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TERMINAL FLOWER2, the Arabidopsis *HETEROCHROMATIN PROTEIN1* Homolog, and its Involvement in Plant Development

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Abstract
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This thesis describes the characterization of the *Arabidopsis thaliana* mutant *terminal flower2* (*tfl2*), the cloning of the corresponding gene, and the analysis of *TFL2* function in plant development. The *tfl2* mutant is pleiotropic, exhibiting early floral induction in both long and short day conditions, a terminating inflorescence and dwarfing. *TFL2* was isolated using a positional cloning strategy, and was found to encode a homolog to HETEROCHROMATIN PROTEIN1 (HP1), previously identified in yeast and animals where it is involved in gene regulation at the level of chromatin, as well as in the structural formation of constitutive heterochromatin.

Investigating the light response during seedling photomorphogenesis I found that the *tfl2* hypocotyl is hypersensitive to red and far-red light and that *tfl2* is impaired in phytochrome mediated light responses such as the shade avoidance response. In the tightly regulated transition to flowering, we have shown that *tfl2* might contribute to the interpretation of both external signals such as light and temperature as well as endogenous cues, via *FCA*, in the autonomous pathway. The Arabidopsis inflorescence meristem is indeterminate, and *TFL2* possibly acts to maintain this indeterminate fate by repression of the floral meristem genes *APETALA1* and *AGAMOUS.* In yeast two hybrid experiments TFL2 was shown to interact with IAA5, a protein with suggested functions in auxin regulation. Further, in *tfl2* mutants the levels of the auxin indole-3-acetic acid decrease with age in aerial tissues, suggesting a function of *TFL2* in regulation of auxin homeostasis and response. In summary, *TFL2* contributes to regulation of several aspects of plant development, in accordance with the mutant phenotype and the identity of the TFL2 protein.

Keywords: Arabidopsis, HETEROCHROMATIN PROTEIN1, flowering, meristem, auxin

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List of papers

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

I. Annika Sundås Larsson, Katarina Landberg and D.R. Meeks-Wagner (1998). **The *TERMINAL FLOWER2* (*TFL2*) gene controls the reproductive transition and meristem identity in *Arabidopsis thaliana***. Genetics 149, 597-605

II. Katarina Landberg, Lars Nilsson and Annika Sundås Larsson. ***TERMINAL FLOWER2* (*TFL2*) regulates the transition to flowering through the autonomous pathway**. (manuscript)

III. Katarina Landberg, Henrik Johannesson, Alessia Para, Lars Nilsson and Annika Sundås Larsson. **TERMINAL FLOWER2, the Arabidopsis HP1 protein, is involved in light-controlled signaling during seedling photomorphogenesis**. (manuscript)

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

* These authors contributed equally to manuscript IV

Contents

Abbreviations

CD	chromo domain
CSD	chromo shadow domain
GA	gibberellic acid
GUS	ß-glucuronidase
IAA	indole 3-acetic acid
LD	long day
mRNA	messenger RNA
ORF	open reading frame
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase PCR
SAM	shoot apical meristem
SD	short day
wt	wild type

The following nomenclature is used in this thesis:

Protein names are written in upper case letters, e.g. TFL2.
Gene names are written in upper case italic letters, e.g. *TFL2*.
Mutant names are written in lower case italic letters, e.g. *tfl2*.

Introduction

Major events during plant growth and development

Plants go through a number of developmental phases during their life cycle, the first being embryogenesis, occurring within the flower. A flower is generally built up by whorls of organs: sepals, petals, stamens bearing pollen, and carpels, the female organ, situated in the center. In the ovule, a specialized structure within the carpel, embryogenesis takes place. During plant embryogenesis the general body plan is laid down and the root and shoot apical meristems, two groups of undifferentiated stem cells, are established. However, most of the plant development occurs postembryonically, through the continuous division and differentiation of the cells in the apical meristems. Hence, the meristems are the primary sites of regulation and execution of developmental change. At the end of embryogenesis physiological changes occur that will enable the embryo to cope with long periods of dormancy, within the seed.

The timing of seed germination is critical for seedling survival and is, depending on species requirements, influenced by multiple factors including ambient temperature, water availability, duration of storage, and degree of vegetational shading. Germinated in darkness, the seedling undergoes skotomorhogenic development, aimed at reaching out of the soil into the light. During this time, the seedling relies on the energy reserves stored in the endosperm or cotyledons. Characteristics of etiolated (dark-grown) seedlings are elongated hypocotyls (embryonic stems) with closed, unexpanded, pale cotyledons, undifferentiated chloroplasts, and an apical hook protecting the shoot apical meristem. Reaching light, the seedling switches to de-etiolated (photomorphogenic) growth, optimal for photosynthesis. Typically, the elongation of the hypocotyl is now repressed, the apical hook straightens, and the cotyledons open and start to expand. Further, chlorophyll pigments are synthesized, chloroplasts develop and stomata open, facilitating photosynthetic activity. Light has the most profound effects on seedling de-etiolation through a complex interplay of photoreceptors, such as phytochromes and cryptochromes (se below), in combination with plant growth hormones.

If the growing seedlings experience shading by other plants they increase hypocotyl elongation, an action that has been termed the shade avoidance response (Vandenbussche *et al*., 2005; Taiz and Zeiger, 2006). In addition to

elongation growth, leaf development is often reduced, and older plants show internode elongation, increased apical dominance, and reduced branching. Prolonged shading often triggers accelerated flowering and reduced seed set as well. The shade avoidance response is triggered by the decrease in the ratio of red to far-red wavelengths of the incoming light, as a consequence of the shading leaves absorbing red light.

During vegetative development the function and maintenance of meristems, providing an inexhaustible source of undifferentiated cells, are crucial for the indeterminate production of lateral organs, and are thus intimately regulated. The vegetative phase can be sub-divided into a juvenile and an adult phase (Baurle and Dean, 2006), reflected in some species by morphological and physiological differences such as the shape and hairiness of the leaves. The most important difference, however, is the ability of the plant to respond to floral inductive signals, acquired during the vegetative phase change. Upon transition to flowering (a process discussed below), the apical meristem initiates floral meristems on its flanks. The floral meristems are determinate, being consumed in the production of floral organs forming the flowers, leading to a new generation.

Plant development in the model species *Arabidopsis thaliana*

Arabidopsis thaliana (Arabidopsis, backtrav in Swedish) is a small weed belonging to the crucifer family (Brassicaceae). It is spread geographically throughout the temperate climate zone of the Northern hemisphere, and has adapted to several different environments, reflected by the existence of many different ecotypes. The plant is small, self-fertilizing, and produces a large number of seeds. These traits, in combination with a short generation time and the ease with which it is manually cross-pollinated makes it particularly suitable for research studies. The main reason why Arabidopsis has developed into the main model plant, however, is its genetics. It is a diploid species with only 5 chromosomes, and the genome is one of the smallest known among angiosperms (Meyerowitz and Pruitt, 1985) with a very low content of repetitive DNA. Arabidopsis responds well to mutagens and is easily transformed, through whole plant infiltration (Bechtold *et al*., 1993) using the plant pathogen *Agrobacterium tumefaciens*, facilitating the generation of large numbers of stably transgenic plants. In the year 2000, the entire Arabidopsis DNA sequence was published, and the basic structure and location of over 25 000 genes was computationally annotated (The *Arabidopsis* Genome Initiative, 2000). Since then, updated versions taking into account full-length cDNA sequence data, ESTs and homology to sequences from other species, have been published (Wortman *et al*., 2003). Several functional

genomic approaches have been undertaken, creating extensive ORF clone collections, a gene expression atlas, whole genome arrays, and knockout mutant collections (Wortman *et al*., 2003), that will further facilitate the research concerning plant form and function.

Following germination, the Arabidopsis shoot apical meristem (SAM) produces leaf primordia in a spiral phyllotaxy, subtending secondary shoot meristems (axillary buds), at its flanks. The nodes do not elongate, which results in a rosette of leaves. Upon transition to reproductive phase the apical meristem produces floral meristems, and the internodes elongate, resulting in an inflorescence. The inflorescence meristem remains indeterminate and continues to form floral meristems in a spiral phyllotaxy until senescence occurs. The floral meristem is determinate, being consumed while producing four sepals, four white petals, six stamens, and two carpels fused to form a pistil.

Transition to reproductive growth

When the environmental conditions are favorable for seed production, the plant initiates flowering. Precise timing of the transition to flowering is crucial for the reproductive success of the plant, and is achieved by a complex genetic network perceiving and coordinating several environmental factors as well as age and developmental stage. Extensive genetic and physiological investigations of induced mutations and natural variations affecting flowering time of Arabidopsis ecotypes have identified many genes involved in this process. Four main pathways are generally pointed out: the long day (LD) pathway, the vernalization pathway, the autonomous pathway and the gibberellic acid (GA) pathway. The pathways converge in the regulation of the flowering time integrators *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), that in turn induce the floral meristem identity genes *LEAFY* (*LFY*), *APETALA1* (*AP1*) and *CAULIFLOWER* (*CAL*) (Parcy, 2005), resulting in the formation of a flower. *LFY* probably has dual functions, apart from being a floral integrator, it mainly acts as an initiator of floral meristem formation (Moon *et al*., 2005). The genetic network leading to flowering is depicted in figure 1.

The autonomous, or constitutive, pathway was revealed by mutants flowering late in both LD and short days (SD), and promotes flowering independently of day length. Seven genes have been identified, belonging to several sub-pathways, that by different biochemical means regulate a common target, the floral inhibitor *FLOWERING LOCUS C* (*FLC*). *LUMINIDEPENDENS* (*LD*) encodes a homeodomain protein of unknown function, and *FLOWERING LOCUS D* (*FLD*) and *FVE* encodes proteins that are predicted to be part of histone deacetylation complexes (Lee *et al*., 1994; He *et al*., 2003; Ausin *et al*., 2004) regulating *FLC* epigenetically. *FCA*, *FPA* and *FLOWERING LATE KH MOTIF* (*FLK*) are predicted to regulate *FLC*

post-transcriptionally through RNA processing, as they all encode proteins containing putative RNA binding domains (Macknight *et al*., 1997; Schomburg *et al*., 2001; Lim *et al*., 2004). The seventh member of the autonomous pathway, *FY*, encodes a mRNA 3´ end processing (polyadenylation) factor, which physically interacts with FCA (Simpson *et al*., 2003). The interaction between FCA and FY is required for the negative regulation of *FLC*, as well as for a negative autoregulation of *FCA*, in which alternative splicing and polyadenylation produces four different transcripts, of which only one encodes active FCA protein (Quesada *et al*., 2003). In addition, FCA was recently shown to be an abscisic acid (ABA) receptor, adding ABA as an important regulating factor of flowering time. Upon ABA binding, the FCA-FY complex dissociates causing accumulation of full-length *FCA* transcript, consequently leading to high *FLC* mRNA levels and inhibition of flowering (Razem *et al*., 2006).

