The Role of Gibberellins in the Regulation of Arabidopsis Flowering Time

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Abstract

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The transition to flowering in the facultative long-day plant Arabidopsis (*Arabidopsis thaliana*) have been thoroughly studied and today it is well understood how flowering in response to changes in photoperiod and cold is integrated at the molecular level. However it is less known how flowering is initiated in response to internal factors in the absence of environmental stimuli. Previously it have been shown that flowering under non inductive short-day conditions is dependent on the plant hormone gibberellin (GA).

In an effort to improve the understanding on how GA promotes flowering we have analysed, which of the many GA's that are florally active, and quantified the developmental changes of florally active GA's in relation to floral initiation under short-day conditions. We found that GA_4 had the highest floral activity and that presiding floral initiation there was a dramatic increase at the shoot apex in the content of GA_4. Intriguingly we found that the expression of the rate limiting GA 20-oxidase remained stable at the time when GA started to increase, indicating that increased accumulation of GA in not caused by local production.

With the help of knockout lines we show that removal of either *GA20ox1* or *GA20ox2* delayed flowering and hence that both genes is participating in the production of GA relevant for the transition to flowering. Analysis of the flowering behaviour of *GA2ox* mutants revealed that some *ga2ox* mutants flowered earlier. Reporter gene analysis showed that expression of the GA2ox gene which had highest impact on flowering time was localised to the shoot apex, indicating that the function of the GA 2-oxidase is to control accumulation of GA's in the shoot apex.

Analysis of the effect of late flowering mutations on flowering time in plants with increased GA signal transduction showed that the LD pathway functions mostly in parallel to GA's and that late flowering in autonomous mutants is partially due to decreased GA signal transduction or decreased GA synthetic capacity.

Keywords: Arabidopsis thaliana, Gibberellin, Flowering time, GA20ox, GA2ox, Sucrose, Floral initiation, photoperiod

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Appendixes

Papers I-IV

The present thesis is based on the following papers which will be referred to by their Roman numerals:

I. Böhlenius, H., Eriksson, S., & Nilsson, O. Regulation of *LEAFY* Transcription by Gibberellins and a Labile Repressor**.** (Manuscript)

II. Eriksson, S. Böhlenius, H., Moritz, T. & Nilsson, O. GA_4 is the Active Gibberellin in the Regulation of *LEAFY* Transcription and Arabidopsis Floral Initiation. *Plant Cell 18,* 2172-2181

III. Eriksson, S., Rieu, I., Nilsson, O., Phillips A. & Hedden, P. GA2ox4 Modulates the Level of Active GA to Control the Transition to Flowering in *Arabidopsis*. (Manuscript)

IV. Eriksson, S. & Nilsson, O. Genetic Interactions between Gibberellin Signaling and Flowering Time Genes. (Manuscript)

Introduction

Most parts that we eat from plants are either from the fruit or the seed, which both are formed as a consequence of the transition from vegetative growth to reproductive development. Plants have evolved to flower at a time when there is the highest possible chance for successful seed production. This requires that flowering is initiated at a time of the year that is favourable for flowering in a specific location. This can produce problems when crops are planted at new locations. Growing crops that is not adapted to the local climate can result in that the plants flowers under non-favourable conditions. If the plant flowers to early there is a risk that the flower freezes before pollination and flowering to late can result in failure to produce fruits before winter. Both situations would lead to reduced yield and result in economic losses for the farmer.

The control of flowering is also a major determinant for the generation time. Short generation time of important crops has made it possible to perform breading, leading to dramatic increased production. However some plats, like trees, have evolved a juvenile face where they do not respond to environmental signals and generation time can be several years. The long generation time of trees hinders effective breeding programs. If it was possible to induce earlier flowering in trees it would be possible to perform faster breading for increased wood growth and improved wood qualities. Understanding how flowering are controlled are therefore important to maximise production.

In this thesis I explore the nature of the internal signals that induce the transition to flowering. No single molecule have so far been isolated that are common for the floral induction in all plants. In many plants flowering are promoted by the plant hormone Gibberellin (GA). Although in some species flowering are instead repressed by GA. I have selected to analyse floral induction in the model species *Arabidopsis thaliana* (Arabidopsis). My main interest was to determine how flowering is induced under non inductive photoperiods and especially the role of GA. I have studied the role of GA by first determining which GA´s promotes flowering and then analysing the changes in the plant. Furthermore I wanted to understand how GA is integrated at the molecular level with other known factors that induce flowering in Arabidopsis.

Background

Development of the flower

Plants have indeterminate development and constantly produce new organs. Embryogenesis leads to the formation of cotyledons and two zones of undifferentiated cells, meristems, which will form the shoot and the root respectively. The shoot meristem consists of a set of dividing undifferentiated cells formed into a dome. On the peripheral zone of the meristem cells are recruited to form outgrowth, primordia in a predefined pattern called phyllotaxy. Recent years

of research have shown that the positioning of the next primordia is determined by local changes in the concentration of the growth regulator Auxin (Reinhardt, 2005; Reinhardt, Mandel & Kuhlemeier, 2000; Reinhardt *et al.*, 2003). Initially primordia develop into leaves with an associated auxiliary meristem (Weigel, 1995). At the switch to reproductive development the fate of the youngest primordia is changed from leaf to flower (Hempel & Feldman, 1995; Hempel, Zambryski & Feldman, 1998). Genetic analysis in the model plant Arabidopsis has identified a set of genes that are involved in determining the fate of the primordia called meristem identity genes. Today four different genes, *LEAFY* (*LFY*), *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*), and *FRUITFULL* (*FUL*) have been shown be involved in initiating and maintaining the floral development program (Bowman *et al.*, 1993; Mandel & Yanofsky, 1995; Weigel *et al.*, 1992) . Of these genes *LFY* play a central role and *lfy* mutants fail to initiate real flowers (Weigel*, et al.*, 1992). The central role for *LFY* is further supported by that the expression of *AP1* and *CAL* is induced after *LFY* and that overexpression of *LFY* is sufficient to induce *AP1* expression in the leaf (Kempin, Savidge & Yanofsky, 1995; Mandel *et al.*, 1992; Parcy *et al.*, 1998). Meristem identity genes induce the expression of floral organ identity genes which control the development of the different flower organs (Pidkowich, Klenz & Haughn, 1999).

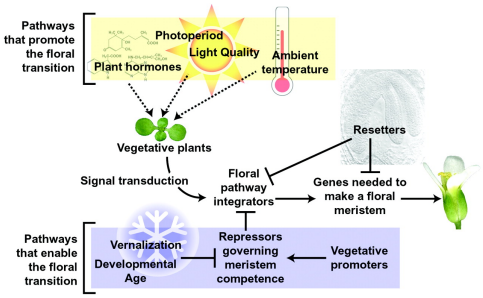

Figure 1. Schematic representation of the different factors determining when flowering is initiated. Flowering is promoted by the environmental factors like photoperiod, light quality and ambient temperature. In the plant there is also a set of factors which determines the competence to respond to environmental stimuli. Picture reproduced from Boss (2004) with the kind permission of the publisher

The transition to flowering is a tightly controlled process that are affected by both internal factors related to the age or size of the plant and environmental stimuli which tells the plant the passing of seasons (Bernier *et al.*, 1993; Bernier & Périlleux, 2005). The ultimate result of all these qualitative factors is the induction of FMI genes (Figure 1). Recent years of research have uncovered how flowering is induced by the environmental factors such as photoperiod and cold. It is less known what other internal factors mediates flowering in Arabidopsis in the absence of environmental stimuli.

Photoperiodic regulation of flowering

For plants grown in temperate regions one predictable environmental factor is the changing duration of daylight. From experiments performed in the beginning of the 20th century it became apparent that flowering of many plants was induced by changes in the day-length. Based on how flowering of plants was affected by changes in day-length they were placed into three groups. Long-day (LD) plants that flowers when the day is longer than a critical length, short-day (SD) plants that

flowers when the day is shorter than a critical time, and day length insensitive plants that flowers at the same time independent of day length (Garner & Allard, 1920). Later it became apparent that the plant do not only simply measure the length of the day, instead there appeared to be an internal rhythm that dictated when the plant was sensitive to light (Bünning, 1936). The initial model proposed by Bünning (1936) has been revised to include the finding that light entrains an internal rhythm (Pittendrigh & Minis, 1964), termed the circadian clock, to the solar cylcle, and is known as the "external coincidence model". (Yanovsky & Kay, 2003). Recent experiments in the facultative LD plant Arabidopsis have illustrated that circadian regulation of *CONSTNAS* expression and stabilisation of the CO protein by light is the central components in photoperiodic induction of flowering (Searle & Coupland, 2004; Yanovsky & Kay, 2003). When high levels of CO protein coincide with light it activates the expression of *FLOWERING LOCUS T* (*FT*) (Figure 2). The same components involved in induction of flowering by LD in Arabidopsis have also been shown to induce flowering of rice (*Oryza sativa*) in response to SD (Izawa *et al.*, 2002).

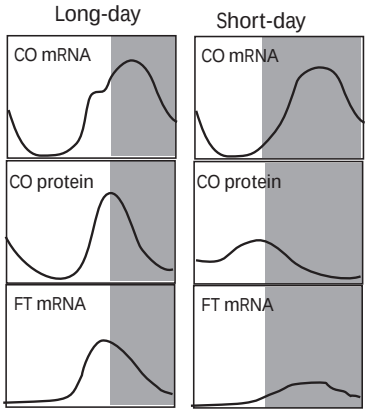

Figure 2 The external coincidence model. Constans mRNA shows diurnal cycling under both long-day and short day conditions. In long-day where CO mRNA coinsides with light CO protein becomes stabilised and induce the expression of flowering locus T. The biphasic expression pattern in LD is caused by light dependent degradation of the CO repressor CDF through FKF1

Light perception and the internal time keeper

One predictable environmental cue is the rhythmic day and night cycle. Plants have evolved a set of photoreceptors in order to be able to adjust development programs to changes in both the length of the light period and changes in light spectra (light quality). The main photoreceptors are the blue light sensing, crypthochromes and phototropins, and the red/far-red sensing phytocromes (Briggs *et al.*, 2001; Cashmore *et al.*, 1999; Smith, 2000). The cryptochromes and phytochromes controls growth and development in response to changing spectrum, light intensity and diurnal duration (Cashmore*, et al.*, 1999; Smith, 2000), whereas the phototropins is mainly involved in determining directional growth in response to directional light signal and chloroplast movement (Briggs & Huala, 1999; Sakai *et al.*, 2001). There are two cryptochromes, *CRYPTOCHROME* (*CRY*)*1* and *CRY2*, and five phytochromes, *PHYTOCHROME* (*PHY*)*A* to *PHYE*, that all are involved in resetting the internal oscillator to the diurnal day and night cycle (Yanovsky & Kay, 2003). It have not been clearly established how the photoreceptors resets the internal oscillator, but one mechanism that has been proposed to be involved is the interaction between light activated phyB with the transcription factor PHYTOCHROME-INTERACTING FACTOR 1 (PIF3), resulting in upregulation

of the morning factors *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*), that are central components of the plant circadian clock (Martínez-García, Huq & Quail, 2000).

