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Meristem Maintenance in *Arabidopsis thaliana*

BY

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Abstract

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The shoot apical meristem (SAM) is the structure that shapes the aerial architecture of the plant, by producing lateral organs throughout development. In the model plant *Arabidopsis thaliana*, the SAM is always identifiable as a characteristic dome, whether it is found in the centre of a rosette of leaves or at the tip of an inflorescence. When senescence occurs and organogenesis ceases, the now inactive SAM still retains its characteristic appearance and it is never consumed into a terminal structure, such as a flower. Mutant plants that undergo termination represent a valuable tool to understand how the SAM structure and function are maintained during plant life.

The aim of this work was to investigate the dynamics of meristem development through morphological and genetic studies of three Arabidopsis mutants that exhibit distinct modes of SAM termination: *distorted architecture 1* (*dar1*), *adenosine kinase 1* (*adk1*) and *terminal flower 2* (*tfl2*). The *dar1* mutation is characterised by a severely distorted cellular architecture within the SAM. We propose that *dar1* affects the pattern of cell differentiation and/or cell proliferation within the SAM apical dome, resulting in termination by meristem consumption. Instead, the *adk1* mutation affects the organogenic potential of the SAM, without altering its structure. The *adk1* mutant has increased levels of cytokinins and, as a consequence of this, cell division is enhanced and cell differentiation is prevented in the apex, causing termination by meristem arrest. Finally, *tfl2* is mutated in the conserved chromatin remodelling factor HP1, a transcriptional repressor with multiple roles during plant development. The *tfl2* SAM terminates by conversion into a floral structure, due to de-repression of floral identity genes. Interestingly, *tfl2* mutants also show an altered response to light, an indication that TFL2 might act as a repressor also in the context of light signalling.

Keywords: Arabidopsis thaliana, development, shoot apical meristem

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C' e' una scuola grande come
il mondo...
Ci insegnano
maestri, professori.
Avvocati, muratori,
televisori, giornali,
cartelli stradali,
il sole
i temporali,
le stelle.
Ci sono
lezioni facili
e lezioni difficili,
brutte, belle
e così così.
Ci si impara a parlare, a giocare,
a dormire,
a svegliarsi,
a voler bene
e perfino
ad arrabbiarsi.
Ci sono esami tutti i momenti,
ma non ci sono ripetenti,
nessuno può fermarsi
a dieci anni,
a quindici, a venti,
a riposare
un pochino.
D'imparare non si finisce mai
e quel che non si sa
é sempre più importante
di quel che si sa già.
Questa scuola
è il mondo intero
quanto è grosso
apri gli occhi
e anche tu
sarai promosso.

G. Rodari

There is a school as big as
the world…
The teachers are
schoolmasters and professors.
Lawyers and carpenters,
television, newspapers
road signs,
the sun,
the storms,
the stars.
The lessons can be
easy,
difficult,
bad, good
and so and so.
There you learn how to talk, play,
sleep,
wake up,
love,
and even
how to get angry.
There are exams at all times,
but nobody fails,
nobody can pause
after ten,
fifteen or twenty years,
to rest
for a while.
There you never stop learning
and what you don't know
is always more important
than what you have already learnt.
This school
is the World, indeed,
as big as it is
open your eyes,
and you also will succeed.

G. Rodari

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

I. Alessia Para and Annika Sundås Larsson (2003). The pleiotropic mutation *dar1* affects plant architecture in *Arabidopsis thaliana*. Dev. Biol. 2003 Feb 15; 254(2): 215-25.

II. Alessia Para, Anders Nordström, Göran Sandberg, Barbara Moffatt and Annika Sundås Larsson. Disruption of the *ADK1* gene causes meristem distortion and cytokinin syndrome in *Arabidopsis thaliana*. (manuscript)

III. Katarina Landberg, Lars Nilsson, Alessia Para and Annika Sundås Larsson. The *TERMINAL FLOWER2* (*TFL2*) Gene Regulates the Transition to Flowering by Repressing Gene Activity. (manuscript)

IV. Alessia Para, Mikael Crona, Katarina Landberg, Sourav Datta, Magnus Holm and Annika Sundås Larsson.TFL2, the Arabidopsis HP1 protein, is required to modulate light signalling during plant development. (manuscript)

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SUMMARY IN SWEDISH

DET APIKALA SKOTT MERISTEMET HOS *ARABIDOPSIS THALIANA*

Det apikala skott meristemet är den struktur som bildar organen som bygger upp de ovanjordiska delarna av en växt d v s bildar blad och nya meristem som ger upphov till sidoskott eller blommor. En population av stamceller finns i den centrala delen av meristemet. När celler i meristemets yttre delar inlemmas i t ex bladanlag ersätts dessa av celler som stamcellerna ger upphov till. Att växten kontinuerligt kan bilda nya organ är alltså beroende av ett konstant nybildande av celler i de centrala delarna av meristemet. Detta i sin tur är beroende av att stamcellerna bibehåller sin identitet som helt odifferentierade celler under hela växtens livscykel. Växthormoner som cytokinin och auxin spelar en betydande roll för meristemets funktion eftersom de är involverade i regleringen av både celldelning och mönsterbildning, d v s var ett organ anläggs.

I många växtarter och i modellväxten *Arabidopsis thaliana*, har skottmeristemet alltid samma struktur vare sig man analyserar det i centrum av en bladrosett eller i toppen av en blomställning. Även när växten åldras och organ inte längre bildas behåller det inaktiva meristemet sin struktur, det omvandlas aldrig till t ex en terminal blomma. Mutanter i vilka skottmeristemet terminerar på något sätt är värdefulla redskap i analysen av hur meristemstrukturen och meristemets funktion bibehålls under växtens livscykel. Målet i detta arbete har varit att studera dynamiken i meristemet under växtens utveckling genom morfologiska och genetiska studier av tre Arabidospsis mutanter som uppvisar tre olika typer av meristemterminering.

I mutanten *distorted architecture1* (*dar1*) konsumeras meristemet i bildandet av flera blommor i toppen av blomställningen. Mutationen påverkar cell differentiering och/eller cell delning inom meristemet och stör på så sätt meristemets struktur och funktion. Genetiska interaktioner med andra kända meristem mutanter konfirmerar att *DAR1* genen är nödvändig för att bibehålla meristemet.

I mutanten *adenosine kinase1* (*adk1*) avslutas meristemet genom att aktiviteten avstannar. I och med att den här genen inaktiveras förändras celldelningskapaciteten i aktivt delande vävnader och i och med det minskar möjligheten till cell differentiering i meristemet. Dessa muterade växter har egenskaper som liknar det som beskrivs som

"cytokinin syndromet" och vi har visat att muterade plantor har förhöjda cytokinin nivåer på grund av ökad cytokininbiosyntes.

Skottmeristemet hos mutanten *terminal flower2* (*tfl2*) terminerar genom att meristemet ombildas till ett blommeristem och sedan en blomstruktur. Kloningen av *TFL2* genen visade att den kodar för en homolog till HETEROKROMATIN PROTEIN1 (HP1), ett protein som påverkar kromatinets struktur. HP1 homologer i olika arter har visat sig spela en viktig roll i den transkriptionella regleringen av gener lokaliserade både inom heterokromatin och eukromatin. TFL2 har också i växter visats reglera olika utvecklingsrelaterade processer som specificeringen av blomanlag och blomställningen utseende. *tfl2* mutanter uppvisar också förändrad respons på ljus vilket indikerar att TFL2 har en roll inom den komplexa regleringen av växters utveckling i relation till olika ljusförhållanden.

Resultaten av detta arbete visar att meristemets struktur och aktivitet regleras på flera olika sätt, t ex både genom växthormoner och kromatinets struktur och det bidrar till förståelsen av det nätverk av signaler som reglerar växters utveckling.

INTRODUCTION

ARABIDOPSIS THALIANA, PROBABLY THE MOST FAMOUS WEED IN THE WORLD

Despite the humble appearance, *Arabidopsis thaliana* (Arabidopsis) is nowadays the most renowned plant in the scientific world. The fame of this small weed has constantly been growing ever since 1943, when F. Laibach recognised the possibilities of this *Brassicacea* as a model organism and started the first collections of mutants. The 1980's were the time of a real boom in Arabidopsis research and the extent of the progress later brought the U.S. Department of Agriculture, the Department of Energy, the National Institutes of Health, and the National Science Foundation collectively to supply $7.5 million in 1990 and $22 million in 1993 towards gaining knowledge on this non-commercial species with great potential.

The advantages of using Arabidopsis as a model organism for plant science became more and more evident as soon as the discovery of the small genome size of Arabidopsis was added to the already exploited features of a short life cycle, abundant seed production and availability of a large mutant collection that make this plant a very suitable green "lab rat". The sequencing and the annotation of the 125 Mb genome of Arabidopsis at the end of the year 2000 represented an authentic milestone for the now large Arabidopsis community, eager to make the most of it by integrating the physical map of the genome with information obtained through long years of genetic analyses.

It is therefore understandable how the use of Arabidopsis played a pivotal role for the genetic and genomic frame of this study and how important it has been to be able to benefit from the massive amount of data collected through several years of investigation by Arabidopsis laboratories all over the world.

ARABIDOPSIS SHOOT APICAL MERISTEM (SAM): STRUCTURE AND FUNCTION

Unlike animals, plants rely on postembryonic development to build their body architecture. As a seedling breaks out from the seed coat, a complex developmental

program is switched on to support the continuous production of aerial organs from a core of self-renewing stem cells that is maintained throughout the life of the plant. The shoot apical meristem (SAM) is the structure wherein the population of stem cells is hosted and the first steps of cell fate acquisition take place, in a harmonic concert of cell division and cell differentiation.