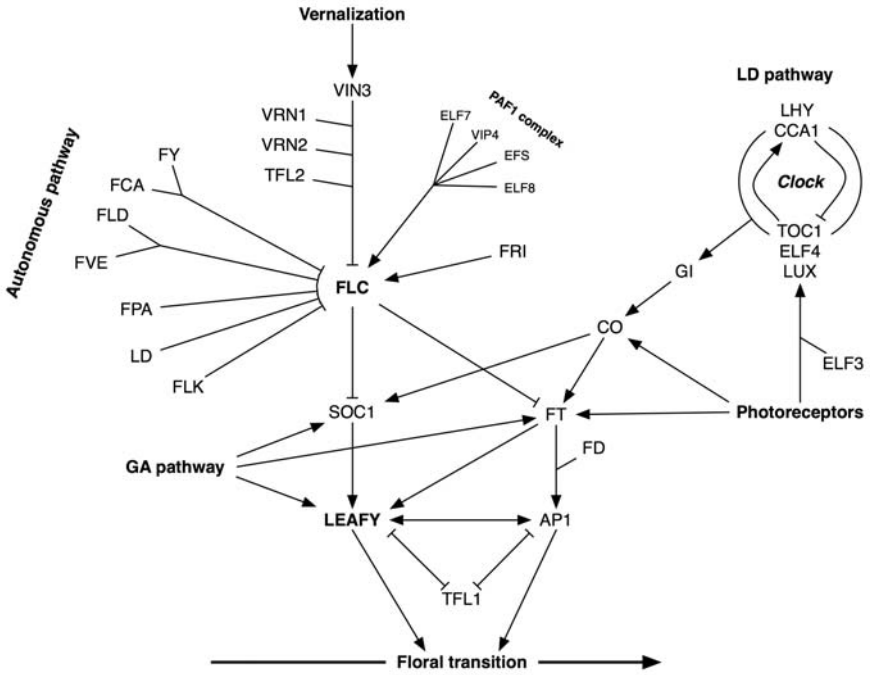

Figure 1. Schematic representation of the major genetic pathways regulating flowering time in Arabidopsis. Abbreviations are explained in the text. Adapted from He and Amasino, (2005).

Vernalization (exposure to an extended cold-period) promotes flowering by making the shoot apical meristem competent to respond to flowering signals. This is also mediated through down-regulation of *FLC*, and the repression is epigenetically maintained after the cold period ceases. Vernalization results in histone modification (deacetylation followed by di-methylation of histone H3) at *FLC*, possibly mediated by VERNALIZATION INSENSITIVE3 (VIN3), VERNALIZATION2 (VRN2), VERNALIZATION1 (VRN1) and TERMINAL FLOWER2 (TFL2) (reviewed by Sung and Amasino, 2005). VRN2 is a homolog of the *Drosophila* Suppressor of Zeste 12 and VIN3 is a PHD finger containing protein, both implicated in chromatin remodeling (Gendall *et al*., 2001; Sung and Amasino, 2004). TFL2 is required for the maintenance of the repressed state of *FLC* (Mylne *et al*., 2006; Sung *et al*., 2006).

The regulation of *FLC* does not only involve negative factors; a number of positively regulating proteins have been identified as well. Studies of natural variation in flowering time of Arabidopsis accessions recognized the *FRIGIDA* (*FRI*) alleles as major enhancers of *FLC* expression. The function of FRI is largely unknown, but five additional *FRI* homologs exist in the Arabidopsis genome (Michaels *et al*., 2004), and FRI has been shown to mediate an increase in trimethylation of H3 at Lys4 (K4), of *FLC* chromatin (He *et al*., 2004). Promotion of trimethylation of H3K4 is also a result of the yeast Paf1 complex, and several Arabidopsis genes related to members of this complex has been identified as factors required for *FLC* mRNA accumulation, including *VIP4*, *VIP6*/*ELF8*, *VIP5* and *ELF7* (Zhang and van Nocker, 2002; He *et al*., 2004; Oh *et al*., 2004). *FLC* itself encodes a MADS box transcription factor, belonging to a sub-family of five additional genes, MADS AFFECTING FLOWERING (MAF1-5), which also probably function as flowering repressors (Michaels and Amasino, 1999; Ratcliffe *et al*., 2001; Ratcliffe *et al*., 2003).

Arabidopsis is a facultative long day plant, initiating flowering in response to increasing day lengths. The LD pathway to flowering was identified by mutants flowering late in LD, not rescued by vernalization. As opposed to the other pathways, a molecular hierarchy has been delineated for the key players in this pathway. Photoreceptors and the circadian clock are pathway inputs, GIGANTEA (GI) works downstream of the clock, activating CONSTANS (CO) that in turn activates FT. GI is a large, nuclear localized, protein of yet unknown biochemical function (Mizoguchi *et al*., 2005). Core components of the Arabidopsis circadian oscillator are the CIRCADIAN CLOCK ASSOCIATED1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY), TIMING OF CAB1 (TOC1), EARLY FLOWERING4 (ELF4), and LUX ARRHYTHMO (LUX) proteins that regulate each other through a transcriptional/translational feedback loop (Gardner *et al*., 2006). The photoreceptors entrain the clock, possibly via EARLY FLOWERING3 (ELF3) (McWatters *et al*., 2000; Covington *et al*., 2001). CO is highly regu-

lated by several promoting and repressing factors, partly through clock-driven signals serving to elevate CO mRNA levels towards the end of the light period during LD (in SD, the CO mRNA levels peak at midnight). This is complemented by cryptochromes and phytochromes antagonistically acting to stabilizing the CO protein at the end of the day, while causing ubiquitination and subsequent degradation of CO in the dark (Valverde *et al*., 2004). In this way, the CO protein levels are high enough to activate floral integrators only during LD, when these two mechanisms coincide. Early grafting experiments concluded that photoperiod is perceived by the leaves, whereas the floral transition takes place in the SAM, meaning that long distance signals must participate in the flowering process (Zeevaart, 1976). CO mRNA is expressed widely at low abundance, however it can only promote flowering when expressed in the leaf vascular tissue (An *et al*., 2004; Ayre and Turgeon, 2004). *FT* and *SOC1* are downstream targets of CO, and FT in turn interacts with FD, a bZIP transcription factor expressed at the flanks of the shoot apex (Abe *et al*., 2005) where it, together with FT, induces *AP1* and *FRUITFUL* (*FUL*) expression. FT is a small globular protein with similarities to Raf kinase inhibitor proteins, although no functional domains have been identified. Recently, it was shown that FT mRNA is actually one of the signals moving from leaf to shoot apex (Huang *et al*., 2005). Further reports show that *FLC*, regulated by the autonomous and vernalization pathways, is expressed both in leaves and in the SAM, repressing the production of systemic signals such as *FT* in leaves, and repressing expression of *SOC1* and *FD* in the meristem (Searle *et al*., 2006).

The GA pathway is required for flowering in non-inductive short day conditions and involves genes with roles in GA biosynthesis or signal transduction. GA can possibly act by upregulating both LFY, SOC1 and FT (Blazquez *et al*., 1998; Gomez-Mena *et al*., 2001; Moon *et al*., 2003; Pineiro *et al*., 2003).

In Arabidopsis, additional factors can have substantial impact on the flowering process. In addition to vernalization, the ambient temperature affects time to flowering; higher temperatures moderately accelerating flowering. Stressful conditions due to suboptimal water and mineral availability, as well as neighboring plants are other examples of parameters that affect flowering. Finally, light quality is believed to regulate the floral integrators separately from the LD pathway (Koornneef *et al*., 1998; Cerdan and Chory, 2003).

The floral integrators ultimately control the activity of meristem identity genes; shoot meristem identity genes that specify the inflorescence as indeterminate and nonfloral, and floral meristem identity genes causing lateral meristems to develop into flowers instead of leaves or shoots. *TERMINAL FLOWER1* (*TFL1*) belongs to the former group while *LFY*, *AP1* and *CAL1* belong to the latter, exerting antagonistic functions. *TFL1* encodes a protein homologous to *FT*, although with opposite functions (Kobayashi *et al*.,

1999). It is hypothesized that, since they do not themself bind to DNA, they would control transcription by interacting with specific proteins targeting them to promoters of floral genes like *AP1* (Ahn *et al*., 2006). *TFL1* delays upregulation, and inhibits the response to *LFY*, *AP1* and *CAL* in the inflorescence meristem, while *LFY*, *AP1* and *CAL* repress *TFL1* expression on the flanks of the inflorescence meristem where floral meristems are formed (Bradley *et al*., 1997; Liljegren *et al*., 1999; Ratcliffe *et al*., 1999). Of the floral meristem genes, *LFY* is expressed earliest, in stage 1 floral meristems, where it in turn directly activates *AP1* and *CAL* expression (Wagner *et al*., 1999; William *et al*., 2004). *AP1* can also be activated independently of *LFY*, most likely by *FT* (Ruiz-Garcia *et al*., 1997; Wigge *et al*., 2005). When the meristem has gained floral identity the ABCDE floral organ identity genes are induced, which through specific molecular combinations define the patterning of the flower (Weigel and Meyerowitz, 1994; Theissen, 2001).

Light control of plant development

Light has profound effects on plant growth and development. Plants are photoautotrophs, using light as a source of energy to synthesize sugars from inorganic substances, through the process of photosynthesis. Besides being the primary energy source, light also provides an important means of sensing the surrounding environment, and has been shown to play a significant role in physiological responses such as seed germination, seedling establishment, phototropism, shade avoidance and transition to flowering. The physiological and molecular responses that plants exhibit in response to light signals, have been termed photomorphogenesis.

Photoreceptors and signal transduction in Arabidopsis

The sedentary nature of plants makes them highly sensitive to the changes in environmental conditions, thus requiring an intrinsic system to detect even subtle variations in light quantity, quality, direction and periodicity. To monitor these variations, higher plants have evolved three major groups of photoreceptors that absorb different wavelengths of light: the phototropins and the cryptochromes that perceive UV-A and blue light (320-500 nm), and the phytochromes that perceive red and far-red light (600-800 nm) (Chen *et al*., 2004; Banerjee and Batschauer, 2005; Rockwell *et al*., 2006).

Two genes encoding phototropins have been isolated in Arabidopsis, *PHOT1* (*NPH1*) and *PHOT2* (*NPL1*) (Huala *et al*., 1997; Sakai *et al*., 2001). The protein structure includes a carboxy-terminal (C-terminal) Ser/Thr protein kinase domain and two amino-terminal (N-terminal) LOV (Light, Oxygen, Voltage) domains. The LOV domains function as the blue light sensors, binding the flavin mononucleotide (FMN) chromophore. Blue light absorption results in receptor autophosphorylation, and subsequent initiation of phototropin signaling. A slower dark reversion completes the phototropin

photocycle (Crosson and Moffat, 2002; Crosson *et al*., 2003; Kennis *et al*., 2004). The PHOT1 and PHOT2 proteins are highly similar, and are both associated with the plasma membrane. Although PHOT1 is specialized for low fluence rates, while PHOT2 is likely more important during high fluence rates of blue light (Briggs and Christie, 2002), their functions overlap in mediating light responses that optimize photosynthesis. This includes phototropism (bending in response to light), stomatal opening, chloroplast accumulation, as well as cotyledon and leaf expansion (reviewed by Christie, 2006). A novel family of LOV domain containing proteins, involved in circadian clock function and photoperiod dependent flowering, has been described that might define additional blue light receptors (Somers *et al*., 2000; Imaizumi *et al*., 2003).