All parts of the central oscillator/internal time keeper are not yet known but like other circadian clocks, rhythmic cycling of mRNA and auto regulation appears to play central role (Barak *et al.*, 2000; Harmer, Panda & Kay, 2001). In addition to the morning factors *CCA1* and *LHY* there are important roles for the evening factors *TIMING OF CAB EXPRESSION 1* (*TOC1*), *EARLY FLOWERING 4 (ELF4)*, and *LUX ARRYTHUMO* (*LUX*) (Doyle *et al.*, 2002; Hazen *et al.*, 2005; Strayer *et al.*, 2000). CCA1 and LHY which are two closely related MYB transcription factors repress the expression of *TOC1* and *LUX* in the morning by binding to a special evening element in their promotes (Alabadi *et al.*, 2001). TOC1 indirectly induces the expression of *CCA1/LHY,* thus repression of *TOC1* leads to decreased expression in the evening of *CCA1*/*LHY*, allowing expression of *TOC1* in the evening. Increased *TOC1* then leads to high levels of *CCA1/LHY* in the morning. Out-put from the circadian clock to the induction of flowering is performed through *GIGANTEA* (*GI*), which shows circadian oscillation, and in a *gi* mutant there is no cycling of *CO* (Fowler *et al.*, 1999; Mizoguchi *et al.*, 2005; Suarez-Lopez *et al.*, 2001).

CO, FT and photoperiodic induction of flowering

CO and *FT* was initially identified as mutants that flowered late in LD, but not in SD (Koornneef, Hanhart & van der Veen, 1991). Cloning of CO showed that it was a zink finger protein and a potential transcription regulator (Putterill *et al.*, 1995). FT is a protein with sequence similarity to the animal phosphatidylethanolamine binding protein or Raf kinase inhibitor protein (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). Ectopic expression of *CO* leads to early flowering, irrespective of day length, in Arabidopsis pointing to central role for *CO* in LD induction of flowering (Onouchi *et al.*, 2000). Removal of *FT* blocks earlier flowering of *CO* overexpressors showing that *CO* induce flowering through activation of *FT* (Moon *et al.*, 2005; Samach *et al.*, 2000). Analysis of *CO* expression was initially reported to be higher in LD than in SD (Putterill*, et al.*, 1995). Closer investigation of *CO* expression in LD showed that CO transcripts showed a diurnal pattern with highest levels at the end of the light period (Suarez-Lopez*, et al.*, 2001). In SD there is a similar circadian expression pattern for *CO*, but instead of a peaking in the light there is a peak in the dark. In LD, where *CO* expression coincides with light, there is a induction of *FT* in the evening (Figure 2). This implies that *CO* expression could be part of a mechanism determining a light sensitive phase of an internal rhythm. In agreement to this moving *CO* expression in SD towards the light, either with non 24-h periods or mutants with changed circadian period, induces earlier flowering by inducing *FT* expression (Blázquez, Trenor & Weigel, 2002; Roden *et al.*, 2002; Yanovsky & Kay, 2002).

Light regulation of CO expression

In addition to an indirect effect of light on *CO* expression through the circadian clock it also affects CO transcription at the end of the light period. In LD, there is a

broad peak of CO expression which is promoted by *FLAVIN-BINDING, KELCH REPEAT, F-BOX* (*FKF1*) (Imaizumi *et al.*, 2003; Nelson *et al.*, 2000). Expression of *FKF1* peaks in the evening and light activated FKF1 protein interacts with *CYCLING DOF FACTOR 1* (*CDF1*), a repressor of *CO*, and induce protein degradation of CDF1. (Imaizumi*, et al.*, 2003). Light also have posttranslational effect on the CO protein, explaining the light sensitive face of the internal rhythm. In plants having constantly high level of *CO* expression, *FT* transcripts still shows circadian cycling due to stabilisation of CO protein by light (Valverde *et al.*, 2004). Light stabilise *CO* through the photoreceptors CRY1, CRY2 and phyA, while phyB induce degradation of CO. In the photoreceptor mutants there are no induction of *FT* by light but also no dramatic difference in CO protein showing that light in addition to control stability of CO potentially activates the protein (Valverde*, et al.*, 2004).

Photoperiod detection in the leaves

Analysis of the temporal expression pattern of *CO* and *FT* shows that they are central components in the induction of flowering. Early experiments on photoperiod induction of flowering indicated that light was sensed in the leaves, where a substance was produced that when transported to the shoot apiece induced the formation of flowers (Chailakhyan, 1936; Knott, 1934). Both *CO* and *FT* have been shown to be expressed in the vasculature of the leaves (Takada & Goto, 2003). Overexpression of *CO* in leaves, but not the shoot apiece, leads to earlier flowering, and to the production of a graft transmissible signal that can induce flowering (An *et al.*, 2004; Ayre & Turgeon, 2004). Earlier flowering by expression of *CO* in leaves requires *FT* and contrary to *CO*, *FT* expressed in the shoot apices can induce flowering. This shows that *CO*/*FT* plays a central role in the production of the leaf derived signal responsible for flower formation in the shoot apices. To increase the requirement for LD induction there is modifications of the *FT* chromatin. Activation of *FT* by CO in the leaf is antagonised by the action of the chromatin associated protein LHC1 (Takada & Goto, 2003). Loss-of-function *lhc1* mutation leads to increased *FT* expression and earlier flowering in SD. Furthermore, *FT* is also repressed by the putative polycomb protein EARLY BOLTING IN SHORTDAYS (EBS) (Gomez-Mena *et al.*, 2001; Pineiro *et al.*, 2003). The *ebs* mutant, like *lhc1*, is early flowering in SD due to increased *FT* expression.

FD a link between FT in leaf and flowering

Recently it has been shown that induction flowering by *FT* requires the FD protein (Abe *et al.*, 2005; Wigge *et al.*, 2005). *FD* was identified independently by two groups in screens for proteins that interacted with FT or in a screen for mutants that suppressed earlier flowering caused by *FT* overexpression. Analysis of the expression pattern showed that the spatial expression of *FT* and *FD* do not overlap. While *FT* is expressed in the vasculature in the leaf (Takada & Goto, 2003), *FD* is predominantly expressed in the apex where it induces expression of the flower meristem identity gene *AP1* (Abe*, et al.*, 2005). That *FT* expressed in the apex but not *CO* can induce flowering suggest that *FT* plays a central role in the production of the elusive florigen (An*, et al.*, 2004). FT is a small protein and GFP, which is a

larger protein, can when expressed from a companion cell specific promoter move to the shoot sink tissue (Imlau, Truernit & Sauer, 1999). This raises the possibility that FT can move from the leaf to the shoot apex and induce flowering. Recently it has been shown that the FT function is conserved in many plant species. Over-expression of *FT* has so far been shown to induce flowering in tomato (*Solanum lycopersicon*), *Citrus* , *populus*, tobacco (*Nicotiana tabacum*), and rice (Böhlenius *et al.*, 2006; Endo *et al.*, 2005; Kojima *et al.*, 2002; Lifschitz *et al.*, 2006). Grafting of tobacco shoots expressing tomato *FT* could induce flowering of the SD tobacco Maryland Mammoth, which can remain vegetative for many years if not exposed to short days (Lifschitz*, et al.*, 2006). Interestingly FT protein has been identified in phloem extracts of the Arabidopsis relative *Brassica napus* (Patrick Giavalisco, 2006). In addition to movement of the FT protein, there is a possibility that the *FT* mRNA could move (Huang *et al.*, 2005). However, in tomato no *FT* mRNA was observed in the apex from the scion (Lifschitz*, et al.*, 2006).

Regulation of flowering by the floral repressor FLC

Many plants from temperate zones require exposure to a long period of cold (vernalization), indicative of passing of winter, before they can respond to photoperiod (Amasino, 2005; Chouard, 1960; Dennis, Helliwell & Peacock, 2006). In many species the role of vernalization are to suppress the expression of a repressor of flowering. Suppression is stable through mitosis even after passing of the cold period, indicative of epigenetic regulation (Sung & Amasino, 2005). From classical studies in henbane it was shown that remembrance of cold was retained during growth in non inductive photoperiods, and that the plants flowered upon transfer to inductive conditions (Lang, 1965). Furthermore it has been shown from experiments in *Lumoria biennis* that cold sensing occurs in the meristem (Wellensiek, 1962; Wellensiek, 1964). By regenerating plants from different cold treated tissue it was found that only plants regenerated from the apex, and not the leaf flowered after transfer to inductive conditions. Similar results have also been shown for Arabidopsis where only plants generated from dividing cells retains vernalization response (Burn *et al.*, 1993). In contrast to photoperiodic induction there is no production of a graft transmissible signal, instead vernalisation regulates the competence to respond to floral stimuli (Sung & Amasino, 2005).