Structure of the SAM

During embryogenesis the region of the embryo that will give rise to the SAM undergoes a process of pattern formation, which lays the foundations of the meristematic activity. The shoot apical meristem has the simple structure of a dome of cells surrounded by emerging primordia. Histological analyses have revealed a more complicated internal organisation of the meristematic dome as the cells in the SAM appear stratified in three clonally distinct layers: L1 and L2 constitute the tunica and L3 the corpus (Fig. 1A). Cells in L1 divide strictly anticlinally and give rise to the epidermis while the cell division plane is less regularly oriented in L2 and the cells from this layer form the procambium, the cortex and part of the pith. In L3, periclinal cell divisions mainly contribute to the formation of the pith.

In addition, cytological features and cell division rates inside the structure revealed the presence of a radial pattern of the SAM: a central zone (CZ) of large, slowly dividing cells at the summit (Steeves and Sussex, 1989), a peripheral zone (PZ) of small and more actively proliferating cells at the flanks (Laufs et al., 1998b) and a submeristematic rib zone (RZ) at the base (Fig. 1B). While proliferation of the cells in the RZ leads to the growth of the stem, the PZ sustains the production of the aerial organs of the plant and the CZ harbours the population of stem cells. Upon cell division, the daughter cells are displaced from the CZ toward the PZ where they undergo differentiation and are recruited as founders of a new organ. The continuity of the organogenic process is dependent on the constant flux of cells from the centre to the flanks of the SAM and that leans on the maintenance of stem cell identity in the CZ.

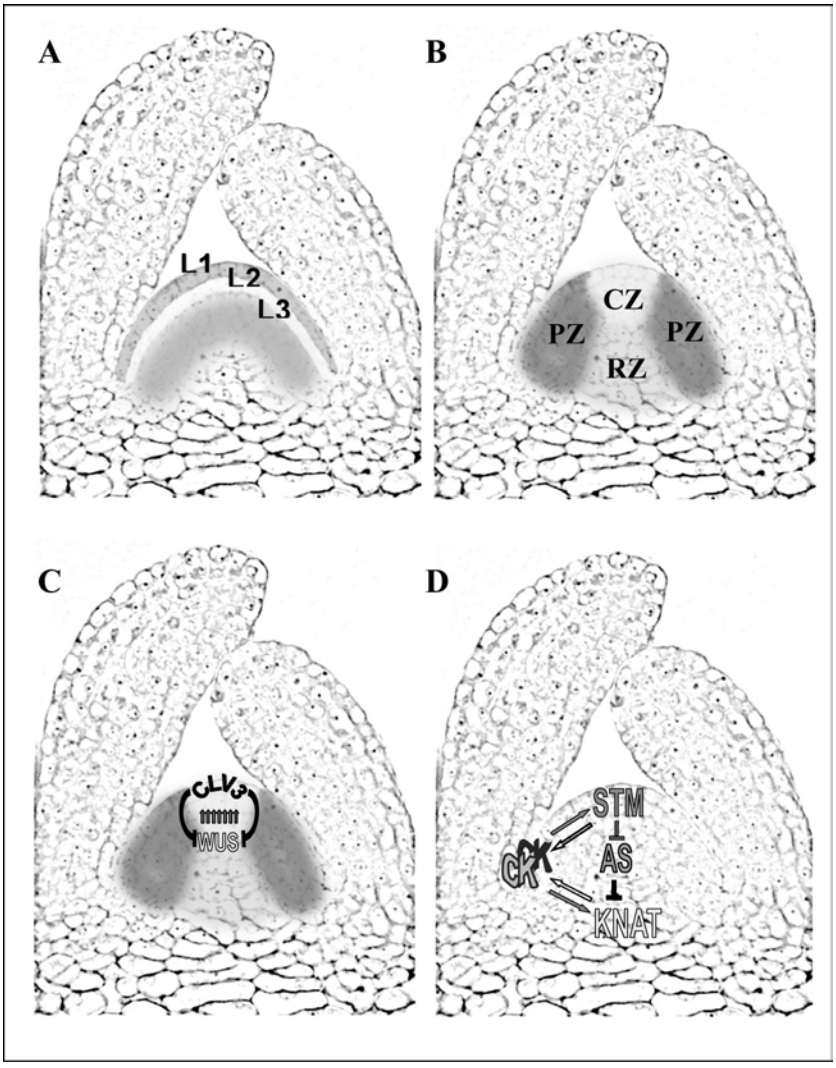

Figure 1: A) and B) Shoot apical meristem (SAM) organization. Schematic view through a section of the vegetative SAM of Arabidopsis.

C) and D) Coordination of cell fate decision across the SAM in Arabidopsis.

Coordination of cell proliferation and cell fate decisions

Dissection of meristem development using genetic, molecular and biochemical methods has revealed a number of signalling pathways that are required for coordinating the rate of cell proliferation and cell fate decisions to preserve the integrity of the SAM. One of the major aspects of meristem economy is the maintenance of stem cell identity in the CZ. Mutations in the *WUSCHEL* (*WUS*) gene cause loss of stem cell identity and the cells in the CZ are therefore incorporated into organ primordia (Laux et al., 1996). *WUS* encodes the founding member of the WOX homeodomain transcription factor family (Haecker et al., 2004) and is exclusively expressed in a subregion of the SAM and of the floral meristem (FM) (Mayer et al., 1998). As source of the signal that confers stem cell identity to the overlying portion of the meristem, the *WUS* expression domain comes to define another functional domain, the organising centre (OC) (Mayer et al., 1998).

The *CLAVATA* (*CLV1*, *CLV2* and *CLV3*) genes encode components of a meristem signal transduction pathway (Clark et al., 1997; Fletcher et al., 1999; Jeong et al., 1999) that is activated upon interaction of the CLV1/CLV2 receptor-like kinase complex (Jeong et al., 1999) with a small secreted polypeptide, CLV3, in the CZ (Fletcher et al., 1999¸ DeYoung, 2001 #16). All *clv* mutants are characterised by an enlarged SAM and morphometric analyses have shown that this is due to an excess of stem cells in the CZ (Laufs et al., 1998). An increase in size of the *WUS* expression domain observed in the SAM of the *clv* mutants indicated that the *CLV* genes are required to contain the stem cell population by delimiting the borders of the OC (Schoof et al., 2000). In turn, WUS can induce the expression of *CLV3* and phenocopy the *clv* defect when expressed in an enlarged domain of the SAM (Schoof et al., 2000). From those data a model is derived in which stem cell homeostasis is regulated by a feedback loop where WUS promotes stem cell identity by increasing *CLV3* expression which results in repression of *WUS* transcription by the CLV signalling pathway to restrain the signal that locks the cells in an undifferentiated state (Brand et al., 2000) (Fig. 1C). *WUS* expression seems to be also under epigenetic control as indicated by the enlargement of *WUS* domain in *fasciata1* (*fas1*) and *fas2* apices (Kaya et al., 2001). *FAS1* and *FAS2* encode two subunits of the CAF-1 (chromatin assembly factor-1) complex, which has been implicated in nucleosome assembly during DNA replication and repair (Adams and Kamakaka, 1999).

Interestingly, the *WUS* domain expanded apically in *fas* mutants, not uniformly as seen in the *clv* mutants, suggesting that FAS1 and FAS2 act through a different mechanism than the CLV pathway.

Once the descendants of the stem cells exit the CZ, they also leave the sphere of WUS signalling, therefore losing stem cell identity. Entering the PZ, however, does not coincide with an instantaneous switch of cell fate but the cells will progressively differentiate while they proceed towards the flanks of the meristematic dome. Premature differentiation in the PZ is prevented by SHOOTMERISTEM LESS (STM), a homeodomain transcription factor belonging the KNOX (KNOTTED-LIKE HOMEODOMAIN) protein family (Long et al., 1996). As its ortholog *KNOTTED* (*KN1*) in maize (Smith et al., 1992), *STM* is expressed throughout the meristematic dome, but down regulated at the site of primordium initiation (Long et al., 1996). Strong *stm* mutants are unable to initiate a SAM whereas in weaker *stm* mutants a few, often fused leaves and flowers lacking internal organs are produced as a result of residual meristematic activity (Endrizzi et al., 1996). The phenotype of the mutant, the *STM* expression pattern as well as the capacity of *KNOX* genes to induce meristem formation when ectopically expressed (Sinha et al., 1993; Lincoln et al., 1994) suggested that STM is required to prevent organ initiation until the organ founder cell population in the PZ has reached a proper size.

Due to the similarities between *stm* and *wus* mutant phenotypes, genetic experiments were conducted to asses the roles of the respective genes in meristem maintenance (Brand et al., 2002; Gallois et al., 2002; Lenhard et al., 2002). The results showed that *STM* and *WUS* control independent pathways that eventually converge to suppress differentiation. Yet, while *STM* acts to antagonise cell differentiation in all meristematic domains, *WUS* is the main promoter of stem cell identity in the CZ. The repressive action of STM is carried out by restricting the expression domain of the *MYB*-related *ASYMMETRIC LEAVES1* (*AS1*) and *AS2* genes to the region where a new organ primordium will arise (Byrne et al., 2000; Semiarti et al., 2001). AS1 and AS2 are able to down regulate the *KNOTTED-LIKE FROM ARABIDOPSIS (KNAT)* genes, *KNAT1* and *KNAT2* (Byrne et al., 2002; Iwakawa et al., 2002). Thus, in presence of STM, the *KNAT* genes are expressed in the SAM and promote meristematic cell fate. Conversely, the *AS1* and *AS2* genes can be expressed where *STM* is turned off, with consequent down regulation of the *KNAT* genes (Ori et al., 2000) (Fig. 1D). The local loss of KNOX gene expression delimits an area in which

those genes will no longer interfere with the organogenic determination signals that promote the specification of primordia.

At the PZ, the *MGOUN* genes *MGO1* and *MGO2* are required to promote the allocation of cells into incipient organ primordia (Laufs et al., 1998a). The *mgo* mutants form an enlarged SAM that is able to initiate only a few primordia and this phenotype has been interpreted as accumulation of cells at the SAM flanks due to defective coordination of cell fate acquisition in the progression from the apex to periphery of the meristematic dome (Laufs et al., 1998b).

