phyllotaxy along the inflorescence, earlier termination of the inflorescence was seen and the plants were not able to set seeds. We interpret the phenotype of the double mutants as an enhancement of traits seen in either of the single mutants, indicating a genetic interaction between *tfl2* and mutants of two genes encoding proteins of the CAF-1 complex, *fas1* and *fas2*.

Early termination of the IFM has been seen in plants with a constitutive expression of *AP1* or *AG* (Mizukami and Ma, 1997; Mandel and Yanofsky, 1995). In leaves of *tfl2-1fas1-1* and *tfl2-1fas2-1* a higher ectopic expression of *AP1* and *AG* was detected compared to *tfl2-1* single mutants. In *fas1-1* and *fas2-1* single mutants or in wild type plants no upregulation of *AP1* or *AG* could be detected. Ectopic expression of floral homeotic genes has also been detected in mutants of genes encoding components of a suggested Polycomb group Repression Complex2 (PRC2) in Arabidopsis (Goodrich et al., 1997; Katz et al., 2004). A member of another predicted PRC2 complex, *VRN2*, has been shown to be crucial for epigenetic gene repression by methylation of histone H3 at Lysine9 and Lysine27 during vernalisation (Bastow et al., 2004). Sung et al. (2006) showed that *TFL2* has a direct role in *VRN2* mediated *FLC* repression by maintaining the epigenetic repression and in the same report they also suggest that *TFL2* could have this function in floral homeotic gene repression as well.

The physical interaction between HP1 and p150 is through a hydrophobic pocket created by the HP1 dimer (Murzina et al., 1999; Brasher et al., 2000). Given that TFL2 has the ability to form a dimer and that the motif known to interact with HP1 proteins is conserved in FAS1, an interaction test by the yeast two hybrid system was conducted with no interaction between the proteins detected. We can not exclude the possibility that the interaction occurs between the two proteins *in planta*. The interaction could be disturbed in the yeast system, be dependent of other factors or rely on post translational modifications carried out *in planta* but not in yeast.

tfl2 display reduced levels of free IAA in aerial tissues (III)

Reduced levels of auxin or an altered response to auxin could be the cause behind several of the traits seen in the *tfl2* mutant phenotype. Among these traits are: short hypocotyl under red and far red light conditions, reduced cell size and epinastic leaves (Sundås Larsson et al. 1998 and unpublished data). Recently, in the report by Kim et al. (2004), the *tu8* mutant was shown to be allelic to *tfl2*. Plants defective in *TU8* have altered levels of indole glucosinolates and altered levels of IAA during different stages of development (Ludwig-Müller et al., 1999).

Free IAA levels were measured in *tfl2* and wild type plants seven, 14 and 21 days after germination and no significant difference in IAA concentration was detected between the genotypes in root tissues. However, reduced levels of free IAA were detected in aerial tissues of *tfl2* plants, a reduction that was more pronounced after 14 and 21 days than after seven days. *tfl2* plants have entered reproductive development 14 days after germination, which is earlier than wild type plants, and to rule out the possibility that the developmental switch is responsible for the reduced levels of IAA, GUS staining in wild type plants were analysed at a time point where wild type has entered reproductive development (21 days after germination). No reduction in GUS staining was detected in wild type at this stage showing that the developmental switch, itself, does not generate reduced auxin levels. Only a low level of auxin accumulated in leaves of 14 days old *tfl2* plants treated with NPA demonstrate that the reduced levels of auxin in aerial tissues are not generated by an elevated polar auxin transport. Thus, our results show that levels of free auxin are reduced in aerial tissues of *tfl2* plants but not in roots. However, we can not conclude if the reduced levels are due to decreased production, enhanced conjugation or enhanced degradation of auxin, all processes known to alter the level of free auxin in plants (Ljung et al., 2002).