The occurrence in a species of plants that show either winter or summer annual habit has made it possible to determine how many locus that are involved in setting winter requirement for flowering. Winter annual behaviour in Arabidopsis are determined by the locuses *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) (Koornneef *et al.*, 1994; Lee *et al.*, 1994; Napp-Zinn, 1955; Napp-Zinn, 1957). FLC prevents flowering by antagonising the activity of *CO* on *FT* and *SUPRESSOR OF CONSTANS OVEREXPRESSION1* (*SOC1*). In addition to vernalization, *FLC* expression is also controlled by members in the autonomous pathway that prevents accumulation of FLC transcripts (Figure 3) (Simpson, 2004). Control of FLC expression has been shown to involve covalent modifications of histones in the *FLC* chromatin and post transcriptional regulation of mRNA (He & Amasino, 2005).

Vernalization

FRI induce high transcription of the MADS box protein FLC that acts to repress flowering (Johanson *et al.*, 2000). Exposure to cold, antagonise activation by FRI and leads to decreased *FLC* expression and earlier flowering (Michaels & Amasino, 1999; Sheldon *et al.*, 1999). Repression of *FLC* is stably maintained through mitosis, indicative of epigenetic regulation of the *FLC* locus. Covalent modifications of histones, also called histone code (Fischle, Wang & David Allis, 2003), are involved in determining transcriptional activity of the *FLC* locus (He & Amasino, 2005). Active transcription before onset of winter is associated with acetylation of lysine, 9 and 27, of histone 3 (H3) and dimethylation of H3 lysine 4, modifications associated with active chromatin (Bastow *et al.*, 2004; He, Michaels & Amasino, 2003). Winter, long exposure to cold, leads to loss of H3 acetylation and induce trimethylation of H3 lysine, 9 and 27, which are hallmark of repressed chromatin (Sung & Amasino, 2005). Initiation of histone modifications in the *FLC* chromatin caused by vernalization requires the action of *VERNALIZATION INSESITIVE3* (*VIN3*) (Sung & Amasino, 2004). Expression of *VIN3* is induced in the shoot apiece after prolonged cold exposure decreasing the risk that fluctuations in temperature during fall induces flowering. The expression pattern of *VIN3* is consistent with sensing of cold in the shoot apex. *VIN3* associates with *FLC* chromatin and initiates removal of acetyl groups from H3 tails leading to decreased *FLC* expression (Sung & Amasino, 2004). Expression of *VIN3* is lost after return to warm temperatures (coming of spring) which shows that *VIN3* only helps establish repressed chromatin and is not involved in stable repression. Instead stable repression is produced by *VERNALISATION1* (*VRN1*) and *VRN2* that probably becomes recruited to the *FLC* chromatin after repression by VIN3 (Gendall *et al.*, 2001; Levy *et al.*, 2002). VRN1 and VRN2 produce dimethylation of histone H3 lysine 9 and 27 which is required for stable repression of *FLC*. Maintenance of stable chromatin also requires binding of LIKE HETROCHROMATIN PROTEIN1 (LHCP1) (also called TERMINAL FLOWER 2, TFL2), an Arabidopsis homolog to *HETROCHROMATIN PROTEIN1* (*HP1*) (Mylne *et al.*, 2006; Sung *et al.*, 2006). *LHP1* in drosophila has been shown to be associated to euchromatin by binding to histone H3 lysine 9 (Liu *et al.*, 2005; Nielsen *et al.*, 2001). In the *lhcp1* mutant there is initial increase in H3 lysine 9 dimethylation but, it disappears after transfer to warm conditions. This shows that *LHCP1* is required for retaining methylation of H3 lysine 9.

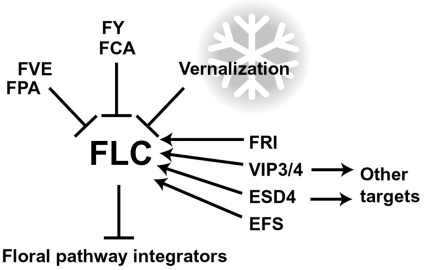

Figure 3. repression of flowering through FLC
There is many factors that dictate the expression of the floral integrator LFC. Accumulation of FLC is prevented by the members of the autonomous pathway and by vernalization. High level of FLC transcription is induced by functional alleles of FRIGIDA. Picture reproduced from Boss (2004) with the kind permission of the publisher

Autonomous pathway

Genes belonging to the autonomous pathway was initially defined by late flowering mutants that responded to photoperiod and had a strong vernalization response (Koornneef, Hanhart & van der Veen, 1991). Today seven genes *FCA* (Macknight *et al.*, 1997), *FPA* (Schomburg *et al.*, 2001), *FY* (Simpson *et al.*, 2003), *LUMINIDEPENDENSE* (*LD*) (Aukerman *et al.*, 1999), *FLOWERING LOCUS D* (*FLD*) (He, Michaels & Amasino, 2003), *FVE* (Ausin *et al.*, 2004), and *FLOWERING LOCUS K* (*FLK*) (Lim *et al.*, 2004), have been shown to belong to this pathway. Result from double mutant analysis indicated that the members of the autonomous pathway controlled flowering independently of each other, with the exception of *FCA* and *FY* (Koornneef *et al.*, 1998). The autonomous pathway genes promotes flowering by preventing accumulation of the of *FLC* (Michaels & Amasino, 2001; Sheldon*, et al.*, 1999). Indications from the additive effect on flowering time in double mutants that most members controlled flowering separately is also supported by increased accumulation of FLC protein in the double mutants (Rouse *et al.*, 2002). This suggests that the genes in the autonomous pathway acts separately to prevent accumulation of *FLC*. Today, the genes corresponding to all the late flowering mutants have been cloned. With this knowledge it is now possible to piece together how accumulation *FLC* is prevented by the autonomous pathway.

Genetic interaction between *FCA* and *FY* was confirmed by interactions at the molecular level (Simpson*, et al.*, 2003). *FCA* encodes a protein with RNA recognition motif and a WW protein interaction domain (Macknight*, et al.*, 1997). *FCA* shows complex post transcriptional regulation with four alternative spicing variants; α, β, γ and δ, where γ corresponds to the full length protein. Interestingly, *FCA* negatively controls it own accumulation by promoting premature cleavage and polyadenylation on a promoter proximal acceptor site, producing transcript β which results in a truncated protein (Quesada, 2003). This requires interaction between FCA and FY, which encodes a 3'-end processing factor; through the WW domain of FCA (Simpson*, et al.*, 2003). In addition to the requirement for *FY* in the autoregulation of FCA pre-mRNA, *fy* mutation also suppress early flowering promoted by *FCA* γ mRNA overepression.

FPA and *FLK* also encode RNA binding proteins and *LD* encodes a homeobox protein, homeobox proteins mostly bind DNA but have on rare occasions been shown to interact with RNA (Rivera-Pomar *et al.*, 1996), a clear indication that post transcriptional regulation is important for repression of *FLC*. However, no alternative splice variants have been reported for *FLC* and thus it remains to be determined how the RNA binding proteins prevents the accumulation of *FLC* transcripts. It could be that the transcripts of one of the many factors that needed for *FLC* expression is regulated by these RNA binding proteins (He & Amasino, 2005).

Resent studies shows that FVE and FLD control *FLC* accumulation by chromatin modification at the *FLC* locus (Ausin*, et al.*, 2004; He, Michaels & Amasino, 2003). In both *fve* and *fld* mutants there is hyperacetylation of H3 tails, which is not observed in the other autonomous mutants. FLD is a deacetylase that together with FVE, a retinoblastoma homolog, promotes removal of histone H3 K4 acetyl

groups which is hallmarks for active chromatin (He, Michaels & Amasino, 2003). Double mutants between *fve* and *fpa* shows no additive delay of flowering time or increased accumulation of FLC suggesting that these proteins worked as a pair (Koornneef*, et al.*, 1998; Rouse*, et al.*, 2002). However in *fpa* mutants there is no hyperacetylation of histones in the *FLC* chromatin, thus it is unclear how *FPA* interacts with *FVE*. In the L*er* background where the double mutant analysis was performed there is no delayed flowering for *fld*, whereby double mutants between *fpa* and *fld* was not investigated (Sanda & Amasino, 1996).

The genes in the autonomous pathway have been proposed to promote flowering in response to endogenous signals. However, no change in regulation of the genes has been shown in relation to the floral transition (Simpson, 2004). It could be that the genes controls flowering in response to yet undefined environmental conditions. Recently it was shown that *FCA* could be responsible for modulating flowering in response to the plant hormone ABA (Razem *et al.*, 2006). It was reported that FCA bound ABA in vitro and that this binding inhibited interaction between FCA and FY. Consistent with this finding they showed that treatment of plants with ABA caused production of more full length *FCA* γ transcripts. There was also delayed flowering time associated with an increased *FLC* expression, following repeated ABA treatments. If ABA binding to FCA is important for the regulation of flowering time then there should exist some growth condition that delays flowering in WT but not in *fca* plants. It would therfore be interesting to analyse endogenous levels of ABA during growth to determine if there is correlation between ABA levels and flowering that is dependent of FCA.

Summer annual growth habit of natural accessions of Arabidopsis has evolved from winter annuals by loss of either *FRI* or *FLC* (Gazzani *et al.*, 2003; Johanson*, et al.*, 2000; Michaels *et al.*, 2003). In winter annuals with dominant variants of *FRI* and *FLC*, *FRI* antagonises the effect of the autonomous genes. Vernalization on the other hand antagonises accumulation of *FLC* by FRI, or by mutation in the autonomous pathway. Before winter there would be high levels of *FLC* due to induction by FRI. After winter there would be low levels of *FLC* due to chromatin modifications produced by the vernalization pathway. Thus it is unclear what the function of the autonomous pathway has in a FRI containing line. Recent identification of null alleles of *fy* highlights a role for *FY* in embryo development. Null *FY* mutants are embryo lethal, showing that *FY* has important roles outside flowering (Henderson *et al.*, 2005).

FLC antagonise action of the LD pathway

FLC has been shown to regulate flowering by preventing induction of *FT* and *SOC1* (Michaels & Amasino, 1999; Sheldon*, et al.*, 1999; Sheldon *et al.*, 2000). FLC interacts with a CArG box in the *SOC1* promoter and in the first intron of *FT* (Helliwell *et al.*, 2006; Hepworth *et al.*, 2002). However, although promoter *FT:GUS* fusions is repressed by FLC there appears to be no binding of *FLC* to the *FT* promoter (Helliwell*, et al.*, 2006), suggesting that FLC also indirectly repress *FT*.