The cryptochrome family of blue/UV-A photoreceptors is widely distributed in bacteria and eukaryotes. The proteins are structurally related to DNA photolyases, blue light induced enzymes that repair UV-damaged DNA, however lacking the DNA-repair activity (Sancar, 2003). In Arabidopsis, three cryptochromes have been isolated, CRY1-3. These proteins contain an N-terminal photolyase homology region (PHR), which binds two light harvesting chromophores, a flavin adenine dinucleotide and a methenyltetrahydrofolate. In CRY1 and CRY2, a C-terminal extension with short conserved stretches is found, but is absent in CRY3, which instead contains an N-terminal transient peptide sequence targeting it to chloroplasts and mitochondria. CRY1 is localized to the nucleus during dark conditions but primarily cytoplastic in light conditions, while CRY2 is constitutively nuclear localized (Guo *et al*., 1999; Yang *et al*., 2000). The regulation and biological function of CRY3 in Arabidopsis is largely unknown. CRY1 and CRY2 are phosphorylated at multiple sites following blue light exposure, which is important for their biological activity (Shalitin *et al*., 2002; Shalitin *et al*., 2003). A few cryptochrome interacting components have been identified, including several phytochromes (Neff and Chory, 1998; Mas *et al*., 2000) and COP1, an E3 ubiquitin ligase involved in light regulated protein degradation. The cryptochromes are important for several light regulated plant responses, including de-etiolation, photoperiod dependent induction of flowering, and resetting of the circadian clock. Although CRY1 mediates high fluence rate responses to blue light, and CRY2 mediates the response to low fluence rate (Ahmad and Cashmore, 1993; Lin *et al*., 1998), both CRY1 and CRY2 are implicated in these physiological responses, and in addition, probably act in concert with the phytochrome photoreceptors.

Phytochromes are by far the most extensively studied plant photoreceptors. They make up a small family of proteins found in plants, cyanobacteria and fungi. In plants, phytochromes sense the ratio of red to far-red light, thereby assessing the quantity of photosynthetically active light. Five phytochromes are present in Arabidopsis, PHYA-PHYE (Sharrock and Quail, 1989; Clack, 1994), classified into two groups. PHYA is light labile, occur-

ring in high quantities in dark grown tissues, and is upon illumination rapidly degraded, while PHYB-E are all light stable. All phytochromes share a common protein structure consisting of an N-terminal photosensory region including four conserved domains which allows attachment of the tetrapyrrole chromophore (phytochromobilin), and a regulatory C-terminal region (reviewed by Rockwell *et al*., 2006). Central to the function of phytochrome is its red/far-red photoconvertibility between two stable states, Pr and Pfr. Synthesized in darkness in its Pr form, red light induces the conversion into Pfr, which for most biological responses is the active form of phytochrome. Pfr can subsequently be converted back to the inactive Pr form by far-red light, or by dark reversion, a slow thermal process in the absence of light. Following light perception the phytochromes are translocated from the cytoplasm into the nucleus, as homodimers (Kircher *et al*., 2002). In the nucleus they are detected as speckles, the function of which is not known. However, a few factors have been shown to co-localize with phytochrome, and it has been hypothesized that the speckles represent sites of interaction between phytochrome and downstream signaling components (Nagatani, 2004). Microarray-based expression profiling of wild type (wt) and phytochrome mutants, grown in different light conditions, has shown that major changes in gene expression occur upon light treatments, and that the phytochromes have both over-lapping and distinct functions in diverse light responses. Several phytochrome-interacting proteins have been isolated, through mutant screening and yeast two-hybrid experiments, including bHLH transcription factors, cryptochromes, nucleoside diphospate kinase-2, and COP1 (reviewed by Schäfer and Bowler, 2002). One of the bHLH proteins, PIF3, which *in vitro* interacts with both PHYA and PHYB, has been shown to bind to the G-box, a motif found in many light responsive genes (Martinez-Garcia *et al*., 2000), indicating that the signal transduction pathway between light perception and gene regulation can be relatively short. Phytochromes possess two histidine kinase domains, suggesting they act as light-regulated kinases, regulating responses through phosphorylation. *In vitro*, both PHYA and PHYB autophosphorylate, as well as phosphorylating PKS1, cryptochromes, and Aux/IAA proteins (Ahmad *et al*., 1998; Fankhauser *et al*., 1999; Colon-Carmona *et al*., 2000). The phytochromes are all involved in regulation of light-responses throughout the plant life cycle, including seed germination and de-etiolation, shade avoidance, circadian rhythm, and floral induction (reviewed by Franklin *et al*., 2005). While PHYA is the main mediator of seedling responses to far-red light, PHYB-E regulate seedling responses to red light and are the main mediators of the shade avoidance response, PHYB having the most pronounced effects.

In addition to the downstream signaling components isolated for each photoreceptor, several genes implicated in multiple receptor pathways have been identified (Cerdan and Chory, 2003; Park *et al*., 2003; Bertrand *et al*., 2005; Kang *et al*., 2005; Ward *et al*., 2005; Lariguet *et al*., 2006), showing

that light perception is regulated and mediated through a complex network combining several photoreceptor functions. Another class of genes functioning downstream of the photoreceptors are the *CONSTITUTIVE PHOTOMORPHOGENESIS/DE-ETIOLATED/FUSCA* (*COP/DET/FUS*) genes, which have repressive functions during photomorphogenic development. Mutations in these genes result in de-etiolated growth in darkness, as they exhibit ectopic expression of light-regulated genes. Several of the gene products are constituents of the COP9 signalosome, a complex involved in ubiquitin dependent proteolysis (Wei and Deng, 2003). COP1 is an E3 ubiquitin protein ligase, and has been shown to act downstream of both phytochromes and cryptochromes. In darkness COP1 is localized to the nucleus and targets HY5 and HYH, positive regulators of photomorphogenesis, for proteasome mediated degradation. DET1 is suggested to link light signaling to chromatin remodeling, since it binds the non-acetylated histone H2B in the context of the nucleosome (Benvenuto *et al*., 2002), as well as UV-damaged DNA Binding Protein1 (DDB1), a protein whose homolog in animals interacts with histone acetyltransferase complexes. Thus it is possible that DET1 inhibits light-inducible genes by binding to the nucleosomes, and upon light exposure is removed through acetylation of the histone tails, enabling gene expression (Schroeder *et al*., 2002).

Auxin control of plant development

Auxin represents one of six major types of hormones found in plants. Plant hormones are small organic molecules that affect a wide range of developmental processes. Unlike animal hormones, they have broad effects, can be synthesized throughout the plant, and can function either at the site of synthesis or at distant targets, by transport. Auxin, the first hormone to be discovered in plants, control responses on a cellular level such as cell division, expansion and differentiation as well as at a whole plant level during developmental processes such as embryogenesis, where auxin affects establishment of apical-basal polarity and formation of the shoot and root apical meristems, hypocotyl growth, lateral root formation, vascular tissue formation, phyllotaxy, tropistic growth, apical dominance and flowering. In the 1930s auxin was identified as indole-3-acetic acid (IAA) (Koegl and Kostermans, 1934; Went and Thimann, 1937). Since then a few other auxins have been found in plants, including 4-Cl-IAA and IBA, however, IAA seems to be the most abundant and physiologically important. The intrinsic regulation of plant development exerted by auxin requires a precise temporal and spatial regulation of the levels of free auxin. This is mediated by a combination of synthesis, transport, degradation and conjugation, acting in concert to regulate auxin homeostasis.

Biosynthesis

All parts of the plant are capable of synthesizing low levels of auxin, but the primary sites are shoot apical meristems, young leaves and root apical meristems (Ljung *et al*., 2001; Ljung *et al*., 2005). Several biosynthetic pathways, making up a large network of intermediate molecules, contribute to IAA synthesis in plants (reviewed by Woodward and Bartel, 2005). Two main pathways are suggested, the tryptophan-dependent and the tryptophan-independent pathway. Little is known about the tryptophan-independent route, and no biosynthetic enzymes have so far been isolated. However, tryptophan-biosynthetic mutants are still able to synthesize IAA, supporting the presence of such a pathway. A number of pathways utilize tryptophan as the precursor, although the relevance of each pathway in plants is still an open question. Even though a number of intermediates have been identified, none of the pathways has yet been completely delineated, due to a high degree of genetic redundancy, in combination with an insufficient number of informative loss-of-function mutants.

Conjugation

Out of the total IAA pool of a plant, only about 1% exists as free IAA, while the rest of the IAA molecules are stored as conjugates (Tam *et al*., 2000). Two types of conjugates can be found, IAA ester-linked to sugars, or amide-linked to amino acids and peptides, amide-linked IAA probably dominating in Arabidopsis. The conjugation of IAA serves several functions. In seeds and other storage organs such as cotyledons, auxin is stored as conjugates. When free IAA is needed, e.g. during seed germination, the conjugates are hydrolyzed to free IAA serving as an IAA source for the developing seedling. At high concentrations, auxin is toxic, and conjugation can serve as a way to protect against high IAA levels. Not all conjugates can be hydrolyzed, but instead permanently inactivate the IAA. For example, when high levels of auxin are exogenously supplied to seedlings, conjugation to Aspartic acid and Glutamic acid occurs, two conjugates that cannot be hydrolyzed. Instead IAA-Aspartic acid, as well as free IAA can be oxidized and thereby permanently degraded. IBA, a naturally occurring auxin contributes to the IAA pool as well, since it can be ß-oxidized to IAA (Bartel *et al*., 2001).

Transport

Auxin moves between cells by diffusion as well as by regulated polar transport. For developmental responses such as gravitropisms and lateral root formation the highly regulated carrier-mediated transport is crucial. In shoots, auxin is transported downwards basipetally, while a two-way transport occurs in the roots: acropetal transport delivering shoot-synthesized IAA through the stele and the phloem, and basipetal transport from the root apex through epidermal cells. IAA enters cells either by membrane diffusion

in its protonated form, or via uptake facilitated by AUX1 and possibly other related proteins. AUX1 is a transmembrane protein related to bacterial amino acid permeases that has been shown to localize asymmetrically in the plasma membrane of auxin-transporting cells (Bennett *et al*., 1996; Marchant *et al*., 1999; Swarup *et al*., 2001; Swarup *et al*., 2004). Several factors regulating auxin efflux have been isolated, most of them belonging to the MDR/PGP (multidrug resistance/P-glycoprotein) and PIN (PIN-FORMED) families. PGP efflux proteins are probably the more generally expressed carriers, out of which some also function as uptake carriers, while the PIN proteins provide the means for highly regulated developmental processes in the meristems (Paponov *et al*., 2005; Geisler and Murphy, 2006; Kramer and Bennett, 2006).

Signaling

The tightly regulated auxin homeostasis very precisely controls cellular auxin levels, within minutes inducing changes in gene transcription. Genetic studies and genome-wide profiling experiments have identified a tremendous number of auxin induced genes, several belonging to the *SMALL AUXIN UP RNA* (*SAUR*), *GRETCHEN HAGEN3* (*GH3*), and *AUXIN/INDOLE-3-ACETIC ACID* (*AUX/IAA*) families of primary responsive genes. In Arabidopsis over 70 *SAUR* genes have been identified, encoding small, short-lived, nuclear proteins, the functions of which in auxin signaling is still elusive (Gil *et al*., 1994; Hagen and Guilfoyle, 2002). As is the case with the *SAUR* genes, *GH3*-like transcripts accumulate upon auxin induction in several plant species. Interestingly, at least some of the *GH3* genes up-regulated by auxin encode enzymes that conjugate IAA to amino acids, and could therefore serve to dampen the auxin signal (Staswick *et al*., 2002; Staswick *et al*., 2005). The Aux/IAA family is well characterized in Arabidopsis and includes 29 nuclear proteins, which share extensive sequence similarities in four conserved domains. Domain I is involved in transcriptional repression (Tiwari *et al*., 2004) and domain II is critical for the instable nature of Aux/IAA proteins (Ramos *et al*., 2001). Domains III and IV are important for homodimerization and heterodimerization with other Aux/IAA proteins as well as with AUXIN RESPONSE FACTORS (ARFs) (Kim *et al*., 1997; Ulmasov *et al*., 1999). The function of Aux/IAA dimers is not clear, but it is hypothesized that they could bind DNA, thus directly regulating gene transcription (Paciorek and Friml, 2006), however the relevance of this is yet to be investigated. Many mutations in Aux/IAA genes have been characterized without detecting any mutant phenotypes, in fact genetic analyses, combining up to three different loss of function mutants, do not show phenotypical alterations, suggesting a substantial functional redundancy among these genes (Overvoorde *et al*., 2005). However, a number of dominant gain-of-function mutations give dramatic auxin resistance phenotypes (Woodward and Bartel, 2005) All dominant mutations are situated in domain II, indicat-

ing the importance of Aux/IAA turnover. Notably, the degradation rates for the members of the Aux/IAA family differ significantly (Dreher *et al*., 2006), allowing a further level of auxin regulation.