The response to auxin is reduced in *tfl2* mutants (III)

Earlier reported phenotypical traits of *tfl2* are in line with altered auxin levels and/or responses and thus we investigated if *tfl2* plants have an altered auxin response in addition to the lower levels of free auxin in the shoot. Root length, number of lateral roots, root gravitropic response and cotyledon vein patterning did not differ between *tfl2* and wild type plants. When treated with auxin a reduced response was evident in *tfl2* plants, both the root length and hypocotyl length was reduced in IAA treated seedlings but to a lower extent in *tfl2* seedlings, as compared to wild type. These results are consistent with a weaker response to auxin in *tfl2* plants.

Auxin causes rapid induction of the *SAUR*, *GH3* and *AUX/IAA* genes (Gil et al., 1994; Reed, 2001; Staswick et al., 2005) and the expression of selected genes from these families were analysed in *tfl2* plants with or without IAA treatment. In untreated plants several but not all of the genes analysed displayed lower expression levels in *tfl2* compared to wild type. Interestingly, *IAA6*, *IAA19* and *SAUR-AC1* were strongly upregulated in wild type leaves between seven and 14 days, not detected in *tfl2*, a result that correlate with the reduced levels of auxin in the *tfl2* mutant at this time point. When induced by auxin the same genes that displayed lower expression levels in untreated plants showed weaker induction in *tfl2* compared to wild type, indicating a weaker response to auxin. Together this shows that several auxin related genes have a reduced expression in *tfl2* plants. This could solely be due to lower levels of free auxin, but both molecular data and data from phenotypical analyses point to an altered response to exogenous supplied auxin. The decreased transcription levels of certain but not all auxin regulated genes analysed in *tfl2*, indicates that *TFL2* is involved in specific processes rather than being a general repressor of transcription in the response to auxin. This is in line with the earlier reported function of *TFL2* in the regulation of specific genes during different developmental programs such as the repression of *FLC* and *FT* and the repression of *AP3*, *AG* and *PI* (Kotake et al., 2003; Sung et al., 2006).

The CSD of TFL2 interacts with the IAA5 protein (III)

Over 50 interacting clones were isolated using TFL2 or the CSD as baits in the yeast two hybrid system. Most of these clones were sorted out as false positives but the interaction between TFL2 and IAA5 mediated by the CSD was further investigated. Traits from the *tfl2* single mutant were enhanced in *tfl2-1iaa5* and one novel trait, reduced apical dominance, was detected corroborating an interaction between the two proteins. *iaa5* single mutant plants display no phenotypical traits deviating from wild type, a phenomenon that has been suggested to depend on functional redundancy of genes within the *AUX/IAA* family (Overvoorde et al., 2005).

The today, in literature, described interactions of the TFL2 protein are the homo dimerisation, the interaction with methylated histone H3 and with CHROMOMETHYLASE3 (Gaudin et al., 2001; Jackson et al., 2002). HP1 proteins from other organisms e.g. yeast, mammals and *Drosophila* interact with several types of proteins with functions in genome organisation, DNA replication and repair together with individual TFs (see Li et al., 2002 and references therein). In human the KRAB/KAP1 complex has been shown to function as transcription repressors (Friedman et al., 1996). In addition to the interaction with KRAB the KAP1 co-repressor has also been shown to bind HP1 and the methyltransferase SETDB1 a interaction that recruit HP1 and

SETDB1 to promoter regions (Ayyanathan et al., 2003). SETDB1 has been shown to methylate lysine 9 at histone H3 that highly increases the affinity of HP1 proteins to the histone. The binding of HP1 to methylated histone H3 in turn generates a facultative heterochromatin structure and creates a stabile repression of the specific gene.

The mechanism behind the repression of AUX/IAA is not known. One possibility of AUX/IAA mediated gene repression is through rearrangements of chromatin structure. Fukaki et al. (2006) recently showed that the chromatin remodelling factor PICKLE is required for the IAA14 mediated repression of ARF7 and ARF19 activity. We suggest a possible model for the described interaction between TFL2 and IAA5 where the ARF-AUX/IAA complex recruits TFL2 and factors that modify the histone code to promoter regions and induce a closed configuration of the surrounding chromatin that represses the specific gene. This could be carried out in a similar way as in the KRAB/KAP1/HP1-complex where HP1 and the histone methyl transferase are recruited to promoters to generate a dense chromatin structure. The function of TFL2 in IAA/AUX mediated gene regulation needs to be further investigated. It is not known which ARF(s) that IAA5 interact with and which genes that these ARF-IAA5 complexes regulate. Once identified, it would be of highest interest to analyse the chromatin structure/methylation level at the actual promoters in wild type and in *tfl2* mutants to evaluate if the chromatin structure is altered.