Cold treatment of the shoot apices is sufficient to induce flowering in vernalization requiring plants (Sung & Amasino, 2005). Consistent with this there is highest expression of *pFLC:GUS* fusions in the apiece (Michaels *et al.*, 2005),

indicating that *FLC* repress flowering mostly in the shoot apex. However, using different tissue specific promoters Sheldon et al (2006) showed that full repression only was achieved by expressing *FLC* in both the phloem and shoot apiece (Searle *et al.*, 2006). They showed that FLC represses, *SOC1* and *FT* in the leaf, and *SOC1* and *FD* in the apex. Binding of FLC to the *SOC1* promoter antagonise activation by the LD pathway (Hepworth*, et al.*, 2002). However, high levels of FLC do not inhibit early flowering of CO overexpression plants.

Internal compounds involved in modulating flowering

For some plants flowering is photoperiod insensitive and for these induction of flowering is believed to be induced in response to factors related to the developmental state of the plant. In Arabidopsis these factors controls flowering under non inductive SD conditions. It has been reported that sugars and the plant gibberellin hormone is involved in promoting flowering (Bernier*, et al.*, 1993; Corbesier & Coupland, 2005).

Control of flowering by sugars

There have been contradictory reports on the role of sugars in the regulation of flowering. Evidence for a role of sugars in the induction of flowering comes experiments where Arabidopsis and *Sinapis alba* grown in SD is induced to flower by one LD or a displayed SD (lights on later then normal) (Corbesier *et al.*, 1996). This has been shown to be associated with an early increase of sucrose at the shoot apices(Corbesier*, et al.*, 1996; Lejeune, Bernier & Kinet, 1991; Lejeune *et al.*, 1993). The increased export of sugars appears not to be due to increased photo-assimilate production; instead export of sucrose comes from starch mobilisation. Evidence for the importance of starch mobilisation also comes from analysis of mutants, impaired in starch synthesis or starch utilisation. The starch-less mutants *adg*, deficient in ADP GLUCOSE PYROPHOSPHORYLASE, and *pgm* deficient in plastid PHOSPHOGLUCOMUTASE, flowers like WT in continuous light, but have decreased growth rate and delayed flowering in SD (Corbesier, Lejeune & Bernier, 1998; Yu *et al.*, 2000). The delayed flowering can be restored by vernalization showing that late flowering is not due to slower growth. Similarly, *sex1* that have excess starch due to inability to utilise starch (Yu *et al.*, 2001), flowers late, especially in SD, and can be rescued by vernalization (Corbesier, Lejeune & Bernier, 1998).

Furthermore it have been shown that sucrose supplied to the shoot apices removed the vernalization requirement from the winter annual ecotypes Stockholm and Leiden (Roldán *et al.*, 1999). Addition of sucrose also accelerates flowering in co, gi fve fpa, and fca, but not in ft mutants. This implies that the sugar effect goes through FT but does not require the LD pathway gene CO.

Promotion of flowering by GA

Initially GAs was identified in Japan as a substance produced by the fungi Gibberella fujikori that caused overgrowth of rice plants (Phinney, 1983). Later research showed that the dwarf pea mutant, initially isolated by Mendel, was

impaired in the GA synthesis, showing that GA is an endogenous compound involved in control of stem elongation. Studies of more mutants impaired in synthesis of GA have shown that GA, in addition to controlling stem elongation, is involved in diverse processes throughout the life cycle of plants. GA deficient mutants are normally dwarf with small dark green leaves and is impaired in germination and development of flower organs (Ross, Murfet & Reid, 1997).

That GA is involved in regulating the transition to flowering was first gained from studies which showed that exogenous application of GA accelerated flowering of many plants (Zeevaart, 1983). Flowering of Arabidopsis was also shown to be influenced by GA application (Langridge, 1957), but the significance was only gained from studies of mutants impaired in either GA synthesis or response. The severe dwarf *ga1-3*, which has a large deletion of *GA1* locus encoding *ent*-copalyl diphosphate synthase (Sun & Kamiya, 1994), is slightly late flowering in LD but failed to flower in SD (Wilson, Heckman & Somerville, 1992). This shows that GA is absolutely required for flowering in SD. Failure of *ga1-3* to flower in SD is at least partially due to a requirement for GA to induce *LFY* and *SOC1* expression (Blázquez *et al.*, 1998; Moon *et al.*, 2003). In WT plants, grown in SD, there is a gradual increase of *LFY* expression until flowering. In *ga1-3* that does not flower in SD, there is no induction of *LFY* expression. Treatment of *ga1-3* plants with GA promotes flowering and restores *LFY* expression (Blázquez*, et al.*, 1998). Interestingly it has been shown that both the GA- and the LD- pathway are integrated at the *LFY* promoter (Blázquez & Weigel, 2000). In *ga1-3* plants grown in LD there is an induction of *LFY*, although delayed compared to WT. Mutations in genes in the LD pathway leads to decreased *LFY* expression in LD (Nilsson *et al.*, 1998). Consistent with that *co ga1* mutants sometimes fails to flower in LD there is no increased *LFY* expression (Blázquez & Weigel, 2000; Putterill*, et al.*, 1995). By analysis of the behaviour of deletions in the *LFY* promoter it was shown that induction by GA and the LD pathway required separate elements of the promoter. Deletion of a region encompassing a potential myb binding site abolished activation by GA and created an obligate requirement for LD to induce *LFY* expression.

In addition to induce *LFY*, GA also has been shown to induce the expression of *SOC1* (Moon*, et al.*, 2003). In *ga1-3* mutant there is no induction of *SOC1* during growth in SD. However it is unclear if the induction of *SOC1* by GA is a direct effect. Although GA appears to play a central role in the induction of flowering little is known how endogenous GA levels are correlated with induction of flowering.

Gibberellin biosynthesis

GA is a product from the diterpenoid pathway and its production start from the common precursor gernalylgernaly diphosphate (GGPP), which is also the precursor of cartenoids and becomes incorporated into chlorophyll, whose levels in plants are much higher than GA (Hedden & Phillips, 2000; Olszewski, Sun & Gubler, 2002). Synthesis of GA must thus be tightly regulated. Elucidation of the steps involved in the production of GA was help by isolation of dwarf mutants which was restored to normal growth after GA application, indicating that the mutant was impaired in a step in the synthesis of bioactive GA. Initially,

Arabidopsis mutants were obtained from five separate loci named *GA1*-*GA5* (Koornneef & Veen, 1980). Today the genes corresponding to all the mutations have been cloned and the specific block in the pathway have been determined (Figure 4)(Hedden & Phillips, 2000; Olszewski, Sun & Gubler, 2002).

Production of bioactive GA starts with the cyclisation of GGPP to *ent*-kaurene via *ent*-copalyl diphosphate catalysed by *ent*-copalyl diphospate synthase (CPS) corresponding to *GA1*, and *ent*-kaurene synthase (KS) corresponding to *GA2*, respectively. These two steps are performed in the proplastids which have been confirmed by import of GFP labelled enzymes into these organelles (Helliwell *et al.*, 2001). The conversion of *ent*-kaurene to GA_{12} aldehyde takes place on the endomembrane outside the plastids, requiring transport of *ent*-kaurene out of the plastid by an unknown mechanism. The conversion of *ent*-kaurene to GA_{12} is catalysed by a set of cytochrome P450 dependent mono-oxygenases where the *ent*-kaurene oxidase (KO), corresponding to *GA3,* catalyse the three successive oxidations of *ent*-kaurne to *ent*-kaurenoic acid. GA_{12} can in some plants be further converted by oxidised on C-13 to form GA_{53}. The last steps in the production of bioactive GA_1 and GA_4 are done in parallel reactions in the cytosol by a set of 2-oxoglutarate dependent dioxygenases. Formation of GA_{20} and $GA_{9,}$ from GA_{12} and GA_{53}, respectively, is done by three successive oxidations resulting in the loss of a carbon catalysed by Gibberellin 20-oxidase (GA20ox) corresponding to *GA5*,. The last step is the addition of a hydroxyl group on C-3 of, GA_{20} and GA_9, leading to the formation of, GA_1 and GA_4, respectively, catalysed by GA 3-oxidase (GA3ox) corresponding to *GA4*. The enzymes up until the formation of GA_{12} are encoded by single genes reflecting the severe GA deficiency phenotype of strong mutant alleles of *ga1*, *ga2* and *ga3*. The latter steps in the production of bioactive GA are encoded by multiple genes explaining the less severe phenotype of *ga4* and *ga5* (Koornneef & Veen, 1980). GA20ox is encoded by five genes, *GA20ox1* to *GA20ox5*, where *GA5* corresponds to GA20ox1. GA3ox is encoded by four genes, *GA3ox1* to *GA3ox4*, where *GA4* corresponds to GA3ox1 (Hedden & Phillips, 2000).

Bioactive GAs, and immediate precursors, can be deactivated by GA 2-oxidase, which also is a 2-oxoglutarate dependent dioxygenase. In Arabidopsis there is eight genes encoding GA2ox called *GA2ox1* to *GA2ox8*.