The role of plant growth regulators in SAM development

Plant growth regulators, or plant hormones, are simple molecules that can influence physiological processes throughout development. A large number of substances are now known to possess hormonal properties in plants and they are divided into different groups according to their chemical structure. After being synthesised in different tissues, such compounds can be transported to the location where they will elicit the response, providing an efficient system for long-distance signalling in the plant body. Growth regulators, like cytokinins and auxin play an important role in the meristematic context of the SAM, as they are involved in the modulation of proliferation and patterning events at cellular level.

The term "cytokinins" defines a large family of N^6 substituted adenine derivatives that generally contain an isoprenoid or an aromatic derivative side chain. The biochemistry of cytokinins has a long history of their own since these growth regulators were isolated in the 1950's on the basis of their ability to promote cell division together with auxin (Miller et al., 1955). This ability has long been exploited for plant propagation through tissue culture techniques but the mode of cytokinin effect in proliferating tissues has only recently become understood. Their entry points in the regulation of the cell cycle are the G_1/S and G_2/M transitions, as well as the progression through S phase (for review see Jacqmard et al., 1994). Upon cytokinin treatment, the expression of *CYCLIN DEPENDENT KINASE A;1* (*CDKA;1)* increases and its kinase activity is induced at the G_2/M transition (Zhang et al., 1996). Cytokinins are also able to induce D-type cyclin transcription at the G1/M transition (Riou-Khamlichi et al., 1999). This transition represents a crucial decision point in the cell cycle at which cells can be committed to progress toward mitosis or to exit cell

division and undergo differentiation (Gutierrez et al., 2002). Other than bypassing the need for cytokinins in tissue culture, constitutive expression of *CycD3;1* results in alteration of cell fate specification in leaf tissues, indicating that the activation CYCD3 pathway not only promotes proliferation but also inhibits differentiation by endorsing the progression to S phase (Dewitte et al., 2003). The finding correlates well with the positive effect of cytokinins on the *KNOX* gene expression (Faiss et al., 1997; Rupp et al., 1999; Nogue et al., 2000) as they have the ability to antagonise cell differentiation throughout the SAM (Lenhard et al., 2002). In turn, ectopic expression of *KNAT1* in lettuce results in higher endogenous cytokinin levels (Ori et al., 1999; Frugis et al., 2001), suggesting the presence of a regulatory loop to control the accumulation of the growth regulator and the induction of meristematic genes.

In addition to the connection with cytokinins, KNOX proteins promote meristematic cell fate by negatively regulating the biosynthesis of another plant hormone, gibberellic acid (GA) (Tanaka-Ueguchi et al., 1998; Sakamoto et al., 2001; Hay et al., 2002). GA is able to enhance transverse cell division and longitudinal cell expansion by affecting the arrangement of newly deposited cellulose microfibrils in the cell wall (Gunning, 1982). Modulation of cell wall extensibility was previously shown to play a key role in plant morphogenesis as induced expression of expansin in the SAM was sufficient to initiate a program that recapitulated at least some aspects of normal leaf development (Pien et al., 2001). Similarly, repression of GA biosynthetic genes by KNOX could prevent biophysical alterations that are normally associated with organ formation in order to preserve meristematic cell identity (Hay et al., 2002).

Another growth regulator that plays a critical role in meristem function is auxin. Auxins effect on plant physiology relies on the distribution of this substance by a non-polar transport in the phloem and a polar transport (PAT) through parenchyma cells surrounding the vascular tissues (Jones, 1998; Muday and DeLong, 2001; Friml and Palme, 2002). Auxin polar transport is mediated by influx and efflux carriers and mutations in the genes coding for such proteins revealed a large number of PAT-related developmental processes. *PIN1* (*PIN-FORMED*) is a member of a family of auxin efflux carriers that controls the initiation of lateral organs in the SAM (Galweiler et al., 1998). The *pin1* phenotype illustrates the consequences of an altered distribution of auxin in the SAM as it develops a pin-shaped apex with no arising primordia at the flanks (Bennett et al., 1995). The *pin1* defect in organ initiation is rescued by local application of auxin (Reinhardt et al., 2000), indicating that PIN is

required for channelling the hormone to defined positions within the meristem where a peak in auxin concentration is able to switch on the genetic program that leads to organogenesis. Nevertheless, the competence to respond to auxin by initiating organ primordia is limited to a meristematic region at fixed distance from the apex that corresponds to a subdomain of the PZ (Reinhardt et al., 2000). The observation implies the presence of an additional auxin-dependent level of zonation in the SAM, which is superimposed on the classical one and defines the site of primordium initiation within the PZ. Together with PIN1, the direction of auxin flux in the SAM is influenced by the auxin influx carrier AUX1 belonging to the LAX (LIKE-AUX1) family (Bennett et al., 1996). The pattern of auxin distribution determines the position of leaf and floral primordia around the stem in a geometrical arrangement known as phyllotaxy (Steeves and Sussex, 1989). A very recent model for the regulation of phyllotaxy in Arabidopsis proposes that intracellular auxin is accumulated in the outer cell layer of the SAM by AUX1 and then diverted toward the tip of the meristem by PIN1. Pre-existing primordia act as a sink, depleting the surroundings from auxin and allowing accumulation of the hormone only at a certain distance where a new primordium can arise, which later becomes a sink itself, thus reiterating the mechanism of organ initiation and maintaining the phyllotactic pattern (Reinhardt et al., 2003). Assuming that each primordium contributes to auxin withdrawal, a divergent angle of 137° can be established between successive organs to determine the spiral pattern found in Arabidopsis.

MERISTEM IDENTITY THROUGHOUT DEVELOPMENT: VEGETATIVE AND REPRODUCTIVE PHASE

The ability to produce different kind of organs is an invariant feature of shoot development but is also regulated by environmental factors that affect the physiology of the plant. The life cycle of Arabidopsis can be roughly divided in two post-embryonic phases according to the identity of the organs that arise from the SAM: a juvenile or vegetative phase and an adult or reproductive phase. During the vegetative phase, the emerging primordia will grow out as leaves while they will develop into leaves subtending axillary meristem and flowers in the later phase.

Vegetative phase

The first cytological sign of organ initiation is a change in the plane of cell division in a restricted area at the flank of the SAM (Laufs et al., 1998; Sinha, 1999). This area corresponds to the site where KNOX genes are repressed to relieve the population of organ founder cells from anti-organogenic signals.

The *AP2*-like gene *AINTEGUMENTA* (*ANT*) shows a complementary expression pattern to STM and is among the first genes to be expressed at the site of organ formation, even before the primordium emerges from the SAM (Elliott et al., 1996). Loss-of-function mutations and ectopic expression of *ANT* causes a reduction or an increase in organ size respectively, indicating that this gene is involved in the control of the total number of cells in lateral organs (Elliott et al., 1996; Krizek, 1999; Mizukami and Fischer, 2000). A similar function was proposed for STRUWWELPETER (SWP) together with a role in pattern formation in the meristem (Autran et al., 2002).

The specification of leaf identity is accompanied by the determination of leaf polarity from the very early stages of organ initiation. The establishment of adaxial/abaxial polarity seems to require signals from different sources since factors both intrinsic and extrinsic to the primordium act together to confer abaxial or adaxial fate to the cells of the incipient lateral organ. In absence of adaxial cues, the abaxial cell fate is taken as the default (Sussex, 1955). The signals that promote adaxialisation come from the SAM and, in turn, the adaxial side of the primordium signals back to contribute in maintenance of the SAM (McConnell and Barton, 1998).

Reproductive phase

The transition from vegetative to reproductive phase is promoted by a floral stimulus or "florigen" (Chailakhyan, 1936). It is now known that the elaboration of the floral stimulus takes place in leaves and that it is driven by photoperiodic signals but the biochemical nature of this signal remains elusive. Nevertheless, remarkable progress has been made in understanding the intricate network of signalling pathways that promote the transition to flowering and the molecular mechanism that bring to the specification of floral identity and floral patterning as a consequence of floral induction.

Arabidopsis is able to respond to the same environmental conditions that are known to stimulate flowering in other plants i.e. light quality, temperature, day length, (Levy and Dean, 1998). Together with external factors, internal cues control flowering by activating distinct signalling pathways that eventually converge on a common set of genes. As a result, the integration of different signals will induce the transition to the reproductive phase. Genetic and molecular dissection of this phenomenon has revealed that environmental control is exerted through the photoperiod and vernalisation pathways, whereas endogenous signals regulate the autonomous and gibberellin pathways (Mouradov et al., 2002) (Fig. 2).

The photoperiod response pathway promote flowering by monitoring light signals. It consists of three parts: the photoreceptors, the circadian clock and an output pathway from the clock. Conformational changes of the photoreceptors and the consequent translocation to the nucleus trigger a signal transduction cascade that synchronises the clock with the duration of the daily light and dark periods to induce circadian regulation of the B-box protein CONSTANS (CO) by GIGANTEA (Suarez-Lopez et al., 2001). Direct transcriptional targets of CO are *FLOWERING LOCUS T* (*FT*) and *SUPRESSOR OF OVEREXPRESSION OF COP 1* (*SOC1*) (Lee et al., 2000; Samach et al., 2000) which participate in the activation of the floral meristem identity genes *APETALA1* (*AP1*) and *LEAFY* (*LFY*) respectively (Ruiz-Garcia et al., 1997). *FT* and *SOC* are considered floral integrators, as they are not specific to the photoperiod response pathway but act also in other pathways (Kobayashi et al., 1999; Onouchi et al., 2000). Both those genes are inhibited by another floral integrator, *FLOWERING LOCUS C* (*FLC*), a MADS box protein whose expression is controlled by the vernalisation and the autonomous pathways (Michaels and Amasino, 1999; Rouse et al., 2002). The vernalisation pathway promotes flowering by lowering the level of *FLC* mRNA in response to extended exposure to low temperatures (Sheldon et al., 2000). Repression of *FLC* by the vernalisation pathway is mediated by an epigenetic mechanism that is thought to change the methylation pattern of *FLC* through VRN2, a polycomb-group protein that switches this gene into a mitotically stable repressed state (Gendall et al., 2001).