Isolation of a third *AP2* class gene from *P. abies* and conservation of the *miRNA172* regulating site (IV)

Two *AP2* class genes, *PaAP2L1* and *PaAP2L2,* from spruce have earlier been described by Vahala et al. (2001). In addition to two *AP2* domains the genes contain other sequence motifs common to the *AP2* gene family.

A third *AP2* gene from spruce was isolated, *PaAP2L3*. Phylogenetic analysis show that all three *AP2* genes from spruce are closely related to *AP2* from Arabidopsis and thus belong to the *euAP2* subgroup of *AP2*/*ERF* genes (Kim et al., 2006; Shigyo et al., 2006). Genes in this group display wide functional diversity regulating also other processes than specification of reproductive organs. In addition to harbouring two *AP2* sequence motifs the genes contain sequence complementary to *miRNA172* known to regulate *AP2* family genes in Arabidopsis and maize (Aukerman and Sakai, 2003; Lauter et al., 2005). *miRNA172* has been described from a wide range of plant species including monocots, gymnosperms and ferns, indicating that the regulation of *AP2* family genes by miRNA is a conserved, ancient process (Axtell and Bartel, 2005). The low sequence complementarity to *miRNA172* in *PaAP2L2*, suggest that the gene has lost this mechanism of

regulation. Alterations in the miRNA complementary site most likely occurred late during evolution, after the split between *Pinus* and *Picea* lineages, since the pine orthologue *PtAP2L2* harbours a complete miRNA site (Shigyo and Ito, 2004).

PaAP2L expression pattern suggest a gene function similar to their angiosperm homologues (IV)

PaAP2L1 and *PaAP2L3* both display a distinct expression pattern during development; however no expression of *PaAP2L2* could be detected by RNA *in situ* hybridisation. *PaAP2L3* is expressed in the VM and in needle primordia indicating a function of the gene in vegetative development of spruce. In the young seed cone *PaAP2L3* is expressed in the ovuliferous scale, in a specific pattern overlapping with *DAL2*, a gene homologous to the C function gene in Arabidopsis (Tandre et al., 1998). The overlapping expression pattern indicates that one of the A class functions described from angiosperms (i.e. Arabidopsis) restricting C class genes probably is regulated by other mechanisms/genes in spruce. A similar situation has been described from *Antirrhinum* were the organ identity genes *LIP1* and *LIP2* do not repress C function (Keck et al., 2003).

After winter dormancy *PaAP2L1* is expressed in the ovuliferous scale displaying an expression pattern complementary to that of *PaAP2L3*, indicating that the genes are active in the differentiation of the ovuliferous scale. Neither of *PaAP2L1* or *PaAP2L3* are expressed in the male cone at any stage of development analysed. That *AP2* genes are expressed in reproductive structures in both gymno- and angiosperms indicates activity of the gene family in the regulation of reproductive development before the split between angiosperms and gymnosperms.

PaAP2L2 shows functional similarities with its Arabidopsis homologues when expressed in Arabidopsis (IV)

Introducing *PaAP2L1* in Arabidopsis generated no phenotypical changes, probably due to repression of the gene by the endogenous *miRNA172*. Constitutive expression of *PaAP2L2* in Arabidopsis generated several phenotypic traits indicating that the gene is involved in several processes during plant development. During vegetative development the *PaAP2L2* expressing plants produced larger leaves and bolted later than the control plants. The later phase transition indicates that the gene has a function as a repressor in the vegetative to reproductive transition, a function also suggested for the

Arabidopsis *AP2*-family gene *TOE1* (Aukerman and Sakai, 2003). A similar function has also been suggested for *AtAP2* when constitutively expressed in *Petunia hybrida* (Maes et al., 1999).