Regulation of GA synthesis

The identification of the genes for the enzymes involved in the production of bioactive GA has made it possible to investigate how the synthesis of GA is controlled at the genetic level. There is only low expression of *CPS* in Arabiopsis which was taken as an indication that the formation of *ent*-kaurene is a rate limiting step in the production of bioactive GAs (Silverstone *et al.*, 1997). However ovexpression of the early genes *CPS* and *KS* have no effect on the level of bioactive GAs (Fleet *et al.*, 2003). Instead there is evidence for that the steps catalysed by GA20ox is an important control point. Expression of genes encoding GA20ox and GA3ox is negatively regulated by active GAs (Chiang, Hwang & Goodman, 1995; Phillips *et al.*, 1995; Xu *et al.*, 1999). Constitutive expression of *GA20ox1* leads to the formation of higher levels of bioactive GAs (Coles *et al.*, 1999; Huang *et al.*, 1998). Consistent with *GA20ox* being an important control

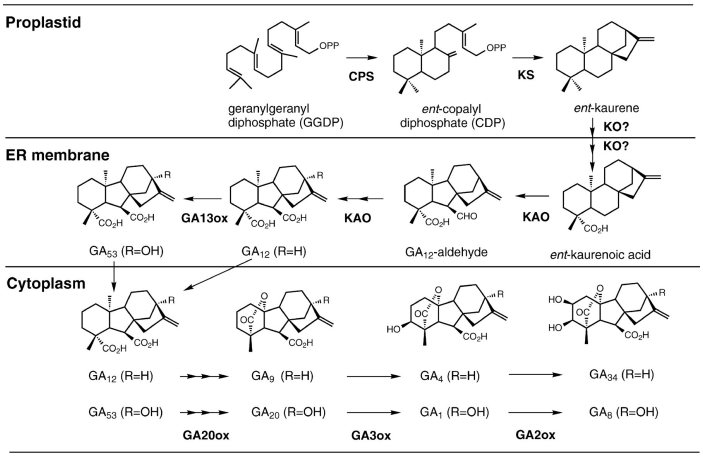

Figure 4. Production of bioactive GA's from the precursor GGDP. The biosynthetic pathway shows sub-cellular compartmentation. The last steps in the formation of bioactive GA_1 and GA_4 occurs in parallel pathways in the cytoplasm. Picture reproduced from Olszewski (2002) witth the permission of the Publisher.

point, expression of *GA20ox* is regulated in response to environmental signals (Garcia-Martinez & Gil, 2001; Wu *et al.*, 1996; Xu, Gage & Zeevaart, 1997). Expression of *GA20ox* in many rosette plants is induced by LD treatment; consistent with induction of stem elongation at flowering. In addition to down regulation of *GA20ox* and *GA3ox* by bioactive GAs, there is induction of genes encoding the deactivation enzyme GA2-ox (Thomas, Phillips & Hedden, 1999). This allows the plant to keep the level of bioactive GAs within a predefined limit.

Site of GA synthesis

The first step in the synthesis of GA was shown to take place in pro-plastids and not in mature chloroplast (Aach *et al.*, 1997; Aach, Böse & Graebe, 1995). Based on these results it was proposed that GA synthesis takes place in young expanding tissue where GA regulated responses takes place. The *ent*-kaurene production activity and the level of bioactive GAs has also been shown to be highest in these tissues (Chung & Coolbaugh, 1986; Smith *et al.*, 1992). In an attempt to determine the site of GA synthesis in rice Kaneko et al (2003) detected that the expression of two *GA20ox* and *GA3ox* genes coincided with the tissue exhibiting GA regulated growth, indicating that GAs was synthesised at the site of action (Kaneko *et al.*, 2003). However, grafting experiments performed in pea shows that GA precursors and active GAs can be transported through the graft junction (Proebsting *et al.*, 1992). Although there are lower level of bioactive GA in older tissue, there is still a high capacity to synthesise active GA (Ross *et al.*, 2003). Lower level of bioactive GA is due to an increased action of GA2ox.

The expression pattern of *CPS* in Arabidopsis gives support for the idea that older leaves retains capacity to produce GA (Silverstone*, et al.*, 1997). *CPS* expression was found to be associated with the major and minor veins in both young and old leaves. Further support for that leaves can act as a source for GAs

comes from analysis of the spatial expression pattern of *GA3ox* genes(Mitchum *et al.*, 2006). It was shown that *GA3ox1* and *GA3ox2* are the most important for production of bioactive GA during vegetative growth and that the two genes had different expression patterns. However, both genes were shown to be expressed in mature rosette leaves. Expression of *GA3ox1* was highest in the base of the leaf while *GA3ox2* expression is associated to the vasculature bundles, similar to the expression pattern of *CPS*. To get the full picture it will be necessary to also analyse the expression of the *GA20ox* genes in detail. Investigation of the expression of *GA2ox* in rice plants gives an indication that regulation of *GA2ox* activity is important in the induction of flowering. During vegetative growth there was *GA2ox* expression in a ring around the shoot apex which became absent at time of floral transition (Sakamoto *et al.*, 2001). This implies that the *GA2ox* prevents GAs from entering the shoot apex. Interestingly similar expression pattern have been shown for two *GA2ox* genes in Arabidopsis (Jasinski *et al.*, 2005).

Gibberellin response pathway

Mutant screening for GA dwarf plants identified a mutant *gibberellin insensitive-1* (*gai-1*) that did not respond to applied GA (Koorneef *et al.*, 1985). The phenotype caused by the mutation is due to a gain of function deletion of 17 amino acids in a region of the protein that is important for proper GA regulation (Peng *et al.*, 1997). *GAI* was shown to belong to the DELLA subfamily of the putative GRAS transcription regulator family (Pysh *et al.*, 1999). In Arabidopsis there are five DELLA proteins, GAI, REPRESSOR OF GA1-3 (RGA); RGA LIKE (RGA)1 (RGL)1, RGL2, and RGL3 that are involved in separate processes (Lee *et al.*, 2002; Peng*, et al.*, 1997; Silverstone, Ciampaglio & Sun, 1998; Wen & Chang, 2002). Loss of function mutations in DELLA genes leads to plants with increased GA response, indicating that DELLA proteins acts as repressors of GA induced responses. In Arabidopsis RGA and GAI are regulating most GA responses during the vegetative phase. Combination of loss function *rga* and *gai* mutations restores most growth defects caused by *ga1-3*, except GA requirement for germination and flower development (Dill & Sun, 2001; King, Moritz & Harberd, 2001). Germination is regulated predominantly by *RGL2* and flower development by *RGA*, *RGL1* and *RGL2* (Cao *et al.*, 2005; Cheng *et al.*, 2004; Tyler *et al.*, 2004). While Arabidopsis contains several DELLA proteins, only one have been identified in rice, SLENDER RICE 1 (SLR1) (Ikeda *et al.*, 2001). Mutations in *SLR1* lead to slender plants of increased height. GA responses have also been shown to be regulated by DELLA proteins in wheat and barley (Chandler *et al.*, 2002; Gubler *et al.*, 2002). Interestingly the "green revolution" varieties introduced in the 60th contains gain of function mutations in the DELLA domain similar to the Arabidopsis gai-1 (Peng *et al.*, 1999).

GA functions as a de-repressor by inducing degradation of DELLA proteins (Thomas & Sun, 2004). Recent cloning of the genes corresponding to two rice dwarf mutants, *gibberellin dwarf1* (*gid1*) and *gid2* has help to unravel how GA is perceived and how the signal is transmitted to induce GA regulated responses. Initially it was found that the *gid2* dwarf mutant was due to mutation in an F-box factor, member of the SCF E3 ubiqutin ligase complex (Gomi *et al.*, 2004). The F-box factor gives the substrate specificity to the SCF complex. SCF^{GID1} interacts

with the rice DELLA protein SLR1 and targets the protein for degradation by ubiqutination and subsequent degradation by the 26S-proteosome. Similarly to the situation in rice it was shown that the unresponsive sleepy1 (sly1) mutant encoded an F-box protein that interacts with RGA and GAI upon GA treatment and targeted them for degradation by the 26S proteosome (Dill *et al.*, 2004; McGinnis *et al.*, 2003).

The rice mutant *gid1* was later shown to be due to a mutation in a protein that acted as a soluble receptor for GA (Ueguchi-Tanaka *et al.*, 2005). GID1 preferentially binds to bioactive GAs. Interestingly, GID1 interacts with SLN1 after GA binding, thus targeting the DELLA protein for degradation. In Arabidopsis there exists three orthologs to the rice GID1 protein, which all shows highest binding affinity to the bioactive GA_4 (Nakajima *et al.*, 2006). Loss-of-function mutations in only one lead to no obvious phenotype indicating that the genes acts redundantly to regulate GA responses.

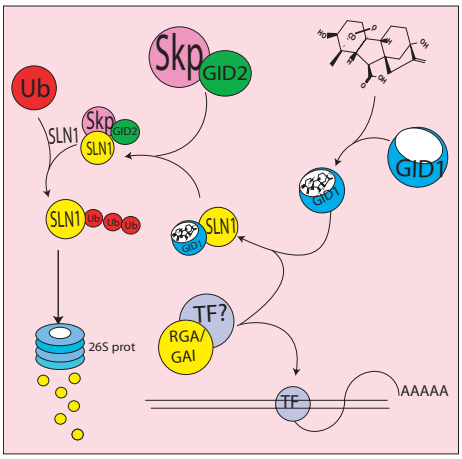

Figure 5. Gibberllin signal transduktion. Gibberellins binds to the soluble GA receptor GID1 resulting in recruitment of DELLA proteins. This potentially release a transcription factor that can activate transcription. Binding of GID1 to DELLA leads to interaction with the SCF^{GID2} E3 ubiqutin ligase complex resulting in ubiqutionation of DELLA proteins and subsequent degradation through the 26S proteosome

Objectives

The broad aim of my research was to improve on the knowledge of the molecular processes controlling Arabidopsis floral initiation under non-inductive SD conditions, and especially the role of the plant hormone gibberellin. The main questions were

- Which of the different GAs are regulating flowering in Arabidopsis?
- What is the molecular mechanism for induction of *LFY* expression by gibberelin signalling?
- How is the synthesis of GAs regulated in SD and where does the GA synthesis take place?
- How is gibberellin signalling or metabolism integrated with the other flower promotion pathways?

Methodological overview

Methods of gene expression analysis

Analysis of the amount of transcripts is often used to determine which genes are involved in regulating a specific process. Many techniques have been developed to detect the transcription of genes. The first method to analyse the level of transcripts was northern blots where transcripts is detected by hybridisation with a labelled complementary nucleic acid. However northern blots have limited sensitivity, thus it is necessary to isolate large amounts of RNA. With northern blot it is difficult to analyse transcripts if you have small samples. To get more sensitive estimates, amplification of transcripts by PCR prior to detection is done. In some cases it is also of interest to get the spatial expression pattern for a gene. For this methods have been developed where the promoter of the gene is fused to reporter transcripts whose product is easier to detect in the tissue.