A conserved Auxin-Responsive-Element (AuxRE) is situated in the regulatory regions of a range of auxin-responsive genes, including Aux/IAA genes. Used in reporter gene constructs, this promoter element confers auxin-induced gene expression (Ulmasov *et al*., 1995; Ulmasov *et al*., 1997b). In a yeast one-hybrid screen, the protein binding the AuxRE was identified (Ulmasov *et al*., 1997a). This auxin response factor (ARF1) belongs to a family of 23 members in Arabidopsis, all sharing a similar protein structure. The N-terminal part contains a large DNA-binding domain that mediates AuxRE binding, and the C-terminal has a dimerization domain, related to domain III and IV of Aux/IAA proteins. ARFs can bind the AuxRE as monomers, homodimers, heterodimers with other ARFs, and as dimers with Aux/IAAs, which repress ARF function. The variable middle region of ARF proteins confers either an active or repressive function to a particular ARF. In protoplast transfection assays, ARFs exhibiting serine-rich middle regions work as transcriptional repressors, while those with glutamine-rich middle regions show activating activities (Tiwari *et al*., 2003).

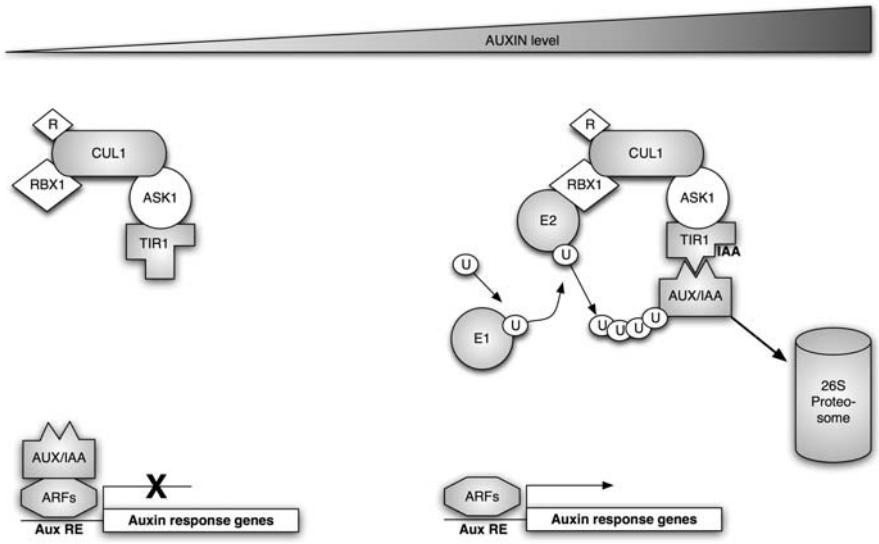

Figure 2. Model depicting auxin regulated gene expression. Abbreviations are explained in the text.

The repression of ARFs, executed by Aux/IAAs, and the degradation of Aux/IAA proteins in response to auxin, thus liberating the ARFs and allowing auxin-induced gene expression, is central to auxin regulation (Figure 2). The phenotype of many of the auxin resistance mutants isolated is caused by Aux/IAA protein stabilization. It was shown that some mutants represent genes encoding proteins involved in ubiquitin-mediated protein degradation. Thus, the high turnover rate of Aux/IAAs is caused by ubiquitination and subsequent 26S proteosomal degradation. This system is found in all eukaryotic cells, and is involved in regulating many processes in plants, including de-etiolation (Wei *et al*., 1994), jasmonate-mediated plant defense responses (Feng *et al*., 2003), and flower development (Wang *et al*., 2003). The attachment of ubiquitin to a substrate is a three-step process where an E1 enzyme activates the ubiquitin C-terminus, an E2 intermediary protein binds the activated ubiquitin, and in concert with the specificity-providing E3 ligase, the target is multiply ubiquitinated. The SCF (Skp1-Cullin-F-box) E3 ligase complexes are the largest family of ubiquitin ligases found in plants. They are made up of the scaffold sub-unit CULLIN that binds RBX1 and the RUB modifier at the C-terminal, and SKP1 at its N-terminal. SKP1 in turn interacts with an F-box protein (Zheng *et al*., 2002). While the SKP1 and RBX1 proteins provide catalytical activity, the F-box proteins confer target specificity (Gray *et al*., 2001). Genetical analysis, including characterization of several auxin resistance mutants, as well as biochemical studies in Arabidopsis, identified genes encoding these subunits (the F-box protein TRANSPORT INHIBITOR RESPONSE1 (TIR1), CULLIN1 (CUL1) ARABIDOPSIS SKP1-LIKE1 (ASK1), and RBX1 (Ruegger *et al*., 1998; Gray *et al*., 1999; Gray *et al*., 2002; Hellmann *et al*., 2003; Quint *et al*., 2005), confirming that an SCF complex is part of the auxin signaling. Further mutant analyses revealed an intricate regulation of SCF complex assembly and regulation, including the COP9 signalosome (Schwechheimer *et al*., 2001).

Not until last year was the model of auxin signaling depicted in figure 2 completed, with the finding that TIR1 is an auxin receptor (Dharmasiri *et al*., 2005a; Kepinski and Leyser, 2005). At high auxin levels, TIR1 binds auxin and is then capable of linking the ubiquitination complex to the Aux/IAA proteins, causing their degradation and subsequent release of ARFs, which in turn induce expression of primary auxin responsive genes. TIR1 belongs to a small sub-family of F-box proteins, including the three AUXIN SIGNALING F-BOX (AFB) proteins. The single *tir1* mutant exhibits only weak auxin response defects. However, combining mutants of all four *TIR1* family members, yielding the quadruple mutant *tir1afb1afb2afb3,* causes dramatic phenotypical auxin defects, most of the individual plants not surviving seedling stage. Analysis of these mutants shows that all four proteins can act as auxin receptors, sharing redundant functions (Dharmasiri *et al*., 2005b).

In addition to the nuclear localized TIR1/AFB auxin receptors, there are indications of a membrane bound auxin receptor, involved in mediating quick non-transcriptional responses such as membrane hyperpolarization and endocytosis. AUXIN-BINDING PROTEIN1 (Steffens *et al*., 2001) has been suggested as a strong candidate for such a receptor, though the mechanism has yet to be demonstrated.

Regulation of gene expression at the level of chromatin

DNA molecules are compacted into a chromatin structure by wrapping around histone proteins forming nucleosomes. One nucleosome consists of eight core histones (two of each H2A, H2B, H3 and H4) around which 147 base pairs of DNA are winded. The tail domains of the histones protrude from the nucleosome, and are subject to extensive post-translational, enzyme-catalyzed modifications including acetylation, methylation, ubiquitination, and phosphorylation. Not only do these modifications cause alteration of the electrostatic charge of the histones, that in turn can alter structural properties and binding to DNA, but they also influence the potential binding of specific protein complexes, affecting both structure and function of the chromatin. Chromatin has been cytologically sub-divided into heterochromatin and euchromatin. Euchromatin is generally less condensed and contains most of the actively transcribed genes. Heterochromatin remains condensed throughout the cell cycle, contains repetitive DNA sequences and few transcriptionally competent genes, is important for proper chromosome segregation, and is found in centromeric as well as telomeric regions. In yeast and animals, general characteristic histone modifications for euchromatin have been identified, and include acetylation of histone H3 and H4 and methylation of H3 lysines (K) 4, 36 and 72. Heterochromatin, in contrast, is enriched in H3 trimethylated at K 9 (H3K9me3) and H4 trimethylated at K 20 (H4K20me3), combined with a general depletion in histone acetylation (Mutskov and Felsenfeld, 2004; Schubeler *et al*., 2004; Pokholok *et al*., 2005). The pattern of modification can function as a signal, recruiting specific proteins or protein complexes to the chromatin. As a well characterized example, it has been shown that H3K9me3 serves as a binding site for HETEROCHROMATIN PROTEIN1 (Bannister *et al*., 2001; Lachner *et al*., 2001; Fischle *et al*., 2005).

HETEROCHROMATIN PROTEIN1

HETEROCHROMATIN PROTEIN1 (HP1) was originally identified in Drosophila as a non-histone component of heterochromatin, involved in stable and heritable transcriptional silencing, required for position effect variegation (PEV) (James and Elgin, 1986; Eissenberg *et al*., 1992). PEV is a tran-

scriptional silencing effect seen when genes that normally resides in euchromatin are translocated to the vicinity of heterochromatin. HP1 is highly conserved throughout the eukaryotes, and homologs have been identified in many organisms, including fission yeast, Xenopus, Drosophila, mouse and human, species in which most studies have been made. Whereas yeast has only one HP1 gene, mouse and human have three. The HP1 proteins are relatively small, less than 200 amino acids, and comprise an N-terminal chromo domain (CD) and a structurally related C-terminal chromo-shadow domain (CSD) separated by a less conserved "hinge" region. The three-dimensional structures of the CD and the CSD of mouse HP1ß have been resolved and shown to be globular domains, consisting of a three-stranded antiparallel ß-sheet folding back against one or two C-terminal α-helices, respectively (Ball *et al*., 1997; Brasher *et al*., 2000). The CD mediates the binding of HP1 to methylated H3, as well as to a core globular domain of H3. A few additional proteins have been reported to interact with the CD; however most protein-protein interactions are thought to occur through the CSD. Possessing a second α-helix, the CSD can form a tight homodimer *in vitro*, and it is hypothesized that this is the state in which HP1 interacts with other proteins (Cowieson *et al*., 2000). By CSD dimerization a hydrophobic pocket is formed that can bind pentapeptides with the consensus Proline-X-Valine-X-Leucine, which indeed has been found in many, but not all, HP1 interacting proteins (Lechner et al., 2005). The less conserved hinge region has been assigned functions during chromatin binding, and is possibly also a target for post-translational phosphorylation (Badugu *et al*., 2005). HP1 proteins from Drosophila as well as from humans have been shown, *in vitro* and *in vivo*, to be post-translationally modified by phosphorylation (Zhao *et al*., 2001 and references therein). Recently, it was shown that HP1 from mammals, just like histones, can be extensively modified, e.g. phosphorylated, acetylated, methylated, sumoylated and ubiquitinated, adding another level of regulation of HP1 proteins (Lomberk *et al*., 2006). Originally identified as a component of heterochromatin, several cases of HP1 function in euchromatin, regulating gene expression, have now been described (reviewed by Hiragami and Festenstein, 2005; Hediger and Gasser, 2006). Thus, HP1 can possess diverse functions, depending on post-translational modifications, protein interaction partners, and chromosomal context.