The inflorescences of the *PaAP2L2* expressing plants were stunted, produced flowers in a disturbed phyllotaxy and some plants displayed inflorescence fasciation. The effects of the gene on developmental processes determined by the apical meristem shows that the spruce gene share functional similarities with *AtAP2,* that regulates the size of the SAM (Würschum et al., 2006). Transgenic Arabidopsis plants showed flowers with an increased number of organs, this trait was more extreme in the two inner whorls, however no homeotic changes were detected. In addition, the flowers were indeterminate with production of several stamens and carpels in a repetitive manner with irregular phyllotaxy. The described phenotype of the flowers indicates a function of *PaAP2L2* in the regulation of meristem maintenance.

PaAP2L2 was able to complement the floral defects of the *ap2-1* mutant. Sepals and petals were produced but like constitutive expression of *PaAP2L2* in wild type background the flowers displayed indeterminate growth. This indicate that at least the gymnosperm gene *PaAP2L2* has the ability to function as an A class gene during reproductive development when expressed in Arabidopsis.

Ectopic expression of *WUS* generates flowers that are indeterminate in constitutive expressors of *PaAP2L2* (IV)

To understand the role of *PaAP2L2* in the regulation of meristem function and FM maintenance, flowers from transgenic Arabidopsis were analysed by *in situ* hybridisation. *clv* mutants show in a similar way as *PaAP2L2* expressing plants an overproduction of stamens and carpels as well as indeterminancy of the flowers (Clark et al., 1995). *CLV3* expression was not altered compared to wildtype flowers; displaying a down regulation after the initiation of two carpels. A continued expression of *WUS* was seen between the latest formed carpels throughout the indeterminate development of flowers. *WUS* down regulation is a process known to involve *AG* and one of the described functions of A class genes is to prevent the expression of *AG* in the two outer whorls of the flower (Bowman et al., 1991). In the *PaAP2L2* expressing plants *AG* was expressed in all the organs normally produced in the two inner whorls, with no expression in the organs of the two outer whorls indicating a mechanism where the action of *PaAP2L2* is stronger than the effect of *AG* in the regulation of *WUS*. Our results suggest a function in meristem regulation of *PaAP2L2* by preventing the down regulation of *WUS* either by negatively regulating the CLV signalling pathway or by independ-

ently antagonising *CLV* effects on *WUS*, corroborating the model presented by Würschum et al., (2006).

PaAP2L1 display similar phenotypical traits as *PaAP2L2* when expressed in Arabidopsis without a non complementary *miRNA172* site (unpublished)

To evaluate if *PaAP2L1* is repressed by *miRNA172* when expressed in Arabidopsis and to analyse the effects of the gene in the heterologous system we mutated the gene, named it *PaAP2L1mod* and expressed it behind the strong 35S promoter in Arabidopsis. The mutations generated were identical to that described by Chen (2004) which does not effect the protein sequence but creates a non complementary *miRNA172* site. As seen in the *PaAP2L2* transformants (Nilsson et al., 2006) the T1 population of constitutively expressing *PaAP2L1mod* plants displayed different strengths of phenotypical traits. The most pronounced traits from the strongest *PaAP2L1mod* expressers were larger leaves, late transition to reproductive phase, disruption in phyllotaxy of the inflorescence and overproduction of flowers as a consequence of an enlarged meristem. In addition indeterminate flowers were seen that like constitutively expressing *PaAP2L2* plants produced additional numbers of stamens in the third whorl as well as a repetitive pattern of stamens and carpels inside the first pair of carpels produced.