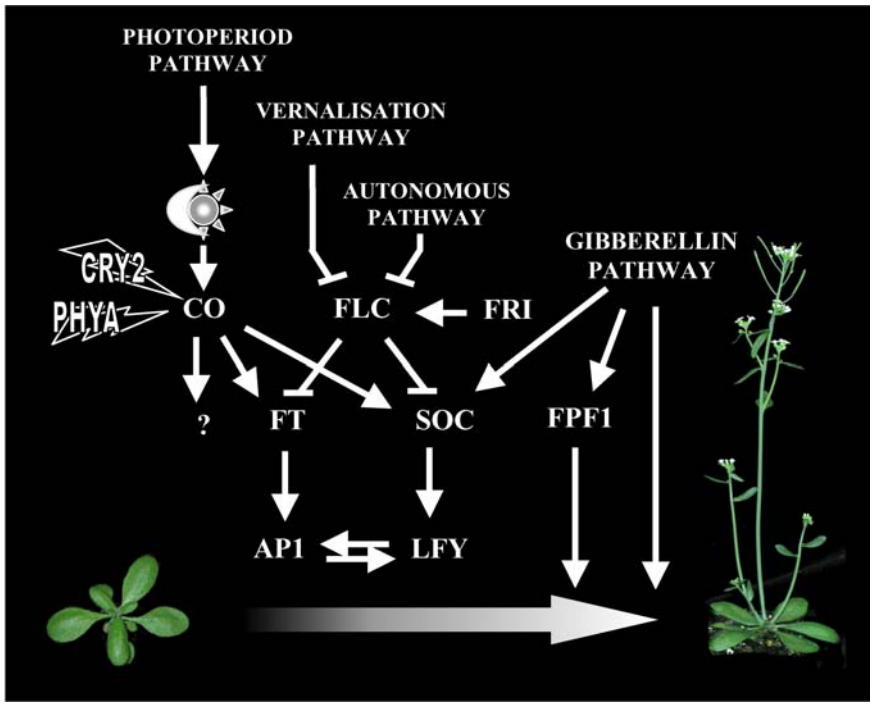

Figure 2: *Arabidopsis thaliana* flowering pathways. The convergence of the flowering pathways on *AP1* through FT and *LFY* though SOC1 gives rise to a punctual and coordinated flowering. Redrawn from Mouradov et al. (2002).

Similarly, components of the autonomous pathway repress *FLC* by altering the chromatin structure of the locus independently for the photoperiod or the vernalisation pathways. In both the autonomous pathway mutants *fld (flowering locus d)* and *fve* mutants *FLC* mRNA levels are increased and the *FLC* locus was found to be enriched in acetylated histones, a hallmark for transcriptionally active chromosomal regions (He et al., 2003; Ausin et al., 2004). This observation suggests that FLD and FVE participate in a mechanism of transcriptional repression mediated by histone deacetylation, as it is also deduced from the function assigned to the deduced proteins: FLD is homologous to a component of the human Histone Deacetylase 1,2 (HDAC 1/2) while *FVE* gene encodes for a chromatin assembly and histone modification proteins similar to yeast MSI (multicopy suppressor of IRA1) and the mammalian retinoblastoma-associated proteins RbAp46 and RbAp48 (He et al., 2003; Ausin et al., 2004). The repressive action of the vernalisation pathway on *FLC* is counteracted by FRIGIDA (FRI) that is able to increase the transcript level of *FLC* (Johanson et al., 2000).

Recently, another cross-talk point has been uncovered upstream to the floral integrators SOC1 and FT as FLC was shown to negatively regulate CRYPTOCHROME 2 (CRY2), one of the blue light photoreceptors, indicating an interaction between the photoperiod and the FLC-dependent pathways (El-Din El-Assal et al., 2003).

The hormonal control of flowering is exerted through the gibberellin (GA) pathway. An increase in GA biosynthesis promotes the transition to reproductive phase under both inductive and non-inductive photoperiods (Xu et al., 1997; Gocal et al., 2001). However, Arabidopsis mutants that fail to produce significant amounts of GA are unable to flower under short days, indicating the requirement for the GA pathway to ensure flowering even in the absence of inductive conditions (Wilson et al., 1992). The molecular mechanism underlying GA regulation of flowering time is the activation of the target genes *FLOWERING PROMOTING FACTOR1* (*FPF1*) (Kania et al., 1997), *GA-MYB* (Gocal et al., 2001) and *SOC1* (Borner et al., 2000) to increase the transcriptional activity of *LFY* (Blazquez et al., 1998; Blazquez and Weigel, 2000).The convergence of the flowering pathways on *AP1* through FT and *LFY* through SOC1 gives rise to a punctual and coordinated flowering response that marks the beginning of a new developmental stage.

Floral development

The progression from vegetative to reproductive phase has a dramatic consequence on the architecture of the plant, as the primordia at the flanks of the SAM develop into flowers and the internodes elongate to build the inflorescence. Such a change is the result of a new developmental program being activated in the SAM to confer floral identity to the organ primordia.

The transcription factor LEAFY (LFY) plays a central role in the acquisition of all the major features that differentiate a flower from an inflorescence branch, by promoting the floral identity switch in the primordia, through the activation the floral homeotic genes (Blazquez et al., 1997). Genetic analyses have shown that these two functions are separate and require different interactive partners (Parcy et al., 1998). LFY gain- and loss-of-function phenotypes demonstrated how this protein is necessary and sufficient to initiate the floral program (Weigel et al., 1992; Weigel and Nilsson, 1995). Similar phenotypes were observed for the MADS-box gene *APETALA1* (*AP1*) (Mandel et al., 1992; Mandel and Yanofsky, 1995) and sterol-inducible activation experiments showed that *AP1* is a direct target of LFY (Wagner et al., 1999), as indicated by previous genetic analyses (Weigel and Nilsson, 1995; Liljegren et al., 1999). The closest homologue of *AP1*, *CAULIFLOWER* (*CAL*) is also a direct target of LFY (William et al., 2004) and participates in the specification of floral identity by acting redundantly to *AP1* (Bowman et al., 1993; Kempin et al., 1995). *LFY* is expressed even before the primordium bulges out from the SAM while *AP1* and *CAL* expression can be detected through well-defined floral primordia (Weigel et al., 1992; Gustafson-Brown et al., 1994; Simon et al., 1996; Parcy et al., 1998). Once LFY has established the expression of its direct targets, in turn AP1 and CAL upregulate *LFY* creating a positive feedback loop that prevents floral reversion (Bowman et al., 1993; Liljegren et al., 1999). Together with LFY, AP1 and CAL, *FRUITFULL* (*FUL*), another MADS box gene, takes part in the specification of meristem identity acting redundantly to AP1 and CAL and in parallel to LFY as it does not appear to be a direct target of LFY activation (Ferrandiz et al., 2000; William et al., 2004).

Although LFY, AP1, CAL and FUL are the main players in the floral developmental pathway, the patterning of the inflorescence is regulated by the interactions between those functions and TERMINAL FLOWER1 (TFL1). Arabidopsis is characterised by indeterminate growth as the SAM retains meristematic activity throughout plant life

and is therefore able to continuously produce lateral primordia. Instead, in the *tfl1*mutant, the SAM is irreversibly transformed into a flower meristem (Shannon and Meeks-Wagner, 1991). AP1 and LFY were found to be ectopically and prematurely expressed in the *tfl1* background, indicating that TFL1 is required to promote indeterminate growth by preventing the expression of floral meristem identity genes in the centre of the SAM (Ratcliffe et al., 1999). Moreover, to further ensure that the floral meristem identity genes remain functional in distinct domains, TFL1 decreases the response to LFY and AP1 as revealed by simultaneous constitutive expression of *TFL1, LFY* and *AP1* (Parcy et al., 2002). The way floral meristem identity genes are repressed is likely to be indirect as TFL1 and its *Anthirrinum majus* ortholog CENTRORADALIS show homology to mammalian phosphatidylethanolamine binding proteins that associate with membrane protein complexes (Bradley et al., 1997). In addition, TFL1 is ectopically expressed in *lfy* and *ap1* backgrounds, whereas it is inhibited when those genes are constitutively expressed (Parcy et al., 2002). Hence, the separation between floral and shoot meristem identity relies on the mutual inhibition of TFL1 and floral meristem identity genes to confine different activities in separated regions of the SAM. The partition is thought to be established by the timing of upregulation of the distinct genes so that *TFL1* expression increases first in the centre of the SAM but not in the flanks where *LFY*, *AP1* and *CAL* can be upregulated and in turn, prevent *TFL1* transcription (Parcy et al., 2002).

Besides their essential role in establishing floral meristem identity, LFY and AP1 together with other factors, are also needed to activate the floral homeotic genes that specify the floral organ types.

According to the ABC model (Coen and Meyerowitz, 1991) and its extensions, like the "quartet model" (Theissen and Saedler, 2001), A-class genes specify sepals, the A and B genes together specify petals, the B and C genes together specify stamens, the C gene specifies carpels and the D genes are necessary for proper development of petals, stamens and carpels (Jack, 2001).

In *ap1* mutants, organs in the first and second whorl fail to develop with the correct identity (Bowman et al., 1989). This mutant trait reveals AP1 floral organ identity properties as it can produce A activity in the outermost whorls. Interestingly, in this context *AP1* activation seems to be independent from LFY as *AP1* expression is still detectable in *lfy* mutant flowers (Parcy et al., 1998; Liljegren et al., 1999). Two other floral homeotic genes, the MADS box *AGAMOUS* (*AG*) and *APETALA3* (*AP3*)

require also LFY for their activation. *AG* belongs to the C class and is required to specify the innermost whorls and to terminate the floral meristem, as suggested by the reiterative production of sepals and petals in the *ag* mutant (Bowman et al., 1989). LFY can bind to enhancer elements in the *AG* promoter but the activation requires the additional binding of WUS to adjacent sites (Lenhard et al., 2001; Lohmann et al., 2001). As a result, *AG* activation is restricted to the area where *LFY* and *WUS* expression domains overlap, i.e. the centre of the floral meristem. Once its domain is established, AG represses WUS before carpel formation to prevent indeterminate growth of the floral meristem (Lenhard et al., 2001; Lohmann et al., 2001). LFY is also participating in the activation of the B-class gene APETALA3 and interaction with the F-box protein UNUSUAL FLORAL ORGANS (UFO) provides regional specificity for the second and third whorl (Lee et al., 1997).