The HP1 protein identified and characterized in plants, TERMINAL FLOWER2 (TFL2), also named LIKE HETEROCHROMATIN PROTEIN1 (LHP1) and TU8 (Gaudin *et al*., 2001; Kim *et al*., 2004) from Arabidopsis, will be presented and discussed in detail in the Result and Discussion part of this thesis. Shortly summarizing work by others, plant HP1 is encoded by a single copy gene, and differs from its animal homologs in that it seems to be mainly localized to euchromatin, although heterochromatic localization has been reported as well (Libault *et al*., 2005; Nakahigashi *et al*., 2005; Zemach *et al*., 2006). TFL2 has been assigned functions in MADS-box gene repres-

sion (Germann *et al*., 2006), including in maintenance of vernalization-mediated mitotically stable repression of *FLC*, a main floral repressor in Arabidopsis (Mylne *et al*., 2006; Sung *et al*., 2006). In addition, ectopic expression of the floral integrator *FT*, as well as altered indole glucosinolate levels has been reported in *tfl2* mutants (Ludwig-Muller *et al*., 1999; Kotake *et al*., 2003).

Map-based cloning

In the pre-genome era

Using forward genetics, you start out with a mutant showing an interesting phenotype. The mutant can be a naturally occurring variant, or induced by chemical treatment or irradiation. To isolate the gene represented by this mutant, a map based cloning technique (also known as positional cloning or chromosome walking) must be used. The initial step in this process is to position the gene as accurately as possible to a small area of the genome, which is accomplished by the use of markers. Classical (i.e. morphological or visible) markers can be used in the initial crude mapping. However, molecular markers are most often preferred in the continued mapping process; extensive maps of molecular markers are available for Arabidopsis. The most commonly used markers include restriction fragment length polymorphism (RFLP) markers (e.g. Chang *et al*., 1988; Liu *et al*., 1996), and PCR-based markers, such as simple sequence length polymorphism (SSLP) markers (Bell and Ecker, 1994) and cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993; Baumbusch *et al*., 2001). The PCR-based markers are obviously the primary choice since these do not require DNA blot hybridization, but can be visualized through electrophoresis and ethidium bromide staining.

To use molecular markers a mapping population is created, by crossing the mutant plant with a plant of a different ecotype. The resulting individual F2 plants, or F3 progeny of these plants, can then be scored for the segregation of the mutation relative to the markers detecting polymorphisms in DNA sequence, between the two ecotypes used. An important factor affecting the resolution of the mapping is the size of the mapping population. For further fine mapping, additional mapping populations enriched in plants with recombination events close to the mutated locus can be made, by crosses to mutants in a different ecotype, representing genes positioned close to the gene of interest. Genetic distances between loci are measured as the probability that a recombination event takes place between them, during meiosis. A genetic distance of 1 % recombination corresponds to an average physical distance of about 250 kb in Arabidopsis (Lukowitz *et al*., 2000).

Following high-resolution mapping, the closest marker detected on each side can be used to screen libraries of yeast artificial chromosomes (YAC), containing inserts of up to 500 kb Arabidopsis genomic DNA, and/or libraries containing shorter DNA inserts, such as bacterial artificial chromosome (BAC), Transformation-competent Artificial Chromosome (TAC), and cosmid libraries. The ends of the identified clones can be isolated by plasmid rescue and inverse PCR, and used to build up a contig of the clones, as well as used as new markers in the cloning process. Once the locus of interest has been located to one specific clone, the last step in the cloning process is to identify the ORF corresponding to the mutant locus. This can be done by subcloning the DNA into an *Agrobacterium tumefaciens* transformation vector and transforming the mutant with these subclones. A complementation of the mutant phenotype reveals the subclone harboring the gene of interest. The final identification of the gene is carried out by sequencing the wt and mutant alleles available, with primers for the potential ORFs identified, and differences in sequence scored.

In the post-genome era

Even before the sequencing of the Arabidopsis genome, many markers, maps, and libraries were available, easing the cloning process. In the post-genome area however, the cloning process can be greatly simplified, and carried out in less than a year. Since both Columbia and Landsberg ecotypes of Arabidopsis has been sequenced, a large collection of predicted Arabidopsis single-nucleotide polymorphisms and small insertions/deletions are publicly available through Monsanto (Jander *et al*., 2002), speeding up the fine-scale mapping. If large mapping populations are created, the availability of such tremendous number of genetic markers make the use of YAC and BAC libraries obsolete. When the gene of interest has been mapped to a small region, the sequence-based map of genes with mutant phenotypes (Meinke *et al*., 2003) can be searched, to confirm that the gene has not already been identified by a mutant. Finally, since binary-BAC transformation libraries are now available, these can be used for complementation tests or, as predicted genes are annotated, directly sequencing suitable candidates and comparing them to their published wt sequence to directly identify the mutated gene.

Results and discussion

Phenotypic characterization of the *tfl2* mutant (I)

We have analyzed two mutant alleles, *tfl2-1* and *tfl2-2* (Figure 3A). Both are inherited as nuclear recessive mutations, and in all characters investigated the mutant phenotypes are identical. The *tfl2* phenotype is pleiotropic, including a dwarfed shoot, a terminating inflorescence meristem, and early flowering (Figure 3B-D), indicating that the *TFL2* gene is involved in several aspects of plant development.

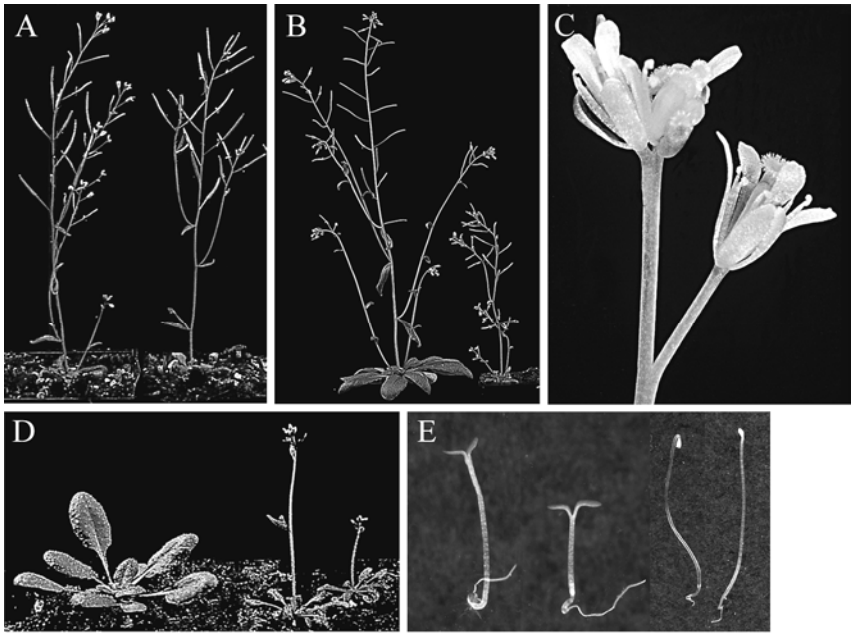

Figure 3. Phenotype of the *tfl2* mutant. **A**, *tfl2-1* and *tfl2-2* **B**, Col (wt) and *tfl2-1* grown for 35 days in LD **C**, terminal flower of *tfl2-1* **D**, Col and *tfl2-1* grown for 18 days in LD **E**, Col and *tfl2-1* seedlings grown for three days in continuous red light (left) and in darkness (right)

The overall size of the shoot is strongly reduced by the *tfl2* mutation, yet the proportions of the shoot architecture are not affected. The roots have not been extensively characterized, however no phenotypic deviation has been detected between *tfl2* and wt comparing primary root length or number of lateral roots. The reduced shoot size is reflected at the cellular level by smaller and fewer cells indicating a defect in cell expansion and division. The shape of the cells is indistinguishable from wt comparing adaxial and abaxial cells of the fifth rosette leaves, nor is patterning of tricomes affected. Hence, cellular organization in, and organogenesis from the vegetative meristem in *tfl2* do not deviate from wt. The vegetative meristem itself was examined by light microscopy, and no visual differences in cell number or cellular organization were detected. The normally indeterminate Arabidopsis inflorescence is terminated in the *tfl2* mutant after producing about 20 secondary meristems, causing an early release of axillary shoots and a rather bushy appearance of the plants. This indicates that the inflorescence meristem identity is lost, and thereby the meristem is consumed while forming an apical floral structure. The terminal flower produced exhibits floral defects of varying severity, for instance carpelloid organs and organs with mixed identities often develop. However, besides the terminal flower, no defects in flower formation or organization have been found.

Arabidopsis is a facultative LD plant, flowering earlier during LD than during SD conditions. *tfl2* initiates flowering after a shorter vegetative period as compared to wt in LD conditions, and considerably earlier than wt during SD. Flowering early in both LD and SD suggests an involvement of TFL2 in the autonomous pathway leading to the onset of flowering. The inhibiting effect of SD is severely reduced in *tfl2*, indicating a malfunction in the sensing of time-period as well, possibly pointing at multiple functions during the floral transition.

Cloning and analysis of the *TFL2* gene (I, II)

The *tfl2-1* and *tfl2-2* alleles originate from fast-neutron and EMS mutagenesis, respectively, hence the mutations had to be identified using a positional cloning strategy. Through genetic linkage to *terminal flower1* we were able to position *tfl2* to the upper arm of chromosome 5. Using PCR based markers and a contig that we created of yeast artificial chromosome clones and bacterial artificial chromosome clones, a small region was delineated containing nine putative open reading frames (ORF). The ORFs were PCR amplified using DNA from the two *tfl2* alleles and wt as templates. A large deletion eliminating two ORFs was detected in *tfl2-2*, and a single base pair substitution in one of these ORFs was found when sequencing the PCR products from the *tfl2-1* allele, pointing to this ORF as the putative *TFL2* gene. Complementation analysis was carried out transforming homozygous

tfl2-1 plants with a fragment containing the candidate ORF. All characters of the *tfl2* phenotype were rescued by this construct, resulting in plants indistinguishable from wt, confirming that this ORF is the *TFL2* gene. The phenotypes of the two *tfl2* alleles are indistinguishable in all characters analyzed, leading to two conclusions. First, the second ORF deleted in the *tfl2-2* allele does not contribute to the phenotype. Second, both mutants most probably represent null alleles, since in *tfl2-2*, the entire *TFL2* gene is deleted. Sequencing of the *TFL2* gene confirmed the sequence published by the Arabidopsis Genome Initiative (www.arabidopsis.org), by Gaudin *et al*. (2001) and Kotake *et al*. (2003).

TFL2 encodes a putative protein consisting of 445 amino acids, with several conserved domains. A CD is found in the N-terminal part, a CSD is situated in the C-terminal, and several nuclear localization signals are present. All these elements indicate a role for the TFL2 protein in gene regulation. The CD is highly conserved, and as the amino acids shown to be important for CD function are conserved in TFL2 as well, it is likely that TFL2 binds chromatin in a similar manner as animal HP1 proteins. The animal CSD mediates the formation of a tight HP1 homodimer *in vitro*, resulting in the formation of a hydrophobic pocket suitable for protein-protein interactions, and indeed many such interacting proteins have been identified. The TFL2 CSD is less conserved; especially the N-terminal part is highly different. However, this does not seem to affect homodimerization, since we have detected homodimerization of TFL2, using the yeast two hybrid system (unpublished results), and similar results have also been reported by Gaudin *et al*. (2001) and Yu *et al*. (2004). However, the differences could still affect the range of protein partners eligible for binding. The hinge region between the CD and the CSD that has been assigned functions in protein interactions, and in post-translational modifications of the HP1 protein, is much longer in TFL2 than in the animal homologs, and the level of conservation is low, meaning that this part of the protein could contribute to differences between animal and plant HP1 protein function.