Analysis of transcripts by RT-PCR

In paper II we wanted to analyse expression of genes involved GA synthesis in micro dissected tissue samples. For this I selected to use a two step reverse transcription (RT) based PCR method. This involves isolation of total RNA followed by RT to get a representative cDNA sample. In the next step transcripts are amplified with specific primers and the product is separated on a gel. In order to get a large linear range I developed a non-radioactive membrane based detection method where DIG-labelled nucleotides are incorporated in the product during the PCR. The products are then detected on membranes with anti-DIG antibodies followed by fluorescent detection. In order to compare expression in different samples it is necessary to normalise the signals obtained for the gene of interest. We selected to use 18s ribosomal RNA as an internal reference gene and calculate expression relative to 18S. To minimise risk of methodological errors we amplified the reference and the gene specific products in the same tube. Since 18S is present at much higher levels it is necessary to inhibit the amplification in order to get similar amount of target gene and reference. We minimized amplification of 18S by use of competimers (primers that compete for binding but that can't be amplified) in combination with normal primers. Under optimal conditions there is doubling of the transcripts after each cycle. With competimers only a fraction of 18S is amplified in each cycle and by modifying the ratio normal primers:competimers it is possible to get the same amount of reference product and target gene. If all PCR reactions are still exponentially amplified, the limiting factor is the dynamic range of selected detection system. Incorporating epitopes into the products that is later detected with antibodies, coupled to fluorescent reaction, gives less background and larger linear range compared to quantification of EtBr stained geles.

Transcript analysis by real-time PCR

With end-point analysis it is not possible to quantitatively compare samples that have bigger difference than the dynamic range of the selected detection method. An improvement to end-point analysis is real-time detection where products are measured at each cycle during the PCR. Expression is then calculated as the cycle when the signal is higher then a threshold level (Wong & Medrano, 2005). Based on the efficiency of the PCR it is possible to calculate the amount of starting transcripts. The principle for real-time detection is that there is increased fluorescence signal with increased products. There are different techniques in which this can be achieved. We have selected to run the PCR with SYBR Green that gives increased signal after binding to double stranded DNA. One drawback with this is that all products, including primer dimmers, is contributes to the signal. By performing a melt-curve analysis, where temperature is gradually increased, it is possible to determine if specific products were amplified. If there is more then one melt peak in the tube it is not possible to calculate the amounts of transcripts. One limit with SYBR is that only one product can be amplified per tube, thus reference gene and target must be amplified in separate tubes. By using fluorescent labelled primers it is possible to detect more then one product per tube, but like with SYBR green all products amplified by the primer gives signal. To increase specificity it is possible to include a probe complementary to a sequence between the binding sites of primers.

To get accurate estimates of the transcript level it is important that the reaction is robust and that the efficiency of the PCR is good (Marino, Cook & Miller, 2003). The efficiency of PCR decrease with increasing length of product and is also affected by secondary structures. Special care should therefore be used when selecting the amplicon to be amplified.

When studying gene expression it is usually not interesting to know the exact amount of transcripts in the sample, instead relative calculations are usually performed. It could be possible to compare absolute transcript amounts between samples but it would be difficult to get relevant results (Bustin, 2000). In absolute quantification values would depend on the sample size, amount of RNA, efficiency of extraction and reverse transcription that have been shown to be highly variable (Mannhalter, Koizar & Mitterbauer, 2000). In order for that to be possible it is necessary to add external control transcripts during extraction of the RNA. The external RNA would then be used to correct for differences introduced during RNA extraction and cDNA synthesis. This is the normal method used for analysis hormones or metabolites. The second approach is to normalise against the total amounts of RNA. This ignores effects introduced by different efficiency of reverse transcription and RNA integrity for different samples. Although, normalisation to total RNA have been shown to be the least unreliable (Bustin, 2002). There is also a concern about the ratio mRNA:total RNA in different cells.

The most common method is to calculate expression relative to a stably expressed gene. In order for this to be possible it is important that the selected reference gene is stably expressed; same expression in all tissue, unaffected by treatment and unchanged with age (Thellin *et al.*, 1999). So far no gene has been reported to have the same expression all the time (Brunner, Yakovlev & Strauss, 2004; Radonic *et al.*, 2004). We have selected to use 18S as an internal standard.

18S is highly expressed and the content is relatively stable. One concern is that 18S is transcribed by RNA polymerase I while mRNA is transcribed by polymerase II. Best normalisation is achieved from analysis of more then one reference gene and then calculates which shows most stable expression in the samples tested. The most stable reference genes are then used to calculate a mean which is later used for normalisation (Pfaffl *et al.*, 2004; Vandesompele *et al.*, 2005).

Methods for detecting spatial expression patterns

The most direct way to analyse the tissue specific expression pattern is to perform in situ hybridisation. This requires time consuming steps and it is difficult to perform large scale analysis. Limitations with in situ are that you have to analyse expression on sections and it is therefore most suited for sub cellular localisation, and not whole plant localisation. There is also limiting sensitivity and it is difficult to get good results with lowly expressed genes.

Instead of investigating localisation of the endogenous transcripts promoter reporter gene constructs are often used. In reporter gene constructs a part of the gene thought to contain all regulatory cis elements are fused to a transcript which product is possible to detect in the tissue. In some cases there are transcriptional or post transcriptional regulatory cis elements in, intron or exons, and in those situations it is necessary to include part of the coding region to get expression pattern that reflect distribution of endogenous transcripts. Reporter genes used in plants include, fluorescent proteins, uidA encoding b-glucoronidase (GUS), fire fly luciferease.

We have selected to use promoter GUS fusions which gives possibility to get whole-mount staining with the use the chromogenic substrate x-gluc resulting in a blue precipitate. With GUS fusion it is also possible to do quantitative analysis of gene expression with substrates that give a fluorescent signal. Reporter gene constructs is introduced into the plant with Agrobacterium that leads to random insertion of the T-DNA, containing the reporter gene, into the chromosome. Depending on where the T-DNA is inserted it is possible to get different expression level. To avoid looking at positional effects it is necessary to analyse the expression of several independent lines.

Discovery of gene function by mutant screening

Classical screen to identify genes involved in regulation of flowering was done with chemical or radiation induced mutagenesis. Recently mutant screening also exploit the almost random insertion of T-DNA into the chromosome. There are pros and cons with all methods. With chemical mutagenesis it is possible to get saturation with relatively few plants due to multiple mutations per plant. There are on the other hand problems to isolate the mutated gene. With T-DNA insertions it is easier to isolate the gene responsible for the phenotype but T-DNA only leads to ~1.5 inserts per plant meaning that more plants needs to be analysed to get saturated screening.

Chemical mutagenesis

I have selected to do mutagenesis with ethylmethane sulfonate (EMS) according to guidelines proposed by Gerge P Rédi (1992). EMS mostly leads to the conversion A to G giving G-C instead of A-T. I performed the mutagenesis with 0.2% EMS in 16h which have around 1 chlorophyll mutant per 200 plants. Screening for mutants is done in two steps: growth of plants from mutagenised seeds and collection of M1 seeds, growth of the M2 generation to isolate mutant plants. Depending of the cost of growing the different generations, different strategies is selected for the size of the different generations. Previous studies have shown that 2 cells in the seeds give rise to all seeds in the next generation. In order to have 88% chance of getting a specific mutant in M2 from one M1 plant it is necessary to plant 16 M1 seeds. Screening for late flowering plants requires large space and it is therefore of interest to get as many different mutant as possible from limited M2 generation. I selected to grow M1 pools of 500 plants. In the M2 1000 seed was planted from each pool leading to that each M1 plant on average is represented by two plants in M2.

Large scale knock-out programs

Analysis of mutations in genes is a powerful tool to determine the function of a gene. In plants it is not possible to do targeted mutagenesis of a gene of interest which previously made it impossible to analyse the function of different members in large gene families. With T-DNA insertions it is possible to create knock out plants if the if the T-DNA is inserted in a gene. This has been exploited in joint large scale screening projects to isolate many T-DNA insertion lines. Flanking sequences of the insert is determined and placed into searchable database. In the database it is possible to search for T-DNA insert lines in your candidate genes and seeds from that plant can then be ordered from a stock centre. We have used this approach to determine the contributions of the different GA2ox genes. There are eight different GA2ox genes in the Arabidopsis genome and they are likely to be redundant. It is unlikely that mutations in any of them could have been identified with traditional screenings.

Results and Discussion

Transcriptional regulation of LFY expression (I)

Early evidence for that *LFY* is involved in promoting flowering in response to GA came from the finding that *LFY* expression was dramatically reduced in GA deficient mutants under SD (Blázquez*, et al.*, 1998). This is also substantiated by that GA treatment induces *LFY* expression (Blázquez*, et al.*, 1998). Analysis of deletions in the *LFY* promoter revealed that GA activation of *LFY* transcription was mediated through a cis-element with similarity to R2R3 MYB binding site (Blázquez & Weigel, 2000). It has been proposed that GA induce *LFY* expression through *AtMYB33* that is similar to *HvGAMYB*, which is responsible for induction

α -amylase in the alurone layer (Gocal *et al.*, 2001). In the alurone GA induction of GAMYB is independent of protein synthesis while induction of α-amylase requires synthesis of new proteins (Gubler *et al.*, 1995). However it have not been shown whether GA directly induce *LFY* expression, or if GA occurs through secondary factors. In paper I we analysed short term GA treatments on *LFY* induction in order to study whether GA induction is an early molecular event. This showed that GA induction of *LFY* was rapid and reaches maximum induction within one hour. Pre-treatment with the translational inhibitor cycloheximide (CHX) did not inhibit GA induction of *LFY*, suggesting that the factors required for induction are already present in the seedlings.