Figure 6. Constitutive expressors of *PaAP2L1mod* and *PaAP2L2mod. PaAP2L2mod* plants (upper left). Inflorescence apex and a flower of *PaAP2L1mod* (middle and lower left, respectively). Three *PaAP2L1mod* plants with different strengths of phenotypic traits (right)

In a similar but opposite way to *PaAP2L1mod* we generated an intact site complementary to *miRNA172* in *PaAP2L2*, named *PaAP2L2mod*. This was done to evaluate if the gene can be regulated by Arabidopsis endogenous *miRNA172* and if so what affects the modified gene generates when ectopically expressed? The *PaAP2L2mod* plants were indistinguishable from wild type plants suggesting a repression of the transcript by *miRNA172*.

Our results suggest that *PaAP2L1mod*, like its sister gene *PaAP2L2* is active in the timing of transition to flowering in Arabidopsis and in the regulation of both the inflorescence and flower meristems, processes known from other *AP2* family genes. The fact that *miRNA172* is present in gymnosperms (Axtell and Bartel, 2005), sequence complementary to *miRNA172* is found in two of the three today known *AP2* genes from Norway spruce (Nilsson et al., 2006) and that the spruce genes are down regulated in Arabidopsis by miRNA strongly suggest a miRNA regulatory function in spruce similar to that described in Arabidopsis.

Sammanfattning på svenska

Från den tidpunkt ett frö gror kommer växten som fröet ger upphov till att sakna möjlighet att förändra sin livssituation genom förflyttning. Levnadsbetingelser skiljer ofta mellan olika platser och dessutom kan de yttre förutsättningarna under en växts livscykel snabbt förändras. På grund av detta måste växter vara kompetenta att anpassa sig till omgivande betingelser. Växter kan genom att styra sin tillväxt förbättra sina livsvillkor, detta kan ske genom anpassning av skottet för att t.ex. söka mer solljus eller anpassning av roten för att aktivt söka vatten och näring. De punkter från vilka tillväxt sker genom celldelning kallas meristem. Skott- och rotmeristem bildas under embryoutvecklingen och ligger vilande till den tidpunkt då fröet börjar gro.

För att lättare och snabbare få en övergripande uppfattning om de mekanismer som styr organismers utveckling arbetar forskare världen över med utvalda modellorganismer. I en av de väl studerade modellväxterna, backtrav (*Arabidopsis thaliana*), kommer skottmeristemet som anlades under embryoutvecklingen att vara aktivt under hela växtens livscykel. Meristemet kan beskrivas som en kupol med olika zoner, dessa zoner är indelade beroende på celldelningshastighet och vilka delar av en växt eller vilka växtorgan de ger upphov till. De centrala delarna av meristemet består av en grupp av ej specialiserade celler med aktiv celldelning, så kallade stamceller, vilka har som funktion att förse andra delar av meristemet med celler då organ bildas vid dess sida.

Under den vegetativa delen av växters livscykel producerar det apikala meristemet blad vilka är aktiva i fotosyntesen. När en växt övergår från vegetativ till reproduktiv fas, dvs. börjar blomma, sker en omställning i meristemet från att bilda blad till att producera sekundära blomställningar och blommeristem utefter blomställningen. Blommeristem avslutas genom bildandet av en pistill till skillnad från det apikala och de sekundära skottmeristemen som är aktiva och producerar blommor under hela växtens livscykel. De processer som styr övergången mellan vegetativ och reproduktiv fas i växter är strikt reglerade. Olika yttre faktorer som t.ex. temperatur, dagslängd, näringstillgång samt inre faktorer som ålder och hormonnivåer styr denna process.

För att anpassa funktionen av cellerna måste inre så väl som yttre signaler kännas av och omvandlas till signaler som styr cellernas aktivitet. Några av de sista stegen i den kedja som ger en förändrad funktion i de olika cellerna sker genom förändrat genuttryck. Delar av organismers DNA kodar för ge-

ner och vid transkription läser enzymer av och översätter den genetiska kod som finns lagrad i generna och därmed bildas budbärar-RNA i en process kallad transkription. RNA står sedan som mall vid bildandet av proteiner, dessa deltar både i organismers cellulära funktioner och strukturella uppbyggnad.