Based on database searches we conclude that only one *HP1* gene exists in Arabidopsis. The same is true for fission yeast, while Drosophila and mammals possess up to five HP1 encoding genes. Interestingly, mutations in *HP1* genes in Drosophila are embryo lethal, while *HP1* mutations in yeast and plants are not, again pointing to partly divergent functions. Summarizing, *TFL2* encodes a HETEROCHROMATIN PROTEIN1 homolog, is possibly nuclear localized, and judging from the known function of HP1 in other organisms, might be involved in several aspects of plant development through gene repression as part of chromatin bound protein complexes.

Expression analysis of the *TFL2* gene (II)

The expression pattern of the *TFL2* gene in wt plants was examined by reverse transcriptase PCR (RT-PCR) and *in situ* RNA hybridization. *TFL2* transcripts were detected in all tissues examined, including seedlings, leaves, inflorescences and siliques. In vegetative apices mRNA levels were high in the meristem, leaf primordia and in young leaves, tissues with high cell division rates and expanding cells. Similarly, *TFL2* transcript levels were high in inflorescence and floral meristems of the reproductive shoot apex. The pattern of expression was homogenous throughout the meristems. In developing floral buds high *TFL2* expression was detected in early stages of all floral organ primordia, and in ovules within the pistil. Considering the high expression in developing flowers it is interesting to note that no deviation in flower phenotype is seen comparing *tfl2* and wt. We detected a general expression in all tissues examined, suggesting that, if *TFL2* is regulated, this probably occurs post-transcriptionally. In animals extensive post-translational regulation of HP1 has been detected (Lomberk *et al*., 2006), and it is possible that the same is true for TFL2. Further, based on homology to animal HP1, a high degree of regulation is likely at the level of protein interaction partners, mediating the binding to DNA.

TFL2 affects seedling photomorphogenesis (III)

The early flowering and reduced sensitivity to daylength exhibited by *tfl2* implicate a participation of TFL2 in light signaling. To address this aspect of TFL2 function we analyzed hypocotyl elongation in response to growth in different intensities of monochromatic red, far-red and blue light. While the fluence rate response curve of *tfl2* did not deviate from that of wt in blue light, the hypocotyl of *tfl2* was significantly shorter in red and far-red light. Under far-red light *tfl2* exhibited a shorter hypocotyl than wt in low light intensities, but was indistinguishable from wt in high light intensities. In red light the hypocotyls of *tfl2* plants were shorter in all intensities tested (Figure 3E), and reached saturation already in low red light intensities. Hypocotyls of light grown seedlings were measured three days after germination. When grown in darkness for the same amount of time, the hypocotyls of *tfl2* were as long as wt hypocotyls (Figure 3E), showing that the inhibition of hypocotyl elongation in *tfl2* is a light-induced developmental process.

The hypersensitivity to inhibition of hypocotyl elongation by red and far-red light exhibited by *tfl2* prompted us to further investigate the interaction between TFL2 and the phytochromes, the photoreceptors for red and far-red light. By double mutant analysis we concluded that the *tfl2* mutant seedling phenotype is dependent on PHYB and PHYA in red and far-red light, respectively. The *tfl2phyB* mutant is indistinguishable from *phyB* after growth

in red light for three days, and in far-red light the *tfl2phyA* mutant hypocotyl length does not differ from that of the *phyA* single mutant. The shade avoidance response, evident as elongation of the hypocotyl in response to a decrease in the ratio of red/far-red light, is mainly controlled by PHYB. Next, we analyzed the shade avoidance response of the *tfl2* mutant. While the hypocotyls of wt plants elongate twice as much when white light is supplemented with far-red light, the elongation of *tfl2* hypocotyls is only slightly increased. These results clearly show that the *tfl2* seedlings exhibit a reduced responsiveness to the change in light quality, indicating a function of TFL2 in PHYB mediated light signaling.

To examine the molecular mechanism underlying the defects in light responses observed in *tfl2* seedlings, we analyzed the relative expression levels of several known marker genes, representing different light regulated processes, by real-time RT-PCR. None of these genes, with one exception, displayed an altered expression in the *tfl2* seedling as compared to wt, either in darkness, after 24 hours, or after three days in red light. In addition, the expression of *TFL2* in wt seedlings was not affected by any of the light conditions tested. However, the expression of *PORA*, encoding an enzyme involved in the early steps of chlorophyll biosynthesis, was 70% lower in *tfl2* as compared to wt when the seedlings were grown in darkness. Following light exposure, the *PORA* expression did not differ between *tfl2* and wt.

In order to investigate whether the reduced *PORA* level in dark grown *tfl2* plants was reflected by reduced biosynthesis of chlorophyll when transferred into light conditions, we measured chlorophyll content of seedlings grown for three days in continuous white or red light, and in seedlings grown in darkness for three days and then transferred to red light for 24 hours. The *tfl2-2* seedlings contained about 30% less chlorophyll, as compared to wt, after 24 hours of red light treatment. When grown in light for three days the chlorophyll levels did not differ between *tfl2-2* and wt. We interpret this as a defect during early chloroplast development in the *tfl2* mutant. A similar defect is evident during de-etiolation in *pif3*, a mutant known to act downstream of phytochrome (Monte *et al*., 2004). It was shown by microarray analysis that the delayed greening of *pif3* is caused by extensive alterations of chlorophyll biogenesis genes. *tfl2* shares additional phenotypic traits with *pif3*, e.g. they are both hypersensitive to red light when it comes to hypocotyl elongation, but neither of them exhibit significant differences in cotyledon size. This further points to a role for *tfl2* in phytochrome mediated light signaling.

COP1 and DET1 are repressors of photomorphogenesis, and to determine a possible relationship with TFL2 we analyzed the *tfl2-1cop1-6* and *tfl2-1det1-1* seedling phenotypes, respectively. In darkness both *cop1* and *det1* exhibit a phenotype resembling a light grown seedling, including a short hypocotyl and unfolded cotyledons. In red light the hypocotyls of *cop1* and *det1* are short, even shorter than *tfl2* hypocotyls. The *tfl2-1cop1-1* and

tfl2-1det1-1 double mutant seedlings displayed a dark-grown phenotype indistinguishable from the *det1* and *cop1* single mutants. The same was true in red light, where the double mutant hypocotyls were as short as the *cop1* and *det1* hypocotyls, showing that both *cop1* and *det1* are epistatic to *tfl2-1* during seedling photomorphogenesis. Additionally, seed germination was severely affected in the *tfl2-1det1-1* double mutant; less than 10% of the seeds germinated, a defect not exhibited by the single mutants. Further, we have noticed that the apical hook of *tfl2* dark grown seedlings is released significantly earlier than the wt hook, indicating that TFL2 is required to repress some aspects of photomorphogenesis in the absence of light. DET1 is suggested to repress gene expression in darkness by interacting with non-acetylated histone H2B. It is possible that TFL2 possesses a similar function in repressing gene transcription during seedling development by binding to methylated histones, together with DET1 or as part of a similar repressive complex.

Roles of TFL2 during the transition to flowering (II)

tfl2 is early flowering in both LD and SD conditions, though the flowering is delayed less than twofold under SD conditions as compared to LD conditions, while wt is delayed three- to fivefold. This suggests that both the light sensing pathway as well as the autonomous pathway might be affected. Ectopic expression of the floral integrator *FT* in the *tfl2* mutant has been reported (Kotake *et al*., 2003), suggesting a function for TFL2 in regulation of *FT*. However, *FT* is a floral integrator, regulated by several of the floral induction pathways. To analyze whether TFL2 exerts its function on *FT* through a specific pathway, we crossed *tfl2* to mutants representing the LD and autonomous pathways, respectively.

No evidence for *TFL2* in the LD pathway

tfl2-1 was crossed with the *elf3-1* and *gi-2* mutants respectively, representing genes acting in the LD pathway. *elf3* is early flowering, photoperiod insensitive and lacks circadian rhythms. *gi* as well is impaired in circadian rhythm and is late flowering mainly under LD conditions. The *tfl2-1elf3-1* double mutant flowered earlier than either of the single mutants during both LD and SD conditions. Further, the double mutant inflorescence meristem was terminated after producing fewer lateral meristems than the *tfl2-1* single mutant. This indicates that *ELF3* might function in regulating meristem identity, although the *elf3* mutant inflorescence meristem is indeterminate. The *gi-1tfl2-1* plants exhibited an intermediate phenotype under LD conditions, flowering almost at the same time as wt plants. Adding the *gi* mutation to the *tfl2* mutant had only minor effects on the flowering time under SD, the dou-

ble mutant flowering only slightly later than the *tfl2* single mutant, probably due to *GI* being involved in promoting flowering mainly during LD conditions. The enhanced phenotype of *tfl2elf3* and the intermediate flowering time phenotype exhibited by *tfl2gi* shows that the single mutant phenotypes are additive. Hence, *TFL2* is probably not involved in the control of flowering time through *GI* or *ELF3*. Downstream of these genes is *CO*. Because of difficulties in generating double mutants, due to the fact that *TFL2* and *CO* are closely linked, we have only investigated the relationship to *CO* at the RNA level. Contrary to Gaudin *et al* (2001), who detected high *CO* expression levels at the two leaves stage in *tfl2*, but in line with the results of Kotake *et al* (2003), we did not find any alterations in *CO* expression levels comparing *tfl2* with wt, either in one, two, or three week old plants (data not shown).

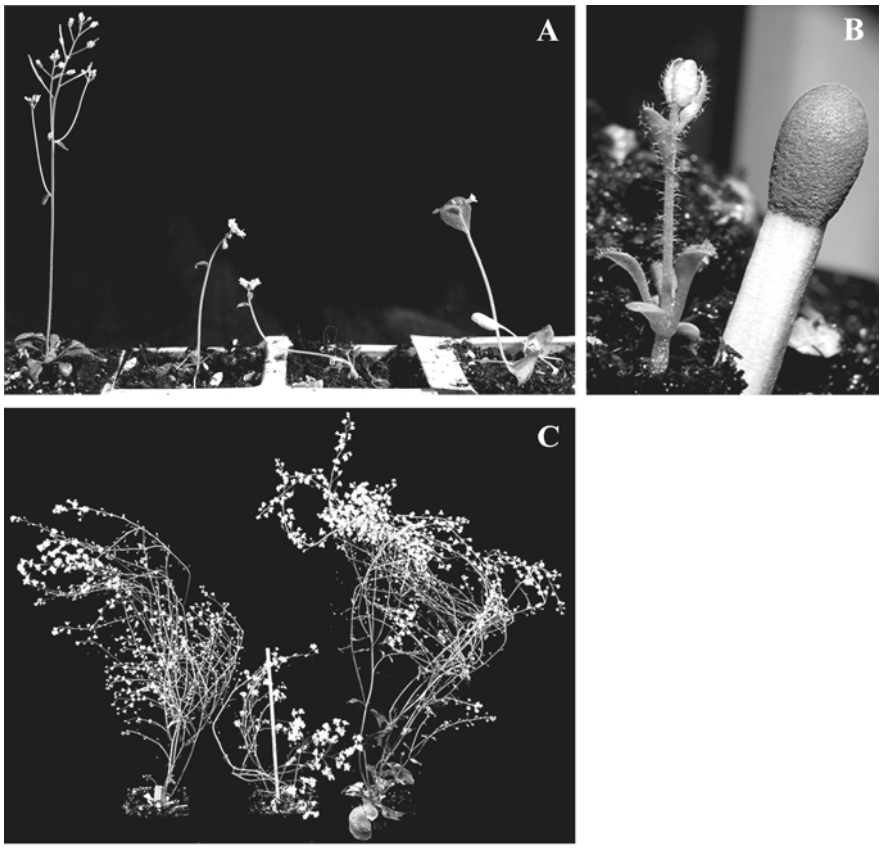

Figure 4. Impact of *phyB-5*, 35S::*AP1* and *ag-1* on *tfl2-1* morphology. A, *tfl2-1*, two *tfl2-1phyB-5* double mutants, *phyB-5*. B, 35S::*AP1 tfl2-1* double homozygous plant. C, Three examples of *tfl2-1ag-1* plants.