LIGHT PERCEPTION AND SIGNALLING IN *ARABIDOPSIS*

Light is the environmental signal that has the greatest impact on plant growth and development. Plants also use light as an energy source to convert simple compounds as carbon dioxide and water to complex organic molecules through the photosynthetic machinery. Hence, light supports all developmental processes whilst at the same time, synchronizes plant development to seasonal and circadian changes. This is carried out by a sophisticated detection system of photosensitive molecules that can detect external changes by monitoring several light parameters such as direction, duration and intensity for different regions of the spectrum. The integration and transduction of light signals via several intracellular pathways converge on the modulation of photoresponsive nuclear genes that orchestrate the response at different levels.

The photoreceptors and their downstream signalling pathways

In *Arabidopsis thaliana*, three major classes of photoreceptor molecules have been classified according to the wavelength they can perceive: the phytochromes (phyA-E) absorb predominantly red/far red light (R/FR) (600-750 nm), the cryptochromes (cry1 and cry2) respond to blue light (B) and UVA (320-500 nm) while phototropins respond to blue light only (for the photoreceptor nomenclature see (Quail et al., 1994).

The PHYA-E are encoded by a small gene family (Sharrock and Quail, 1989). The phytochromes are synthesised in the dark in the physiologically inactive P_r form that is photoconverted into the active P_{fr} form upon absorption of a photon while absorption of FR transform back to the P_r form (Quail, 1997). phyB is the most abundant phytochrome in light grown plants as phyB-E P_{fr} is stable in white light (W) (Clack et al., 1994; Hirschfeld et al., 1998) while phyA P_{fr} form is degraded by R or W light and is activated only in the far-red portion of the spectrum (Hennig et al., 1999). Light also modulates the nucleo/cytoplasmic subcellular localisation of phyA-E in a light-quality-dependent fashion (Kircher et al., 2002) and mediates autophosphorylation of phyA as well as phosphorylation of other proteins by the phytochromes (Fankhauser et al., 1999).

In Arabidopsis, two genes, *CRY1* and *CRY2*, encoding B light photoreceptors have been identified (Ahmad and Cashmore, 1993; Hoffman et al., 1996; Lin et al., 1996). Both CRY1 and CRY2 are nuclear protein but while CRY2 is constitutively imported to the nucleus, CRY1 is prevalently cytosolic in light (Cashmore et al., 1999; Guo et al., 1999; Kleiner et al., 1999).

Phototropins (PHOT1 and PHOT2 in Arabidopsis) are very similar to cryptochrome in structure but display different photosensitivity (Briggs et al., 2001; Kasahara et al., 2002). PHOT1 localises in close proximity to the plasma membrane (Sakamoto and Briggs, 2002).

Upon light exposure, a signal transduction cascade propagates the signal downstream of the photoreceptors. In order for the plant to rapidly adapt to the changes in light conditions, the photoreceptors are translocated to the nucleus where they interact with signalling intermediates to regulate light-modulated gene expression. Classical genetic screens have revealed that distinct signalling pathways branch off from each photoreceptor although the signalling intermediates are organised in a light-signalling network, thus allowing cross talk among the pathways (Fig. 3A).

The phytochrome mode of action at the level of transcription became more clear when phyA and phyB where found to bind to the PHYTOCHROME-INTERACTING FACTOR 3 (PIF3) in a conformation-dependent manner (Martinez-Garcia et al., 2000). The basic helix-loop-helix (bHLH) protein PIF3 can bind to light-responsive promoters but binding-site selection and transcriptional activation are achieved through PIF3 heterodimerisation with other factors, such as the bHLH proteins HFR1 and PIF4 (Fairchild et al., 2000; Huq and Quail, 2002). phyA and phyB can also

interact with protein kinases like NDPK2 (nucleoside diphosphate kinase 2) in the cytosol or phosphatases like PRP1 (phytochrome related phosphatase 1) (Choi et al., 1999), indicating that post-translational modifications modulate phytochrome signalling. Mutants affecting the phytochrome signalling processes have been identified for both phyA and phyB pathways. In particular, EID1 (EMPFINDLICHER IM DUNKELROTEN LICHT1) and SPA1 (SUPPRESSOR OF PHYTOCHROME A 1) seems to be involved in different but interacting phyA-dependent signal transduction chains as negatively acting factors, possibly mediating the degradation of phyA signalling intermediates (Zhou et al., 2002). From the early intermediates, the light signals are tranduced to integration components that modulate the activity of downstream effects: the pleiotropic COP/DET/FUS loci. The eleven members of this group share the same photomorphogenic phenotype in darkness and can be divided in two classes: eight of these loci constitute the COP9 signalosome while the other three, COP1, DET1 and COP10 have independent roles (Deng et al., 1991; Wei and Deng, 1992; Suzuki et al., 2002; Wei and Deng, 2003). Nevertheless, all the COP/DET/FUS loci function in a protein ubiquitination pathway, the 26S-proteasome-related activity of the COP9 signalosome triggering proteolysis of the ubiquitinated targets of COP1, which acts as E3 ubiquitine ligase (Osterlund et al., 2000; Schwechheimer and Deng, 2000). In light, the nucleus is slowly depleted of COP1, allowing transcriptional activation of light-responsive promoters, and COP1 nuclear exclusion is promoted by the phytochrome signalling pathway. In addition, the B light receptors cry1 and cry2 are thought to interact with COP1 to suppress its activity in a light-dependent manner, although a direct *in vivo* binding has been shown only in the case of cry1, in accordance with cry1 dark/light nuclear/cytosol partitioning (Wang et al., 2001). The interaction with COP1 and the phytochromes might account for part of B light mediate gene expression although other mechanisms must be active in cryptochrome signalling, microarray experiments having shown that B light is able to modulate the transcription of almost as many genes as R light (Ma et al., 2001).

The role of photoreceptors in Arabidopsis development

The perception of light signals through the photoreceptors provides the plant with vital information about its surroundings to modulate physiological responses thereafter.

Seed germination is entirely mediated by phytochromes as it can be induced under low quantity of R or FR light as well as under continuous FR light (phyA), enabling the seeds to detect the soil surface and to sense apertures in the R depleted shade of chlorophyllous vegetation (Shinomura et al., 1994; Shinomura et al., 1996; Hennig et al., 2002). In order to prevent photomorphogenesis in darkness, the COP/DET/FUS proteins patrol the nucleus targeting light-responsive regulators downstream of the photoreceptor signalling pathways for degradation (Deshaies and Meyerowitz, 2000). The central role of COP/DET/FUS proteins is clearly indicated by the dark growth phenotype displayed by mutant seedlings of the COP/DET/FUS loci, as they exhibit all the traits associated with active light perception (Deng et al., 1991). Photoreceptors mediate changes in gene expression, cellular and subcellular differentiation, and organ morphology that are associated with development under light. Both phytochromes and cryptochromes contribute to the inhibition of hypocotyl elongation in the wavelength of light they can absorb, but phyA controls the development under low light intensity. PhyB, phyC and the cryptochromes take over when phyA is rapidly degraded by light (Fankhauser, 1997). R, FR and B light promote the opening of the apical hook, which indicates that multiple photoreceptors intervene in this response while both phyA and phyB are required for cotyledon expansion in W light but only phyB in B and R light (Liscum and Hangarter, 1993; Neff and Chory, 1998).

Together with shade avoid responses, plants maximise energy uptake by orientating the photosynthetic organs toward light. Phototropism, the directional curvature of plant organs in response to light, requires growth increase on the shaded side with simultaneous growth decrease on light exposed side one through a differential cell elongation/expansion program mediated by auxin distribution and B light signalling (Muday, 2001; Parks et al., 2001). PHOT1 and PHOT2 are also active during phototropic response but PHOT1 functions under all light intensities while PHOT2 functions under high intensity B (Jarillo et al., 1998; Sakai et al., 2001).

As previously described, the photoperiod response pathway is one of the major pathways regulating flowering time. In night break experiments FR, B and R light can promote flowering upon light exposure in the middle of a long night but plants grown under a high R/FR ratio or under continuous R light flower later than plants grown under a low R/FR ratio or under B light (Goto et al., 1991; Bagnall et al., 1995).

Since FR induces flowering, the phyA signalling pathway is thought to positively effect floral initiation and the *PHYA* loss- and gain-of function phenotype in both long

day (LD) and short day (SD) conditions, matching the assumption as the mutant plants are late and early flowering respectively (Johnson et al., 1994; Bagnall et al., 1995; Neff and Chory, 1998). B light also promotes flowering through cry2 while cry1 effect is not fully understood. *cry2* mutants flower late in LD but not in SD conditions and transgenic plants overexpressing *CRY2* flowers early in SD but not in LD conditions (Guo et al., 1998; Koornneef et al., 1998). Interestingly, *cry2* late flowering phenotype can be phenocopied by growing Arabidopsis plants in B-plus-R light whereas *cry2* does not show a late flower phenotype in continuous B or R light, indicating CRY2 promotion of flowering is dependent on these regions of the spectrum (Guo et al., 1998; Mockler et al., 1999). Differently from phyA and cry2, phyB and the redundant phyD and phyE functions activate the R signal transduction cascade that inhibits flowering. Combining phytochrome and cryptochrome mutants has provided a genetic framework to illustrate functions of the photoreceptors in the regulation flowering time (Fig. 3B). These account for the R light dependent early flowering phenotype of *cry2* despite the opposite effect of *cry2* and *phyB* mutants on flowering time (Guo et al., 1998; Mockler et al., 1999). Moreover, the *phyB* early flowering phenotype is enhanced in R and *phyB* is partially epistatic to *cry2* in R-plus-B light due to the redundant function of phyD and phyE (Guo et al., 1998; Mockler et al., 1999). Similarly, phyA seems to promote flowering in response to FR by inhibiting phyB function as indicated by *phyAphyB* early flowering phenotype (Devlin et al., 1996; Neff and Chory, 1998). Phytochrome signalling can directly activate floral initiation but the implementation of the signal transduction in different daylenghts requires interaction with the circadian clock. Under low light intensities of R and B light, cry2 and phyA have been found to give a modest contribution to the entrainment of the clock i.e. the resetting of the pacemaker by light signals and no contribution at all under high intensities where cry1 and phyB are actively involved (Somers et al., 1998). However, mutations in *PHYA* and *CRY2* affect the photoperiodic control of flowering, indicating that the effect of phyA and cry2 signalling is likely modulated by an output of the circadian clock (Johnson et al., 1994; Guo et al., 1998).