The CHX treatments uncovered that *LFY* was controlled by a labile negative regulator. Treatment with CHX resulted in accumulation of more *LFY* mRNA than treatment with GA. Similar results has been reported for *GAMYB*, where CHX induction is likely the result of degradation of DELLA proteins (Gubler*, et al.*, 2002; Gubler*, et al.*, 1995). However, we found that CHX induction of *LFY* is not mediated through the action of DELLA proteins. Evidence for this comes from that CHX treatment resulted in increased transcription of *pLFY:GUS* reporter gene constructs where the GA-response-element is removed. Furthermore, CHX induced transcription of *LFY* in plants with loss-of-function *gai* and *rga* mutations, the DELLA proteins responsible for repressing GA response during vegetative growth. Together these data show that the negative regulator is functioning independently of the GA pathway.

Initial CHX treatment was performed on whole seedlings, thus increased *LFY* mRNA accumulation could result from ectopic induction. However when CHX treatment was performed on different tissue we only detected accumulation of *LFY* in shoot apex samples. This suggests that the function of the negative regulator is to control the action of an activator. In an attempt to determine which factors controls *LFY* expression through the activator/negative regulator we analysed the CHX effect in mutants where *LFY* transcription is known to be affected (Blázquez & Weigel, 2000; Nilsson*, et al.*, 1998). Treatment with CHX induced transcription in all tested mutants, indicating that the activator/negative regulator controls *LFY* transcription by an undiscovered mechanism. Analysis of *LFY* reporter genes constructs showed that the CHX effect on *LFY* transcription requires an element in the first 246 bases of the promoter. Previously no factors have been identified that control *LFY* in this region.

Gibberellin and floral initiation in SD (II)

In Arabidopsis grown under SD there is a gradual increased of *LFY* expression until it reaches a threshold level where a newly formed primordia is formed into a flower. In the *ga1* GA deficient mutant flowering under SD is abolished, at least partially, due to failure to upregulate *LFY* expression. However, although there is a clear genetic link between GA and *LFY* expression it has not been shown whether gradual increased *LFY* expression is caused by an increased accumulation of bioactive GAs (Blázquez*, et al.*, 1998; Blázquez & Weigel, 2000). Previously GA quantifications required gram size samples making it impractical to analyse tissue specific analysis. With the help of improved Gas chromatography mass spectroscopy (GC-MS) protocols using heavy weight isotope labelled standard we

were able to quantify GAs in micro dissected shoot apical samples corresponding to the tissue expressing *LFY*. In order to establish connection between GA, *LFY*, and floral induction it is also necessary to establish which GAs is responsible for inducing flowering in Arabidopsis.

GA_4 is the active GA in control of Arabidopsis flowering

In total 126 different GAs have been identified in plants, fungi and bacteria (MacMillan, 2001). Although, most are either precursors, or deactivation products, of bioactive GAs and only a few possesses intrinsic biological activity. Previously it have been reported that GA_4 is the most active in regulating cell elongation, shoot growth, and feed-back regulation of GA3ox1 expression in Arabidopsis (Cowling *et al.*, 1998; Talon, Koornneef & Zeevaart, 1990; Xu, Gage & Zeevaart, 1997). However, it has not been shown which GAs is active in regulating Arabidopis flowering. In *Lolium temulentum* it has been shown that GA_5 and GA_6 are the active GAs involved in regulating flowering, whereas they have only low activity in regulation of stem elongation (King *et al.*, 2003; King *et al.*, 2001). Through the use of dose-response experiments for induction of *LFY* expression we show that GA_3 and GA_4 is equally active when applied to older plants, while GA_4 is almost 10 times more active when applied to seedlings grown in liquid culture. Together with that GA_3 and GA_4 was the most efficient in inducing flowering in the *ga1* mutant these data suggests that GA_4 is the most active in promoting flowering in Arabidopsis. These findings are supported by analysis of the binding specificity for the newly identified GA receptor GID1 (Ueguchi-Tanaka*, et al.*, 2005). In Arabidopsis there are three orthologs to the rice protein GID1 that all show highest affinity to GA_4.(Nakajima*, et al.*, 2006).

GA_4 accumulates in the shoot apex at the time of floral initiation

In order to investigate whether there is any change in GA content related to floral initiation in SD we quantified GAs in micro dissected shoot apices. The samples were delimited by the tissue expressing *LFY* during vegetative growth. In addition to analysing the developmental changes in GAs in the apex we were also interested in whether there was any change in sugar content in relation to floral initiation.

We selected to base transition to flowering on the expression of *AP1* and *AP3* which is only expressed in developing flowers (Hempel *et al.*, 1997; Jack, Brockman & Meyerowitz, 1992). Expression of *AP1* was first detected in samples collected form 42 day old plants, whereas that of *AP3* was detected in 49 day old plants. This indicates that, in a majority of the plants, floral initiation occurs between day 42 and 49.

The quantification of GAs showed that GA_4 was the most abundant, of all tested GAs, at all time points. Initially there was a high level of GA_4 in the shoot apical region, which subsequently decreased and remained low until day 42. Interestingly the level of GA_4 increased about 30 times between day 35 and 42. At the same time as the levels of GA_4 started to increase we also detected increased accumulation of sucrose, but not that of the monosaccharides glucose and fructose. This suggests that Arabidopsis floral initiation in SD is presided by a dramatic increase in GA and sucrose at the shoot apex. The dramatic increase is surprising

considering that the plants were induced to flower under constant conditions without environmental trigger. This suggests that the rapid increase in bioactive GA in the shoot apex is triggered when the plants reach a critical age or size.
It should be noted that we did not detect a clear correlation between GA content in the apex and *LFY* expression. In 14- and 21- day old plants there is low *LFY* expression even though there were high levels of GA_4 at these time points. Furthermore there is almost no change in *LFY* expression at the time when GAs started to increase in the shoot apex. This discrepancy can be explained by that local change in the expression of *LFY* in primordia could be masked by *LFY* expression in the base of young leaves.

Transport of GAs from leaves to shoot apex

An interesting issue, raised by the sharp increase of GA in the shoot apex before floral initiation is whether the GAs are produced locally or transported from outside sources. The last steps in the formation of bioactive GAs performed by GA 20-oxidase and GA 3-oxidase have been shown to be rate limiting steps in the formation of bioactive GAs (Eriksson *et al.*, 2000; Huang*, et al.*, 1998). The expression of many of the *GA20ox* and the *GA3ox* genes is subjected to feed-back regulation by bioactive GAs (Cowling*, et al.*, 1998; Xu *et al.*, 1995; Yamaguchi *et al.*, 1998). Thus, analysis of the expression pattern of these genes can give valuable information about where the increased GA content in the shoot apex is originating from. At the time when GA started to increase there was unchanged expression of *GA20ox* and *GA3ox* genes, suggesting that increased GA levels is not caused by local induction of *GA20ox* or *GA3ox*. However, although *GA20ox1* was unchanged when GAs started to increased there was increased expression after flower initiation prior to bolting. This is surprising considering that the expression of *GA20ox* is down regulated by high levels of bioactive GA, or increased GA signal transduction (Xu*, et al.*, 1999). That GA20ox1 expression is induced even though there is high levels of bioactive GA in the tissue suggests uncoupling of feed-back regulation after transition to flowering. Similar mechanism must function in plants shifted from SD to LD where there is increased *GA20ox* expression and increased GA production (Wu*, et al.*, 1996; Xu, Gage & Zeevaart, 1997).
The level of bioactive GAs are also affected by the activity the deactivation enzyme GA2-oxidase. Increased GA content in the apex could therefore be the result of decreased expression of *GA2ox* genes in the apex. However, we detected increased expression of *GA2ox2* and *GA2ox4* when GA started to accumulate in the apex. This is in agreement with previous

Figure 6. Aerial after GA application to one leaf. The *ga1* mutant was grown in SD and 10µl of 10µM GA_4 was applied to a single leaf. The inactivation product GA8 was used as control

experiments which show that GA2ox genes often is induced by high level of bioactive GAs (Thomas, Phillips & Hedden, 1999). Together with unchanged expression of *GA20ox* and *GA3ox* these data suggests that accumulation of GA_4 in the shoot apex is not caused by local changes in expression of GA metabolism genes. Instead GAs is likely to originate from outside the shoot apex. We detected that application of GA to one leaf of *ga1* mutant plants resulted in an expansion of all leaves that could be observed already one day after application (Fig 6). Repeated GA application resulted in flowering indicating that GA can be transported from the leaf to the shoot apex and induce earlier flowering.

Modified expression of GA metabolism genes affects time to flowering (III)

In paper II we detected a dramatic increase in GA_4 at the shoot apex presiding initiation of flowering in SD. Analysis of the expression of GA metabolism genes suggested that the GAs is originating form outside the shoot apex. The site of GA synthesis can be investigated by analysis of the expression pattern for genes involved in the formation of bioactive GAs. However in order for that to be fruitful it will be necessary to determine exactly which genes that are involved in maintaining GA homeostasis during vegetative growth in SD. It can be hypothesised that removal of the gene involved in controlling GA levels relevant for floral initiation should affect the time to flowering. We therefore selected to analyse the effect of mutations in different members the *GA20ox* and *GA2ox* gene families, on flowering time under SD conditions.
The only *GA20ox* gene for which mutants have been described is *GA20ox1* (Koornneef & Veen, 1980; Sponsel *et al.*, 1997). For the *GA2ox* genes no loss of function mutation has been identified by mutagenesis in Arabidopsis. We therefore selected to analyse T-DNA insertion lines in different *GA20ox* and *GA2ox* genes obtained in the Columbia background.

Removal of either *GA20ox1* or *GA20ox2* resulted in delayed flowering indicating that both genes participate in the control of GA homeostasis during vegetative growth. While *GA20ox1* and *GA20ox2* have overlapping function in the production of bioactive GA during vegetative growth, *GA20ox1* has a distinct role in formation of GAs required for stem elongation after flowering. The specific defect for *ga20ox1* in stem elongation is supported by findings in paper II where expression of *GA20ox1*, but not *GA20ox2*, started to increase in the shoot apical samples after transition to flowering. These findings suggest that GAs required for stem elongation is formed at the shoot tip. The double mutant *ga20ox1 ga20ox2* is almost dwarfed to the same extent as *ga1* mutants. This suggests that *GA20ox1* and *GA20ox2* are the major *GA20ox* genes controlling synthesis of bioactive GAs during vegetative growth. In contrast to *ga1*, *ga20ox1 ga20ox2* initiated flowering showing that some of the other *GA20ox* genes manages to produce sufficient amounts of GAs required for flowering. These data shows that the expression pattern of both *GA20ox1* and *GA20ox2* has to be considered before it can be determined were GAs relevant for flowering is being formed.