Our results on seedling photomorphogenesis indicate that *TFL2* is involved in *PHYB* regulated processes. We measured time to flowering of *tfl2-1phyB-5* plants under LD conditions. Interestingly, the double mutant plants flowered after producing the same number of rosette leaves as the *phyB* single mutants (Figure 4A and data not shown), suggesting that *phyB* is epistatic to *tfl2* in flowering time as well as in the hypocotyl response to red light. Cerdan and Chory (2003) suggest the existence of a light quality pathway regulating flowering time, in which *PHYB*, through *PHYTOCHROME AND FLOWERING TIME1* (*PFT1*), can trigger flowering by directly upregulating *FT*, and it is possible that *TFL2* is acting in this pathway.

TFL2 is a regulator of the autonomous pathway

We investigated crosses between *tfl2-1* and three of the mutants representing the autonomous pathway, *fca-1*, *fve-1* and *ld-1*, which are all late flowering in both LD and SD conditions. When combining *tfl2* with either *fve-1* or *ld-1*, the resulting double mutant plants displayed an intermediate phenotype, flowering almost at the same time as wt plants, both when grown in LD and in SD. Thus, the single mutant phenotypes are additive, revealing no interaction between the corresponding genes. However, the *tfl2-1fca-1* double mutant flowered as late as the *fca-1* single mutant under LD conditions, and in SD a few days earlier than *fca-1* but significantly later than wt. This epistatic relationship indicates that TFL2 has a function in the FCA part of the autonomous pathway to floral induction.

To further investigate this interaction, we analyzed the relative expression level of *FLC*, *CO*, *FT* and *SOC1* in single and double mutant plants grown for two weeks in LD conditions, using real time RT-PCR (se Table 2 in Paper II). We did not detect any significant difference in expression levels of *CO* or *SOC1*, comparing wt, *tfl2-1*, *fca-1* and *tfl2-1fca-1*. The expression of *FT* was slightly lower in *fca-1* mutants, but more than 10 times higher than wt in *tfl2-1*. In *tfl2-1fca-1* plants the high expression of *FT* found in *tfl2-1* was strongly reduced, indicating that high *FT* expression is at least partly dependent on *FCA*.

The expression of *FLC* in *tfl2-1* did not differ from wt while in *fca-1* it was about three times as high compared to wt. Interestingly, in the *tfl2-1fca-1* double mutant the expression of *FLC* was extremly high, more than five times higher than in the *fca-1* single mutant. This suggests that, although the *tfl2-1* single mutant does not exhibit an altered *FLC* expression, TFL2 can affect the expression of *FLC*. Recently this was shown to be the case during vernalization where an extended cold period mediates downregulation of *FLC* expression through histone modifications, including histone H3 deacetylation and increased levels of H3K9 dimethylation. In a vernalization responsive genetic background where the *tfl2* mutant is introduced, the expression levels of *FLC* still decline in response to cold, as they

do in wt. However, when returned to warm conditions *FLC* mRNA increase again in a *tfl2* background, in contrast to wt where *FLC* mRNA levels remain low, showing that *TFL2* is required for maintenance of the repressed state of FLC (Mylne *et al*., 2006; Sung *et al*., 2006). Further, TFL2 might also act directly on the *FT* locus, and thus directly regulate the expression of *FT* (Germann *et al*., 2006). What interaction partners are required for this binding to occur is not clear. In summary, my and others results support that TFL2 regulates time to flowering through the repressive action on more than one locus, within several of the pathways leading to flowering.

TFL2 regulates meristem identity (I)

The *tfl2* mutant inflorescence terminates in a floral structure, showing that the normally indeterminate fate is lost, indicating a role for *TFL2* in maintenance of inflorescence meristem identity. We have analyzed crosses between *tfl2* and mutants representing the meristem identity genes *LFY*, *AP1* and *TFL1*. TFL1 encodes a protein closely related to FT, however since *tfl1* is early flowering and *ft* is late flowering, it is suggested that they have antagonistic functions on promoters of floral genes through interaction with proteins such as FD (Ahn *et al*., 2006). The *tfl2-2* allele was isolated as an enhancer of *tfl1*, which in addition to early flowering in both LD and SD also exhibit a terminating inflorescence meristem. Both these characters were enhanced when adding the *tfl2* mutation, revealing additive functions and no clear interaction between TFL1 and TFL2 in preventing premature flowering. During flowering time regulation both genes have been shown to work on the level of *FT*, however probably through different interaction partners and mechanisms.

LFY and *AP1* define floral meristems and are repressed in the inflorescence meristem; in plants overexpressing either *LFY* or *AP1* a floral structure terminating the inflorescence is produced (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). When combining *lfy* and *tfl2* a completely additive phenotype was seen, including early flowering, a terminating apical meristem and dwarfing typical of *tfl2*, and a higher number of lateral branches as well as the leafy flowers characteristic of the *lfy* mutant. Thus, removing *lfy* expression is not enough to restore inflorescence meristem identity in the *tfl2* mutant. The same is true for *tfl2ap1* double mutants, where the inflorescence meristem still terminated. However in *tfl2ap1* plants, a novel character was seen. Except for a lack of petals, in about 30% of *ap1* flowers secondary flowers develop in the axil of first whorl organs. The production of secondary flowers was strongly suppressed in the *tfl2ap1* double mutant, indicating an antagonistic function between *tfl2* and *ap1* in meristem identity, and suggesting a function of *tfl2* in the floral meristems in line with the *in situ* expression analysis showing expression of *TFL2* mRNA through-

out early stages of the floral bud. The lack of suppression of termination of the inflorescence meristem seen in both *tfl2lfy* and *tfl2ap1* can be due to ectopic expression of additional flower meristem identity genes in the *tfl2* mutant. To further examine the possible interaction of *TFL2* with *AP1*, we crossed *tfl2* to a weak overexpressor of *AP1* (35S::*AP1*) (Liljegren *et al*., 1999). The 35S::*AP1* plants are early flowering, and exhibit terminating inflorescences. Combined with *tfl2* the resulting plants showed an enhanced phenotype, entering reproductive phase after producing three rosette leaves and terminating the inflorescence after producing on average only one secondary meristem (Figure 4B). This phenotype resembles that of strong *AP1* overexpressing plants (Liljegren *et al*., 1999), suggesting that loss of *TFL2* causes elevated ectopic *AP1* expression, consistent with a hypothesis where *TFL2* is a regulator of the *AP1* part of flowering time control. Corroborating this, we have by GUS staining and *in situ* experiments found an ectopic expression of *AP1* in the *tfl2* mutant (data not shown).

The *AGAMOUS* (*AG*) gene is required for termination of the floral meristem and when mutated indeterminate flowers are formed, while when overexpressed precocious flowering and a terminating inflorescence is seen (Mizukami and Ma, 1997). Combining *tfl2* with *ag*, the *tfl2* phenotype was partially rescued; plants flowered later than *tfl2* single mutants and the inflorescence terminated after producing 20 to 70 flowers (Figure 4C). It is possible that *AG* is one of the targets for the repressive action of *TFL2* in the inflorescence meristem. This is supported by *in situ* hybridizations where we have detected ectopic expression of *AG* in the *tfl2* mutant (data not shown), and results by Kotake *et al*. (2003) who found elevated levels of *AG*, *PI*, *AP3* and *SEP3* in *tfl2* mutants by DNA microarray experiments.

TFL2 interacts with IAA5 through the chromo shadow domain (IV)

In a yeast two hybrid experiment, using full length TFL2 as well as a partial TFL2 protein consisting of the chromo shadow domain, we isolated the early auxin response protein IAA5 as an interaction partner to TFL2. The truncated protein, consisting of only the chromo shadow domain, was sufficient for the IAA5 interaction. To further investigate the possible interaction between TFL2 and IAA5, we crossed the corresponding mutants. IAA5 loss of function mutants exhibited no detectable phenotype. Since IAA5 is a member of the large AUX/IAA protein family, lack of mutant phenotype is probably due to genetic redundancy (Overvoorde *et al*., 2005). In many characters, the *tfl2iaa5* mutant was indistinguishable from the *tfl2-1* single mutant. Although the *tfl2iaa5* inflorescence was terminated after producing the same number of lateral meristems as *tfl2*, the height of the double mutant

plant was further reduced. In addition, an early release of a higher number of axillary inflorescences was detected in the double mutant, not seen in either single mutant or wt. The reduced apical dominance of the double mutant is a novel auxin-related trait that indicates an interaction between the two genes.

In animals, a large number of HP1 interacting proteins have been identified. In plants, so far only a few proteins have been isolated, including methylated histone H3, CHROMOMETHYLASE3, and HP1 homodimerization. We can now likely add IAA5 to this list, as a protein interacting with the CSD of TFL2. Recently, evidence for AUX/IAA mediated epigenetic gene silencing was reported, when the chromatin remodeling factor PICKLE was shown to be required for IAA14 mediated repression of ARF7 and ARF19 (Fukaki *et al*., 2006). In view of the possible interaction between TFL2 and IAA5 in plants, an interesting HP1 interacting protein complex in humans is the KRAB/KAP1 repression system. Here KAP-1, a corepressor of KRAB domain containing zinc-finger proteins that bind specific gene promotors within euchromatin, coordinates both histone H3 methylation and HP1 binding, resulting in a denser chromatin structure, repressing transcription. Involvement of TFL2 in maintenance of repression through methylated histone H3 has been reported in the vernalization response, and TFL2 could execute a similar function in auxin regulation, maintaining a repressed state of promotor regions of a sub-set of IAA induced genes. It is not yet known which ARFs are bound by IAA5.

Auxin homeostasis is affected by TFL2 (IV)

The possible interaction between TFL2 and IAA5 led us to further characterize the auxin status of the *tfl2* mutant. Auxin levels and auxin distribution was analyzed in *tfl2* and wt plants harboring the DR5::GUS construct. DR5 is a synthetic auxin response element coupled to a minimal promoter-ß-glucuronidase (GUS) reporter gene (Ulmasov *et al*., 1997b). We did not detect any visible differences in distribution or intensity of GUS activity (blue staining) at any time point, analyzing the roots of *tfl2* and wt. At the seedling stage, GUS staining was analyzed after growth in darkness or red light for three to five days, but no differences in staining pattern between *tfl2* and wt were found. This was the case also in plants grown for seven days in white light. However, after two and three weeks of growth in continuous white light, the GUS staining in the *tfl2* rosette was strongly reduced as compared to wt. The GUS activity in *tfl2* rosettes was strongly decreased at the point in time where floral transition occurs in *tfl2* mutants. In wt plants, flowering was induced after three weeks, and GUS staining was still high in wt rosettes at this stage, showing that the level of free auxin detected by GUS staining does not influence time to flowering. When *tfl2* and wt plants were exogenously supplied with auxin, the staining pattern was the same for

both genotypes, indicating that *tfl2* is still able to respond to auxin. When grown on the polar auxin transport inhibitor NPA, a low level of staining was visible in the rosettes of two weeks old *tfl2* plants, although GUS activity was still much higher in wt plants. This indicates that a certain amount of auxin is present in the *tfl2* rosette and that the lack of GUS staining in the rosette of *tfl2* after two weeks is not due to auxin transport away from the leaves.