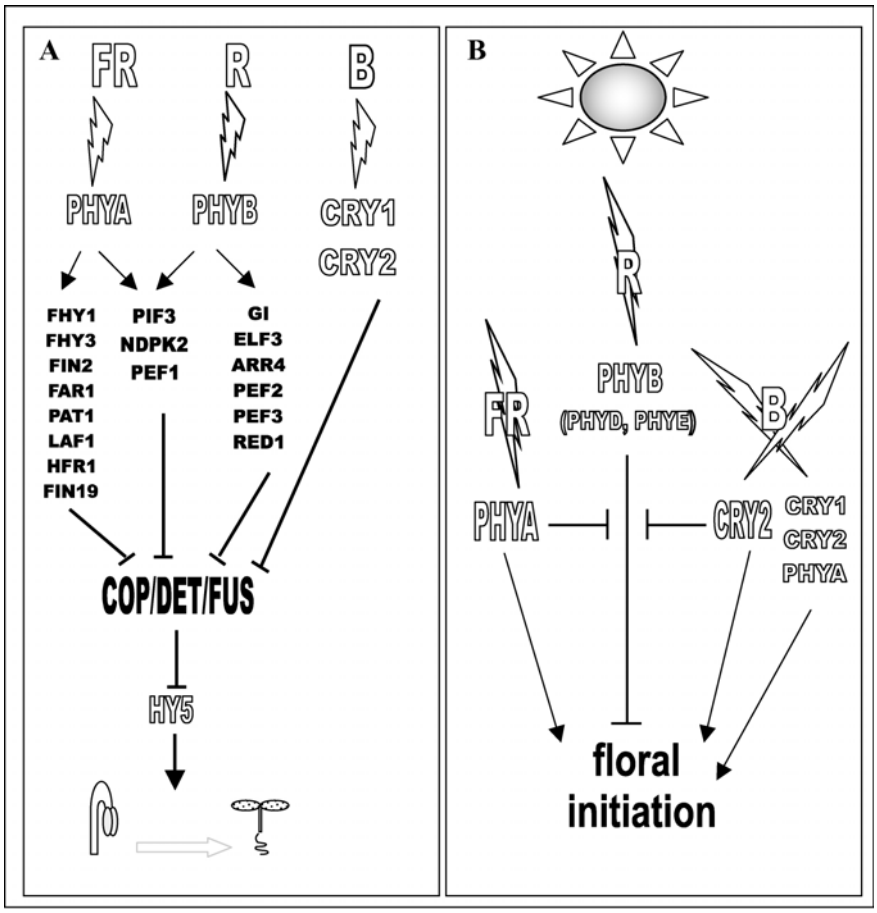

Figure 3:A) Model of genetic interactions regulating de-etiolation in *Arabidopsis thaliana.* Redrawn from Sullivan and Deng (2003). B) Model depicting functions of photoreceptors regulating floral initiation in *Arabidopsis* grown in continuous lights. Redraw from Mockler et al. (2003).

AIM OF THE WORK

The aim of this work was to investigate the dynamics of meristem development through morphological and genetic studies of three Arabidopsis mutants that exhibit distinct modes of SAM termination. Isolation of the corresponding genes for two of the mutants has been completed and helped to unravel the molecular mechanism underlying abnormal meristem behaviour.

The results from this work uncover the existence of additional pathways that are active at different levels of SAM activity, therefore contributing to elucidate one of the most intricate regulatory network that governs plant development.

RESULTS AND DISCUSSION

TERMINATION BY MERISTEM CONSUMPTION: THE *dar1* MUTANT (**I**)

dar1 affects multiple aspects of plant development

The *dar1* mutant was isolated in a greenhouse screen for *Arabidopsis* mutants showing developmental defects. A small fraction of the *dar1* seeds failed to germinate and a number of *dar1* seedlings bore three cotyledons, suggesting a possible role for DAR1 in embryo development. After germination, the *dar1* mutation disturbed vegetative development affecting the rate of primordium initiation as well as the size and the shape of the rosette. However, the mutant and the wild type were found to flower approximately at the same time, indicating that the SAM is still able to respond to external signals and developmental cues to start flowering. Compared to the wild type, *dar1* adult plants are short in size and showed reduced apical dominance as well as distorted phyllotaxy of the inflorescence which would eventually terminate after the production of 10 to 12 flowers with narrower petals and distorted stamens. The *dar1* primary root is shorter than the wild type and the production of lateral roots is severely reduced.

dar1 affects SAM and RAM morphology

The *dar1* mutation perturbs the pattern of cell differentiation and/or cell proliferation within the SAM as abnormal cellular and sub-cellular organisation was observed. Upon the transition to the reproductive phase, the *dar1* meristem develops into an abnormal structure showing various degrees of fasciation.

During *dar1* inflorescence development the mutant apex was exposed, lacking the typical scale of surrounding flower primordia at different and temporally consequent stages (Smyth et al., 1990). *dar1* SAM terminated in a cluster of 2-3 flowers long before the wild type SAM would undergo senescence. Altogether, the morphological observations on *dar1* meristem development pointed to a distortion in SAM function

already present at early stages of plant growth, but highly enhanced at the onset of flowering, probably concomitant with the increase in mitotic rate.

The SAM, however, is not the only meristematic structure affected by the *dar1* mutation. Morphological observation of the *dar1* root tip revealed an abnormal organisation of the structure of the root apical meristem (RAM) together with a reduction in size of the elongation zone, indicating that DAR1 is required for the correct execution of the cell division pattern that results in an almost invariant organisation of the root tip.

dar1 shows genetic interaction with known meristem mutants

Several aspects of the *dar1* phenotype suggest a role of DAR1 in the context of meristem function. To assess which regulatory pathway DAR1 is part of, double mutant combinations of *dar1* and other mutants showing disruption in meristem organisation or maintenance were analysed.

The most telling interaction was observed with *mgo1* mutation, as it was not complemented by *dar1*. MGO1 and DAR1 loci are mapped to two different chromosomes (Laufs et al., 1998a, and our unpublished results), ruling out the possibility that *dar1* and *mgo1* would represent distinct alleles of the same gene. The *mgo* mutants exhibit an enlarged SAM due to accumulation of cells in the PZ and are defective in primordia initiation (Laufs et al., 1998a; Laufs et al., 1998b). Thus, the *MGO* genes are thought to be required to repress cell division and promote cell differentiation at the periphery of the SAM. The combination of *dar1* and *mgo1* mutations caused fasciation of double heterozygous plants and lethality of double homozygous plants, strongly suggesting a close interaction between *DAR1* and *MGO1* and leading to the speculation that DAR1 is likely to play a role in the same meristematic context as MGO1. The result of the cross between *dar1* and *fas2* supports the assumption that *DAR1* and *MGO1* are active in the same pathway. As the *mgo* mutations, *fas1* and *fas2* cause a defect in primordium initiation and meristem size (Leyser and Furner, 1992; Kaya et al., 2001). In addition, *FAS1* and *FAS2* seem to be required in *mgo1* background for organ production (Laufs et al., 1998). *dar1fas2* double homozygotes resembled the double mutant between *mgo1* and *fas2,* regarding the meristem enlargement and the presence of abnormal leaf primordia, although the plants were still capable of forming leaves and flowers.

The *FAS* genes are involved in a mechanism that regulates the extent of the *WUS* domain, as it expanded apically in *fas* mutants, not uniformly as seen in the *clv* mutants (Kaya et al., 2001). WUS is required to confer stem cell fate to the CZ of the meristem and *dar1* enhances the already severe *wus* phenotype, suggesting that DAR1 and WUS are likely to work in parallel pathways. Interestingly, the *dar1* mutation does not seem to interact with *clv3* as the double mutant phenotype showed an additive effect, although the CLV3 expression domain was found to be reduced in the *dar1* background (our unpublished results). The enhancement of the *fas2* phenotype by *dar1* indicates that DAR1 and FAS2 are involved in a CLV-independent mechanism(s), controlling the dimension of the stem cell population versus cell fate acquisition. However, they are likely to be active in different pathways because it is improbable that DAR1 could cooperate with FAS2 to regulate the extent of WUS domain since it has the same dimension in *dar1* background as in the wild type (our unpublished results). Instead, *DAR1* could be involved in an additional genetic pathway together with *MGO1*. Only the characterisation of *DAR1* and *MGO1* genes and the nature of *dar1* and *mgo1* mutations will allow to assign a more precise role to these genes.

DAR1 mapping by positional cloning

The *DAR1* locus was mapped using CAPS and SSLP PCR markers distributed over the Arabidopsis genome. The chromosome position of the locus was assigned to the bottom arm of chromosome 1 and the area was circumscribed to a region defined by the SGCSNP69 and nF19K23 markers positioned 1,25 cM and 2,25 cM respectively from the *DAR1* locus. To create a high density map of the area, sequences from the Landsberg *erecta* random sequence database were compared with Columbia genome sequences from bacterial artificial chromosomes (BAC), to score DNA polymorphisms. More recently, the Cereon collection of predicted Arabidopsis single-nucleotide polymorphisms (SNP) and insertions/deletions (INDELs) has been made available (http://www.arabidopsis.org/Cereon) (Jander et al., 2002). Based on this information, 50 putative CAPS and SSLP were designed for the area and were used to score an enlarged mapping population to find increasingly tight linkage to the mutation (our unpublished results). This last step defined an interval of 381 Kb that contains 105 open reading frames (ORFs) predicted and annotated by the Arabidopsis

Genome Project. Sequencing of this area is currently in progress in order to identify the genomic modification that caused the *dar1* phenotype.