De processer som sker i en organism är kontrollerade av dess gener. För att kunna behålla ett aktivt meristem under hela växtens livscykel eller för att växten skall blomma vid en för den fördelaktig tidpunkt krävs det att gener som är specifika för dessa processer är aktiva i rätt position, tidpunkt, ordning och styrka. Dessutom skall andra gener som inte har en funktion i denna eller andra pågående processer i cellen vara inaktiva. Genernas aktivitet styrs genom en process kallad genreglering. Genreglering sker på flera olika sätt och på olika nivåer, både före och efter bildandet av RNA. För att starta transkription finns transkriptionsfaktorer vilka reglerar geners uttrycksnivåer genom att binda in till genens promotorsekvens och därmed förändra genens uttryck. För att stänga av en gens uttryck kan det DNA-segment som bildar och omger genen vara otillgängligt för transkription genom täta DNA-protein strukturer, så kallat heterokromatin. Denna process är reversibel och genen kan åter bli tillgänglig för transkription.

Syftet med det arbete vilket resulterat i denna doktorsavhandling har varit att få fördjupad förståelse i delar av de processer som styr växters utveckling genom genreglering och då speciellt meristemets utveckling och fasövergången mellan vegetativt och reproduktivt stadium.

Från backtrav har sedan tidigare mutanten *terminal flower2* (*tfl2*) beskrivits. Mutanten uppvisar bland många karaktärer dvärgväxt, tidig övergång till reproduktiv fas och avslutande, så kallad terminering, av det apikala meristemet i bildandet av en blomma. I den första delen av avhandlingen beskriver jag arbetet med att klona genen som kodar för *tfl2* och djupare utreda några av de processer i vilka genen är aktiv. Genen som kodar för proteinet TFL2 tillhör familjen *HETEROCHROMATIN PROTEIN1* till vilken representanter isolerats från såväl jäst, växter, djur som människa. HP1 proteiner har visat sig vara aktiva i genreglering som en komponent i bildandet av heterokromatin. På samma sätt som andra beskrivna proteiner från denna familj har TFL2 förmågan att binda ett annat TFL2-protein och därmed vara funktionell som en av flera komponenter i regleringen av andra gener. I backtrav är *TFL2* aktiv i många olika vävnader under hela växtens livscykel vilket tyder på att genen deltar i regleringen av flera olika processer från det att ett frö gror till det att frö åter bildas. *TFL2* reglerar aktiviteten av andra gener med en funktion i att främja övergången till reproduktiv fas och gener med en funktion i att behålla ett aktivt skottmeristem fram till det att växten vissnar och dör. *tfl2* mutanten visar också en lägre halt av och en svagare respons på växthormonet auxin. Dessa resultat tyder på att genen är aktiv som en komponent i att både reglera bildandet av och agera på responsen av

auxin. De resultat som jag presenterar ger en djupare förståelse av vilka funktioner som ligger bakom delar av mutantens synliga karaktärer.

I den andra delen av min avhandling beskriver jag hur jag analyserat genen *Picea abies APETALA2 LIKE3* (*PaAP2L3*) en av tre hittills identifierade gener från gran (*Picea abies*) tillhörande genfamiljen *APETALA2*. I blomväxter deltar gener från denna familj i flera processer som till exempel meristemfunktion, övergång mellan olika utvecklingsfaser och mönsterbildning av blomman. Funktionen av generna *PaAP2L1*, *PaAP2L2* och *PaAP2L3* har studerats med uttrycksstudier i gran. Genernas uttrycksmönster visar att de är aktiva i meristem och reproduktiv vävnad vilket tyder på att de också reglerar processer i dessa vävnader. För att studera funktionen av generna *PaAP2L1* och *PaAP2L2* har dessa överförts till och uttryckts kontinuerligt i Arabidopsis. Det kontinuerliga uttrycket i Arabidopsis försenar övergången till reproduktiv utveckling och förändrar meristemets funktion i såväl det apikala skottmeristemet som blommeristemet. De sammanlagda resultaten indikerar starkt att funktionen av gener inom *AP2* gruppen från så vitt skilda växtarter som Arabidopsis och gran är konserverad trots cirka 300 miljoner år av skild evolution.

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