To confirm the differences in free auxin levels we analyzed the absolute levels of free IAA in root and shoot samples from one and two weeks old plants. Consistent with the DR5::GUS results, we did not detect statistically significant differences in auxin levels in the roots comparing *tfl2* with wt plants. However, when measuring IAA in the shoots, a significant difference was found between *tfl2* and wt already in the samples collected after seven days of growth, and in 14 days old tissue the IAA levels were much lower in *tfl2* as compared to wt. Since the level of free IAA is the outcome of synthesis, transport, conjugation and degradation we cannot simply conclude that the auxin synthesis is low in the *tfl2* mutant, but since the NPA treatment did not greatly change the GUS activity pattern in *tfl2*, we can probably rule out the possibility that the polar auxin transport system is defective. Our preliminary data, measuring the incorporation of fed deuterium, points at a lower rate of IAA synthesis in the shoot of the *tfl2* mutant as compared to wt (data not shown). Supporting low auxin levels in the *tfl2* mutant, *TU8* has been shown to be allelic to *TFL2*. The *tu8* mutant was isolated due to developmentally altered levels of leaf glucosinolates. The synthesis of glucosinolates is related to the synthesis of IAA, and altered IAA levels have been reported for *tu8* (Ludwig-Muller *et al*., 1999).

Expression of auxin regulated genes in *tfl2* (III, IV)

To analyze to what extent the reduced levels of IAA in *tfl2* is reflected on the gene expression level, we measured RNA levels of genes representing the *GH3*, *AUX/IAA* and *SAUR* families, known to be auxin regulated, using real time RT-PCR. RNA samples were extracted from seedlings grown for three days in darkness, red and white light, and from seven and 14 days old *tfl2* and wt plants. We did not detect differences in expression levels of the GH3 genes *DFL1* and *DFL2* comparing *tfl2* and wt. The *SAUR-AC1* gene was expressed at lower levels in *tfl2*, at all time points tested. Nothing is known about the function of *SAUR-AC1* in plants, but the expression is induced after only a few minutes of altered auxin levels. Of the *AUX/IAA* genes we analyzed *IAA5* and the closely related *IAA6* and *IAA19*, as well as the distantly related *IAA12*. At the seedling stage the expression of *IAA19* was high in dark grown plants, but is strongly reduced upon light treatment. In the *tfl2* mutant the expression of *IAA19* differed from wt only in dark grown plants,

where the expression levels were lower in *tfl2*. The expression of *IAA5* was transiently induced in wt seedlings after one hour of red light, and this peak was absent in *tfl2*. The *SAUR-AC1*, *IAA5* and *IAA19* genes are categorized as early red light responsive genes in a global microarray analysis (Tepperman *et al*., 2006). Our results showing an altered response of these genes in *tfl2* thus corroborate our hypothesis of a function of *TFL2* in seedling deetiolation as part of a phytochrome signal transduction pathway.

In seven and 14 day old plants grown in white light the expression of *IAA6* and *IAA19* was much lower in *tfl2* as compare to wt, *IAA5* slightly lower, while the expression of *IAA12* did not differ between *tfl2* and wt. When dividing the tissue into root and shoot, we found that the genes showing the largest differences in expression level between *tfl2* and wt, *IAA6*, *IAA19* and *SAUR-AC1,* were highly expressed specifically in the leaves of wt plants, in contrast to *tfl2* which expresses these genes at a low level also in the leaves. Thus, the reduced expression levels of a subset of auxin regulated genes, with a high expression in leaves, correlates well with the low levels of free IAA detected in *tfl2* leaves.

tfl2 mutants display a reduced sensitivity to supplied auxin (IV)

The *tfl2* phenotypic traits, such as the reduced size of the *tfl2* mutant, a short hypocotyl in light and curled, epinastic leaves, are all characters known to be influenced by auxin levels and/or response. Other well-characterized auxin related phenotypic traits include root length and lateral root initiation, gravitropic growth responses and vascular patterning. We analyzed these aspects of the *tfl2* phenotype without detecting any deviations from wt.

Additionally, we analyzed the response to exogenously supplied auxin. Two simple tests of auxin response are length of the hypocotyl, and primary root length when grown on different concentrations of auxin. *tfl2* seedlings exhibited the same response pattern as wt seedlings in both tests, except for high IAA levels where *tfl2* was less affected than wt, indicating a reduced auxin sensitivity of *tfl2*. We analyzed the expression pattern of a set of auxin-induced genes after auxin treatment, and the expression of all tested genes was induced following auxin treatment in both *tfl2* and wt plants. However, the expression of *IAA5*, *IAA6* and *SAUR-AC1* did not increase to the same extent in *tfl2* as compared to wt, further supporting the notion that the auxin response is affected in the *tfl2* mutant.

Svensk sammanfattning

Detta projekt startade med en mutant av modellorganismen *Arabidopsis thaliana*, eller backtrav på svenska. Backtrav är liten, lättodlad, har kort generationscykel och god frösättning. Dessutom har den ett litet genom med stor andel kodande DNA, är diploid samt kan transformeras med främmande DNA med hjälp av en växtpatogen. Backtrav har därför använts världen över för molekylärbiologiska studier, och var den första växt vars hela genom sekvenserades, år 2000.

Vår mutant uppvisar flera intressanta karaktärer, varav en gav den dess namn; *terminal flower2* (*tfl2*). För att förstå betydelsen av dessa följer en kort sammanfattning av huvuddragen i växters utveckling. Större delen av växters organbildning sker efter embryoutvecklingen, då fröet grott, till skillnad från hos djuren där alla organ bildas innan födseln. I tillväxtzoner, s.k. meristem, sker all celldelning och härifrån bildas alla nya organ såsom rötter, blad och ståndare. De primära meristemen är apikala d.v.s. finns i toppen av skottet respektive roten. Det är mycket viktigt att meristemcellerna inte konsumeras; poolen av odifferentierade celler hålls konstant för att en kontinuerlig organbildning ska kunna ske. Meristemet är därför starkt reglerat på gen- och proteinnivå. Från skottmeristemet bildas stam, nya sidoskott och blad under den vegetativa fasen av livscykeln. Då de yttre och inre livsbetingelserna är optimala för reproduktion går växten in i blomningsfas. Backtrav är en fakultativ långdagsväxt, vilket betyder att den går in i reproduktiv fas betydligt tidigare då dagarna är långa än då de är korta. Toppmeristemet övergår till att bilda blomställnings- och blommeristem på flankerna, vilka i sin tur ger upphov till de olika blomorganen; foderblad, kronblad, ståndare och karpeller. Cellerna i blommeristemet konsumeras därmed och blomman terminerar i och med bildandet av karpellerna som växer ihop till en pistill. Under den vegetativa fasen av livscykeln bildar backtrav en rosett av blad som en konsekvens av att ingen sträckning (elongering) av cellerna i stammen sker. Vid övergången till reproduktiv fas börjar dock stammen elongera och en blomställning bildas, vilken producerar sekundära blomställningar och blommor. Cellerna i blomställningens apikala meristem behåller sin odifferentierade identitet livscykeln ut.

tfl2-mutanten avviker från vildtyp på flera sätt (se Figure 3). Det apikala meristemet konsumeras, efter att ha producerat ungefär tjugo sidomeristem, i bildandet av en terminal blomma. *tfl2* blommar tidigare under långa dagar, och då dagarna är korta blommar den nästan lika tidigt som under långa da-

gar. Dessutom är mutanten dvärgväxt vilket avspeglas på cellnivå; *tfl2* har både färre och mindre celler.

Korsningsanalyser har visat att det förmodligen endast är en defekt gen som orsakar alla *tfl2*s avvikande karaktärer. Gener är de delar av DNAt, organismers arvsmassa, som via RNA kodar för proteiner. Proteinerna i sin tur kan fungera som byggstenar, enzymer eller regulatorer av processer i organismens livscykel. En grupp proteiner, s.k. transkriptionsfaktorer, reglerar avkodningen av DNAt, så att rätt proteiner bildas i rätt celler vid rätt tidpunkt. DNAt är dessutom packat till en tätare struktur, kromatin, också detta m.h.a. proteiner. Hur tätt packat DNAt är påverkar hur tillgängliga generna är för avkodning, och möjliggör också den precisa kopiering och uppdelning av genomet som sker vid celldelning.

Vi har isolerat genen som orsakar de avvikande karaktärerna hos *tfl2* m.h.a. en positionell kloningsteknik. Det visade sig att genen, *TFL2*, kodar för ett protein som uppvisar stora likheter med ett protein som redan isolerats från både jäst, bananfluga, mus och människa, HETEROCHROMATIN PROTEIN1 (HP1). I dessa organismer har man visat att HP1 har flera viktiga funktioner; dels har det en strukturell funktion vid bildandet av mycket tätt packat kromatin, sk heterokromatin, dels deltar det vid regleringen av uttrycket av kodande gener. HP1/TFL2 har inga DNA-bindande domäner, däremot två protein-interaktionsdomäner och deltar därför förmodligen vid genreglering som del av ett proteinkomplex. *TFL2*-genen är aktiv i många olika organ och utvecklingsstadier, vilket är i linje med att en mutation i den här genen påverkar flera av växtens karaktärer. Att *tfl2* uppvisar en minskad, men inte obefintlig, känslighet för dagslängd vid induktion av blomningsfas, indikerar att *TFL2* medverkar vid regleringen av blomningstid, dels genom att delta i tolkningen av ljussignalerna, dels genom att motverka igångsättandet av blomning utan påverkan av externa signaler. Genom att korsa *tfl2* med andra mutanter har vi styrkt hypotesen att *TFL2* är delaktig i regleringen av blomning vid icke-induktiva förhållanden, men inga uppenbara interaktioner med de gener som styr blominduktion som respons på långa dagar detekterades. Däremot har vi undersökt hur *tfl2* reagerar på olika våglängder av ljus vid groning, och då hittade vi indikationer på att *TFL2* deltar i ljusreglering, via en eller flera av receptorerna för rött ljus, fytokrom. Vidare har vi funnit att TFL2 reglerar två gener som ger blommeristemet dess identitet. Vi förmodar att TFL2 nedreglerar uttrycket av dessa gener i det apikala meristemet för att hindra att det terminerar.

I djur har man identifierat flera olika proteiner som interagerar med HP1. I växter har andra forskargrupper visat att TFL2 binder till ett metylas och till histon H3, ett protein som är en del av kromatin. Vi har påvisat en interaktion med ytterligare ett protein, IAA5, vilket är involverat i auxinsignalering. Auxin är ett växthormon som påverkar många av växtens funktioner. Vidare har vi mätt mängderna auxin i *tfl2* mutanten och det visade sig att nivåerna av fritt auxin sjunker i skottet med stigande ålder, vilket avviker

från hur det normalt förhåller sig. Vi drar slutsatsen att TFL2 deltar i regleringen av vissa gener som är involverade i syntes av auxin och/eller respons på auxin. De många avvikande karaktärerna som uppvisas av *tfl2*, och studierna av *TFL2*s funktion, visar att HP1 i växter kan reglera flera olika processer i växten, liksom det gör även i andra organismer. Däremot är inte mutationer i *TFL2* genen letala, vilket är fallet då HP1 muteras i bananfluga. Det visar att TFL2 troligen inte har en vital funktion som strukturell komponent av heterokromatin, vilket skiljer TFL2 hos växter från HP1 homologer hos djur.

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