TERMINATION BY MERISTEM ARREST: *adk1* MUTANT AND CYTOKININ ACTION (**II**)

The GT6-2 line was isolated in a gene trap screen (Wilson et al., 1996) and it was selected based on a morphological phenotype suggestive of altered meristematic activity. The T-DNA insertion causing the mutation was found to disrupt the *ADK1* locus and thus the mutant will be referred to as *adk1*.

Cytokinin syndrome and meristem distortion in *adk1* plants

The *adk1* phenotype displayed a peculiar arrest of the inflorescence SAM along with several developmental abnormalities that were previously related to the "cytokinin syndrome" (Hewelt et al., 1994; Redig et al., 1996; Faiss et al., 1997). The adult mutant plants were short in size with deformed rosette and cauline leaves and all green tissues showed a darker shade than the wild type plants. *adk1* flowers had an irregular number of floral organs and they clustered at the top of the inflorescence. The siliques were shorter than the wild type but had longer petioles. Root growth was also affected by the mutation as *adk1* the root was reduced in length with few lateral roots.

The *adk1* mutation caused a dramatic alteration of meristem morphology. The meristematic dome appeared enlarged in *adk1* plants before the transition to the reproductive stage, a feature that indicates overproliferation as an early event preceding the arrest. After the transition to reproductive phase, the *adk1* inflorescence SAM grows into a pinhead with a spiral of arrested primordia on the flanks. Histological analyses showed that the organisation of the SAM was not affected in *adk1* apices, suggesting that the mutation compromised the function of the SAM without altering the structure.

adk1 meristem defect is due to overproliferation

The phenotype of the *adk1* SAM indicates that the distortion is likely to be caused by overproliferation. Indeed, cell proliferation markers were found to be misregulated in the *adk1* background, indicating that the mutation was likely to alter the proliferative capacity of meristematic cells. In the mutant, the promoter activity of the mitotic *cyclin B1;2* gene was higher than in the wild type in the tissues where cell division is active such as the SAM and the RAM. This observation suggests that in the *adk1* background cells are maintained in an actively dividing state, implying that competence for cell proliferation is increased. The high level of *KNAT1::GUS* expression in *adk1* root and shoot apices confirmed the correlation between increased proliferative behaviour and inhibition of differentiation, since the *KNOX* genes play an important role in meristem maintenance (Endrizzi et al., 1996; Lenhard et al., 2002). Upregulation of KNAT genes could also explain the suppression of the *stm-1* proliferation defect in the *adk1stm-1*. The double mutant phenotype displayed a restored meristematic function confirming the ability of the *adk1* mutation to, not only enhance, but also induce cell proliferation since the *stm-1* mutant completely lacks a shoot apical meristem (Endrizzi et al., 1996). A similar conclusion has been also drawn for the double mutant combination between *stm-1* and *pasticcino* (*pas*), a negative regulator of hormonal response in the control of cell division and differentiation (Harrar et al., 2003).

The T-DNA is inserted in the *ADK1* gene

Cloning of the regions flanking the T-DNA revealed that it was inserted in the *ADENOSINE KINASE 1* (*ADK1*) gene. ADK1 is an enzyme that phosphorylates adenosine (Ado) and adenosylic substrates as previously characterised (Moffatt et al., 2002). RT-PCR analyses showed the *adk1* mRNA lacks the terminal part of the coding region. The last few amino acids are therefore excluded from the C-terminal end of the truncated protein, likely compromising both the formation of the ATP binding site and the stability of the protein (Maj et al., 2000). The 40% ADK activity in *adk1* plants is possibly due to the activity of the other Arabidopsis ADK protein, ADK2 (Moffatt et al., 2002).

Altered hormone sensitivity and cytokinin levels in *adk1*

Several lines of evidence indicate that ADK participates in cytokinin interconversion, that is, the interchange between cytokinin base, riboside and ribotide. The cytokinin interconversion pathway has been indicated as a mechanism for regulating the level of active cytokinins (McGawn and Burch, 1995; Auer, 2002; Lexa et al., 2003). ADK converts Ado to AMP and based on *in vitro* assays and feeding experiments, plant ADKs are proposed to act on cytokinin substrates as well (Moffatt and Ashihara, 2002). For example the first plant gene encoding ADK was isolated from the moss *Physcomitrella patens* and the authors proposed that it was likely to play a role in cytokinin interconversion based on its ability to convert isopentenyladenosine, a cytokinin riboside, to the monophosphate form (von Schwartzenberg et al., 1998). Later, two isoforms of ADK were identified in Arabidopsis and an *in vitro* assay of recombinant versions of the enzyme showed their preference for adenosine rather than isopentanyladenosine but a role for these enzyme in cytokinin interconversion was not ruled out (Moffatt et al., 2000). Recently, an adenosine kinase from tobacco BY-2 has been isolated using a zeatin affinity column and it was shown that ADK interaction with the bound zeatin could be disrupted by several other adenine-based purine derivatives, supporting the conclusion that ADK is able to bind to cytokinin and thus may be involved in cytokinin interconversion (Laukens et al., 2003).

The effect of adenosine kinase deficiency on plant development was investigated by Moffatt et al. by creating lines with reduced expression of both ADK isoforms by transgene silencing (Moffatt et al., 2002). The most ADK deficient plants had less than 7% of the ADK activity present in wt plants and they displayed a pleiotropic phenotype with altered root growth, loss of apical dominance, wavy leaves and reduced internode elongation. This phenotype was attributed to an inhibition of S-adenosylmethionine-dependent methylation activities due to Ado accumulation, rather than a lack of AMP synthesis since other purine salvage mutants are vegetatively normal. The delayed senescence phenotype of the ADK-deficient lines suggests that they may also have cytokinin defects, in addition to changes in methylation yet to be investigated (Moffatt B.A., unpublished).

In agreement with this, the *adk1* mutation was found to alter the sensitivity to cytokinin since the mutant root showed a decreased sensitivity to the cytokinin benzyladenine (BA), suggesting that the mutant was very likely to have altered

cytokinin levels and/or response. *adk1* plants were also less sensitive to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), this effect fitting well with the previously reported increase of the ACC synthase activity, the key enzyme in ethylene biosynthesis, upon cytokinin treatment in the root (Vogel et al., 1998).

HPLC measurements of several cytokinin compounds in *adk1* adult plants showed a considerable increase in some cytokinin types. In particular, the amount of *N*-glucoside cytokinin conjugates was higher in the mutant, strongly suggesting that a decreased ADK activity caused an imbalance in endogenous cytokinin levels. As a matter of fact, these specific metabolites accumulate as a consequence of enhanced cytokinin biosynthesis and are probably involved in the mechanisms that control the homeostasis for this hormone (Werner et al., 2003). Further, support for the assumption that the mutant phenotype is due to an increase in endogenous cytokinin levels comes from the analysis of biosynthetic rate of IPMP and ZMP that were found to be elevated in the *adk1* mutant. How the ADK1 deficiency results in increased levels is at this point unclear.

The connection between *adk1* phenotype and increased levels of cytokinin is also corroborated by the characterisation of cytokinin–deficient Arabidopsis and tobacco plants which have elevated cytokinin breakdown (Werner et al., 2001; Werner et al., 2003). Those plants are affected in the same developmental processes as *adk1* but with an opposite effect. Cytokinin deficiency resulted in a diminished activity of the SAM that showed a strong reduction in size although the structure was not altered. In contrast, *adk1* SAM exhibited signs of overproliferation. In the plants with low levels of cytokinins root growth was enhanced as well as the production of lateral roots while *adk1* root growth was severely reduced and only a few lateral roots were visible. Cytokinins have been reported to regulate the progression through mitosis (Redig et al., 1996; Zhang et al., 1996) and thus, higher levels of cytokinins likely result in higher mitotic activity in *adk1* plants compared to the wild type, as revealed by *cyclin At1::GUS* pattern in the SAM and RAM.

Taken together, our analyses of the *adk1* mutant shows that the disruption of the *ADK1* gene severely affects plant development by altering the proliferative behaviour of actively dividing tissues and possibly reducing cell differentiation in the SAM and RAM. As a result, the mutant plants display a pleiotropic phenotype with traits that are reminiscent of the cytokinin syndrome, suggesting a connection with a defect in cytokinin metabolism. Indeed, we could show that *adk1* mutant plants exhibit an

altered sensitivity to the plant hormone and contain higher levels of cytokinin due to increased cytokinin biosynthesis.

TERMINATION BY LOSS OF MERISTEM IDENTITY: *tfl2* MUTANT AND GENE REPRESSION (**III**)

The *tfl2* mutant has been previously described (Larsson et al., 1998). The *tfl2* mutation caused premature termination of the SAM in a floral structure, early flowering and photoperiod hyposensitivity. Genetic analyses revealed that TFL2 had a role in floral primordium specification and inflorescence patterning.

Cloning, characterisation and expression pattern of *TFL2*

The *TFL2* locus was previously mapped to the upper arm of chromosome 5 (Larsson et al., 1998). The smallest region that could be defined by positional cloning contained nine putative ORFs that were amplified by PCR, using DNA isolated from the two *tfl2* alleles, *tfl2-1* and *tfl2-2*, and wild type as template. The sequence of the chromo box containing ORF MVA3.4 revealed a single base pair substitution in *tfl2-1*, introducing an in-frame stop codon while in the *tfl2-2* mutant the gene is deleted. Complementation analysis with a fragment containing the candidate ORF confirmed the identity of *TFL2* gene. The phenotypes of the two *tfl2* mutants are highly similar, implying that both are null alleles since the entire gene is deleted in *tfl2-2*.