For the analysed *GA2ox* T-DNA insertion lines only insertion in *GA2ox4* caused significantly earlier flowering. In combination with *ga2ox2* there was even earlier flowering, suggesting that *GA2ox2* and *GA2ox4* have partially redundant function.

Reporter gene studies showed that *GA2ox4* was only expression in, and around, the shoot apex. This expression pattern has previously been shown for both *GA2ox2* and *GA2ox4* in seedlings grown in LD (Jasinski*, et al.*, 2005). Together these data points to that GA2ox2 and GA2ox4 have redundant function in controlling the entry of GA into the shoot apex. This sets a requirement for a large increased GA production in order to overcome deactivation by GA2ox in the apex.

Interaction between GAs and other factors known to induce flowering (IV)

Although GAs has a central role in the induction of flowering in SD, little is known about the how the GA signal is integrated with the other known factors inducing flowering. The only connection that has been thoroughly established is the activation of *LFY* by GAs. Analysis of interactions between late flowering mutants and plants that have either impaired GA synthesis, or reduced GA response, have revealed that GAs mostly functions in parallel to the LD- and the autonomous- pathway (Putterill*, et al.*, 1995; Reeves & Coupland, 2001). However these analysis has been hampered by that severe GA deficient mutants and plants impaired in GA signalling fails to initiate flowering in SD conditions, therefore making double mutant analysis in SD conditions non informative.

We selected to take a different genetic approach to characterize how the GA pathway is integrated with the other pathways. We analysed the effect of increased GA signal transduction, through loss of the major DELLA proteins GAI and RGA, coupled to a severe reduction of the biosynthesis of GAs through mutation in *GA1*, on flowering of mutants from the different pathways. These plants are locked in maximal GA response and have lost the ability to regulate GA signalling both through changes in GA production and GA signal transduction. As a starting point we chose to analyse the effect of mutations in, *CO* and *FT* from the LD pathway, *FCA* from the autonomous pathway, and *SOC1* from the floral pathway integrator group. In addition to analyse the effect of the single mutants in the constitutive GA signalling background we analysed the effect of all combinations between the late flowering mutations.

Based on the findings we propose that *SOC1* is not an important target for induction of flowering by GA. The *soc1* mutation caused the same delayed flowering in both WT background and in the constitutive GA signal transduction background. Previously *SOC1* have been suggested to integrate signals from the GA pathway. This is based on that *SOC1* expression is lower in ga1 mutants and that GA treatment resulted in increased SOC1 expression (Moon*, et al.*, 2003). However if *SOC1* is an important target for GA then there should have been a larger delay in plants where flowering is promoted by increased GA signalling.

The autonomous pathway mutation *fca* delayed flowering to a lesser extent in plants with increased GA signal transduction. This is an indication that the activity of the GA pathway is decreased in autonomous pathway mutants. From analysis of dose-response curves on *LFY* activation by GAs, there appears to be slightly reduced sensitivity towards GA in *fca* mutants. These findings suggest that the autonomous pathway partially inhibits flowering by repressing GA action.

In agreement to previous reports we detected that the LD pathway works mostly in parallel to the GA pathway (Reeves & Coupland, 2001). However, analysis of mutant combinations in WT background revealed that *CO* in addition to promoting flowering in LD, can function as a repressor of flowering under SD in *fca* mutants. Earlier flowering by the *co* mutation was not observed in plants with increased GA signal transduction, suggesting that *CO* potentially reduces the activity of the GA pathway. A repressive role has previously been suggested for the rice CO homologue Hd1. In SD, which induce flowering in rice, *hd1* flowers late, while it flowers earlier in non inductive LD (Yano *et al.*, 2000).

Except for in *fca* mutants there was no acceleration of flowering by increased GA signalling in LD. Previously it have been suggested that *co* mutants is daylength insensitive, meaning that it flowers after the same time irrespective of photoperiod. However *co* mutants flowers earlier under LD conditions than under SD. We propose that earlier flowering of co mutants in LD potentially is caused by increased activity of the GA pathway. The expression of the rate limiting *GA20ox* is induced in plants shifted from SD to LD resulting in increased GA levels (Xu, Gage & Zeevaart, 1997). The missing effect of increased GA singling in LD could thus be caused by that GA signalling is already saturated under LD.

Mutant screen for identification of SD floral activators (-)

The classical mutant screens for genes affecting transition to flowering in Arabidopsis was performed under inductive LD condition in summer annual ecotypes. These screen identified genes that promoted flowering in response to changes in photoperiod and genes that promoted flowering independent of photoperiod. The latter class of genes, belonging to the autonomous pathway, have been shown to promote flowering by preventing accumulation of the floral repressor FLC. However, *flc* mutants only flowers slightly earlier than WT plants under SD conditions, indicating that other factors must be involved in promoting flowering in SD. The result in paper IV also highlights that it remains to be discovered what factors that are inducing flowering in response to GAs. Furthermore there must be some factors that dicate when the synthesis of GA should be induced.

Therefore it remains to be discovered how flowering is promoted under SD conditions. We selected to set up a screen for identification of genes involved in promoting flowering in plants grown under SD conditions. In order to prevent isolation of new alleles of the autonomous pathway genes we performed the mutant screen in flc-3 mutant background, where mutation of autonomous genes has no affect on flowering time.

Up until now 5000 M2 plants, corresponding to seeds from ~2500 individual M1 plants, have been analysed for late flowering under SD conditions. This resulted in 18 potentially late flowering mutants. Seeds were collected from these plants and flowering time was analysed for individual plants in the next generation. Out of the 18 selected M2 plants five resulted in late flowering M3 plants (Figure 7B). Because we were specifically interested in genes involved in promoting flowering in SD flowering time was also analysed in LD. Only M3 plants from line 4.7 flowered with the same amount of leaves as *flc-3* (Figure 7A). This suggests that the mutation in 4.7 specifically affects flowering under SD conditions. From some

of the M2 plants it was apparent that the there was different flowering time classes in LD. This can either be caused by that the M2 plant was heterozygous for a dominant or semi-dominant mutations or that there is other mutations that antagonise the action of the late flowering mutation.

Plants from pool 1 to 3 showed an increased tendency for late flowering in LD while there was unchanged flowering in SD. This is the results of a faulty timer in the growth room where these M2 parents were selected. Upon analysis of the light profile it was discovered that the light was turned on in the middle of the dark period. This resulted in acceleration of flowering by night brake treatment.

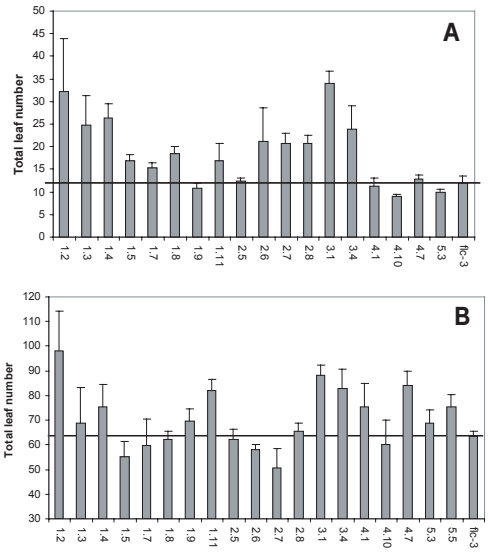

Figure 7. Flowering time for isolated late flowering mutants
(A) and (B) Total leaf number at flowering for M3 plants under (A) LD and (B) SD conditions. Numbers expressed as the mean +/_ 2xSE error of the mean

However, the identification of 4.7 shows that this is a fruitful approach to identify potentially new factors controlling transition to flowering specifically in non inductive conditions. The class of mutants that are flowering late in both LD and SD can also not be placed in any of current pathways. Almost all mutants delaying flowering in both SD and LD have been shown to belong to the autonomous pathway. However we performed the mutant screen in a flc mutant background where autonomous pathway mutations should have no effect on flowering.

Conclusions

The work presented in this thesis shows that floral initiation in Arabidopsis under non inductive SD conditions is presided by a dramatic increase of the bioactive gibberellin GA_4 and sucrose at the shoot apex. Together with that the decision whether to initiate flowers ultimately occurs in the developing organs at the shoot apex this suggest that Arabidopsis flowering under SD is induced by accumulation gibberellin. Decreasing the activity of the rate limiting GA 20-oxidase by loss-of-function mutation in either of the two predominantly expressed GA20ox genes, GA20ox1 and GA20ox2, resulted in delayed flowering. In contrast, decreasing the level of the deactivation enzyme GA 2-oxidase results in earlier flowering. Reporter gene studies showed removal of GA 2-oxidase activity at the shoot apex

is the cause for earlier flowering. It still remains to be discovered where the GA20ox genes are expressed to produce GA's required for initiation of flowering. However one attractive model is that decreasing *GA20ox* expression reduces the export of GA from the leaves. Based on hypothesis it is possible to postulate that the level of GA in the shoot apex depends on the total biosynthetic capacity in the plant and the activity of GA 2-oxidase in the shoot apex. In order compensate for the decreased biosynthetic capacity in the leaf of *ga20ox* mutants the plant have to reach a larger size before the level of GA exported from the whole plant surpass the activity of GA 2-oxidase in the shoot apex. Conversely reducing GA 2-oxidase activity allows GA to accumulate earlier leading to that the plants initiates flowers at a smaller size. However, this model have to be verified by quantification of changes in GA content in the shoot apex of the mutants. There is also a question where the two GA20ox genes are active in order to produce GA required for flowering. The specific shoot elongation defect for the *ga20ox1* indicates that GA required for stem elongation after bolting is localised in to the shoot tip.
Analysis of interaction between the GA pathway and the other known flower promotion pathways revealed that GA promotes flowering mostly independently of the other pathways.

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