Database searches did not reveal overall sequence similarity to any other Arabidopsis gene, suggesting that *TFL2* is a single copy gene. Several functional domains can be identified in the TFL2 protein; of particular interest are a chromo domain (CD) and a chromo shadow domain (CSD) The proteins showing highest overall amino acid sequence similarities to TFL2 are all plant chromo domain containing proteins; a chromo domain protein from rice (35% identity, 14% similarity), a chromo domain protein from carrot (Kiyosue et al., 1998; 33% identity, 15% similarity), and a Heterochromatin protein1-like protein from tomato (33% identity, 15% similarity). Comparing the four plant homologues, the aa sequence is most highly conserved in the chromo and chromo shadow domains.

Chromo domain proteins are traditionally divided into three major groups. The HP1-like proteins contain a CD and a CSD separated by a short hinge region. These

proteins are small, generally less than 200 aa. The PC-like proteins are longer, over 300 aa, and contain a CD and a C-terminal PC-box. The third group consists of proteins with two CD in tandem. TFL2 clearly belongs to the HP1-like group, as it contains both a CD and a CSD. However, TFL2 and other plant HP1 differ from HP1 proteins of other organisms in that the hinge and acidic regions are longer. In addition, part of the CSD diverges from other HP1 proteins.

The expression pattern of the *TFL2* gene in the wild type plant was investigated by *in situ* hybridisation and RT-PCR experiments. *TFL2* was found to be expressed in several different tissues and stages including root, leaves, inflorescences and siliques as indicated by RT-PCR results. *In situ* hybridisation on shoot apices revealed high levels of *TFL2* mRNA in proliferating tissues of both inflorescence and floral meristems and the *TFL2* signal was homogenous throughout the entire meristem, as previously described (Kotake et al., 2003).

TFL2 regulates plant development through gene repression

HP1 proteins from different organisms are part of multi-protein complexes that are thought to mediate silencing of heterochromatic genes. As homologous of HP1, TFL2 can act as a transcriptional repressor to affect several developmental processes.

TFL2 repressive function is required for maintaining inflorescence meristem identity since *tfl2* inflorescence meristem terminates by the conversion into a floral structure, a trait that was previously associated to ectopic expression of meristem identity genes, such as *AP1*, *AG* and *LFY* (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995; Mizukami and Ma, 1997). Enhancement of the *35S::AP1* phenotype was observed in combination with *tfl2* and interpreted as an indication of ectopic AP1 promoter activity in *tfl2* background. This was confirmed by *in situ* hybridisation and the use of a reporter gene construct (*AP1::GUS*) as well as RT-PCR analyses of various tissues of *tfl2* plants. In addition, ubiquitous expression of *AG* was detected by *in situ* hybridisation and RT-PCR suggesting that TFL2 has a negative effect on *AG* transcription. The partial rescue of *tfl2* termination in *ag1* background further corroborated the hypothesis that *TFL2* functions as a repressor of *AG*.

Yet, the effect of TFL2 on *AP1* and *AG* expression seems to be indirect, as speculated by Kotake et al. The authors proposed that the floral integrator FT is the immediate target of TFL2 since *FT* expression is upregulated in a stronger and earlier fashion

than the floral and meristem identity genes in *tfl2* background. In addition, *ft* could rescue the early flowering phenotype of *tfl2* (Kotake et al., 2003). Nevertheless, the combination with *ft* could not completely rescue other features of *tfl2* phenotype like curly leaves and loss of apical dominance. In particular, curly leaves were previously observed upon ectopic expression of *SEP3*, *AG* and both *PI* and *AP3* in wt background, suggesting that TFL2 could be more directly involved in the transcriptional activation of those genes (Goodrich et al., 1997).

TFL2 is involved in both the photoperiod sensitive and the autonomous pathway of flowering

The photoperiod insensitivity previously reported for *tfl2* assigned TFL2 function to the photoperiod sensing pathway and phenotypic analyses of the combination of *tfl2* and two mutants affecting this pathway provide genetic support this conclusion
elf3 is an early flowering mutant and is involved in mediating transduction of light signals to the circadian clock (Zagotta et al., 1996; Hicks et al., 2001). When combining *tfl2* and *elf3-1* mutations an enhancement of early flowering both in LD and in SD conditions as well as a premature termination of the SAM is seen, revealing that *elf3* affects also pathways regulating meristem identity. Mutations in the *GI* gene cause late flowering in LD due to the disruption of circadian regulation of *CO* transcription (Suarez-Lopez et al., 2001). When adding *gi-1* to *tfl2-1*, the double mutant flowered later than *tfl2* in LD, almost as late as wild type, indicating a partial rescue of *gi* late flowering phenotype in *tfl2* background possibly by upregulation of FT even without activation of CO (Takada and Goto, 2003). In SD, the double mutant plant flowers only slightly later than the *tfl2* single mutant, a phenotype that might be explained by the counteracting repression of *FT* by EARLY BOLTING IN SHORTDAYS (EBS) under non-inductive conditions (Gomez-Mena et al., 2001).
The photoperiod pathway merges with the autonomous pathways at the level of FT regulation, so *tfl2* was tested for genetic interaction with the late flowering mutant *fca*, a member of the autonomous pathway (Macknight et al., 1997; Samach et al., 2000). *tfl2-1fca-1* and *fca-1* flowered at the same time showing that *fca-1* is completely epistatic to *tfl2-1*. This result indicates that the two genes work in the same pathway.
In summary, these results show that disruption of *TFL2* gene, the plant *HP1* homologue, causes derepression of genes involved in flower development and

flowering time. The additive phenotype of *tfl2elf3* and *tfl2gi* indicates that TFL2 plays a role in controlling flowering time according to light signals, but that it is likely to act in a different pathway than ELF3 and GI. Moreover, the epistatic relationship with *fca* mutant suggests a possible involvement of TFL2 in the cross talk between the photoperiod and the autonomous pathway. Further investigation of downstream genes regulated by TFL2 repressive activity will help to clarify the role of TFL2 in the regulation of flowering time and meristem maintenance.

TFL2 AND THE MODULATION OF LIGHT SIGNALLING (**VI**)

The *tfl2* mutant was previously described as early flowering and photoperiod insensitive, suggesting a connection with light responsiveness. We could show that the *tfl2* mutation causes, in fact, hypersensitivity to various wavelengths of light and partial derepression of photomorphogenesis in the dark. In addition *TFL2* interacts with another known repressor of light signalling, *COP1*, uncovering new aspects of TFL2 repressive action during development.

TFL2 plays a role as a repressor in the modulation of light signalling

The earliest indication that the *tfl2* mutant has a defect in light perception is the absence of photoperiodic response of hypocotyl elongation. In addition, the *tfl2* mutation affects the sensitivity to different wavelengths of light; the *tfl2* hypocotyl is hypersensitive regarding inhibition of elongation, under red, blue and far-red light. Red light had the strongest effect on *tfl2* seedlings and the concomitant enhancement of cotyledon expansion and inhibition of hypocotyl elongation grown in red light (our unpublished data) confirmed that the *tfl2* mutation enhances normal light-induced photomorphogenic development, as opposed to causing a general defect in seedling growth and development (Quail, 2002). *tfl2* hypersensitivity to red light suggests that TFL2 is likely to play a role in the negative regulation of phyB signalling. Both the *tfl2* phenotype and its sensitivity to red light could be explained by overexpression of *PHYB* or hyperactivation of phyB signalling in the mutant. Nevertheless, the *tfl2* hypocotyl showed some degree of hypersensitivity to blue and far-red light as well, which might be due to the cross talk between different phytochrome signalling

pathways (Hennig et al., 1999). Although the hypocotyl elongation was not significantly reduced in darkness, etiolated *tfl2* seedlings showed a partial photomorphogenic phenotype with release of the apical hook and the slightly expanded cotyledons. The observation suggests that *TFL2* is required to repress some aspects of photomorphogenesis in the absence of light.

TFL2 is required to maintain the expression of light regulate genes

Expression analyses revealed that the expression of light regulated genes is not maintained in the *tfl2* background under white light, suggesting that TFL2 participates in the molecular mechanism that controls gene expression according to light signals. Interestingly, a transient increase in mRNA levels after a short exposure to white light was observed for all the light responsive genes that showed a difference in expression level in darkness and light in *tfl2* background compared to wild type. The expression decreased within 24h, indicating that *tfl2* mutation does not prevent the induction of those genes but, rather, affects the maintenance of their expression state. A subtle effect of the *tfl2* mutation could be noticed in the expression of *COP1*, *PHYA* and *PORA* and this difference could account for the partial photomorphogenic phenotype displayed by the *tfl2* mutant in darkness.

Complex genetic interactions between *TFL2* and *COP1* during plant development

The phenotype of the double mutant combination of *tfl2* and *cop1* grown in light suggests a complex interaction between TFL2 and COP1, key repressor of plant photomorphogenesis and light responses. In darkness, an additive effect was observed in the *tfl2cop1-6* double mutant seedlings, suggesting that TFL2 has a role in skotomorphogenesis and that TFL2 and COP1 act independently to repress photomorphogenesis. In contrast, a partial rescue of *cop1* and the reciprocal rescue of *tfl2* and *cop1* early flowering phenotypes in light, indicate that TFL2 and COP1 might function in the same pathway in repressing flowering.
Similarly to *COP1*, the phytochromes and the cryptochromes, *TFL2* was previously reported to localise to speckles in the nucleus where it is likely to participate in the transcriptional regulation of euchromatic genes (Gaudin et al., 2001). This specific subnuclear distribution, together with the genetic interaction, might represent an

additional evidence of an involvement of COP1 and TFL2 in the regulation of a common set of targets during development.

In conclusion, we have presented molecular and genetic evidence that TFL2 is required for modulating light signalling during development. Disruption of the *TFL2* gene causes hypersensitivity to different wavelengths and misregulation of light responsive genes without affecting the early regulative events. The partial deetiolated phenotype of *tfl2* seedlings in the dark suggests an additional role for TFL2 in repression of photomorphogenesis as also supported by the genetic interaction with COP1.

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