Analysis of the function of the CONSTANS protein and the transcriptional regulation of *FLOWERING LOCUS T*

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“Nature doesn’t care what anyone thinks.”
Richard Feynman (1918-1988)

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Abstract

The transition to flowering is one of the most important developmental switches in the life of a plant. For some species this switch appears only once in life and is tightly regulated. Flowering is directly coupled to reproductive success and therefore has to occur under optimal conditions. Several genetic pathways regulate the transition to flowering. CO is a central component of the photoperiod pathway and mediates flowering in response to day length by regulating the expression of *FT*. CO encodes a B-Box transcription factor that also contains a plant specific CCT-domain. Since CO does not contain a known DNA-binding motif I conducted yeast-two-hybrid screening to identify proteins that recruit CO to DNA. In addition, I conducted yeast-one-hybrid screening to identify proteins regulating *FT* expression. Here I present evidence that CO interacts with all members of the heterotrimeric CCAAT-box-binding factor (CBF). The CBF-complex consists of three subunits named HAP2, HAP3 and HAP5. HAP3 and HAP5 dimerize and associate with the DNA-binding subunit HAP2. Both CCT-domain and HAP2 proteins contain the HAP2-DNA-binding motif. Mutations affecting conserved residues in this domain cause loss-of-function phenotypes in CCT-domain proteins, which might be due to an impaired interaction with HAP2. The *HAP3a* subunit is co-regulated with *CO* by *GIGANTEA* and expression of a putative dominant negative transgene causes late flowering. MtN19 was found to interact with the CCT-domain of CO and with the *FT* promoter in yeast. Studies on this gene suggest a possible regulation by natural antisense transcription since transgenic plants expressing a dsRNAi construct are late or early flowering. The early flowering lines express *MtN19*, *CO* and *FT* at high levels. Late flowering plants can not be rescued by overexpression of *CO* by the *35S*-promoter. *MtN19* and *CO* are both expressed at the end of the light period in long days implying that they might function together to regulate *FT* expression. *FIDGET* (*FIT*) was isolated by yeast-one-hybrid screening and found to bind the *FT* promoter. It encodes an APETALA2-like protein. Misexpression of *FIT* in the phloem, the place where *FT* is naturally expressed, accelerates the floral transition. Moreover, I present evidence that *FIT* is expressed in vascular tissue upon UV-light induction. Finally, I show that UV-light is able to accelerate the floral transition. Two other AP2-like proteins delay the floral transition when expressed in the phloem and can also interact with the *FT* promoter. In summary, this thesis presents the first insight how CO may regulate expression of the floral integrator *FT* and proposes evidence for a novel flowering time pathway involving stress-induced AP2-like transcription factors.

Zusammenfassung

Die Entscheidung zur Ausbildung der Blüte ist einer der wichtigsten Prozesse in Pflanzen. Viele Pflanzen blühen nur einmal während ihres Lebens weshalb dieser Prozess einer strengen Kontrolle unterliegt. Da das Ausbilden der Blüte mit dem reproduktiven Erfolg des Organismus gekoppelt ist, muss dieser Entwicklungsschritt unter optimalen Bedingungen erfolgen. Vier verschiedene genetische Signalwege, die in die Regulation der Blütenbildung involviert sind, wurden bisher beschrieben. Einer der wichtigsten Signalwege, der die Ausbildung von Blüten und somit den Wechsel von vegetativem zu reproduktivem Wachstum einleitet ist der photoperiodische Signalweg. Licht bewirkt das Einstellen der inneren cirkadianen Uhr, welche die zyklische Expression des *CONSTANS* (*CO*) Gens bewirkt. Die Stabilität des CONSTANS Protein wird durch Licht unterschiedlicher Wellenlängen reguliert. *Arabidopsis thaliana* ist eine fakultative Langtagpflanze, was bedeutet, dass sie in Lagtagbedingungen schneller zur Blüte kommt. CO bewirkt die florale Transition in Erwiderung auf eine lange Photoperiode durch Aktivierung von *FLOWERING LOCUS T* (*FT*). CO ist ein B-Box Transkriptionsfaktor mit einer pflanzenspezifischen, carboxyterminalen CCT-domäne. Da CO keine bekannte DNA-Bindedomäne besitzt haben wir Yeast-two-Hybridscreens durchgeführt um Proteine zu isolieren durch die CO an DNA binden kann. Darüber hinaus haben wir Yeast-one-Hybridscreens durchgeführt um Proteine zu finden die mit dem *FT*-Promoter interagieren und dessen Aktivität beeinflussen können. Diese Arbeit zeigt, dass CONSTANS mit dem trimeren CCAAT-box-Bindefaktor (HAP-Komplex) interagiert. Der HAP-Komplex besteht aus drei Untereinheiten von denen zwei (HAP3 und HAP5) dimerisieren. Nach Bildung des HAP3/5 Dimers bindet die HAP2-Untereinheit, die eine DNA-Bindungsdomäne besitzt. Der ternäre Komplex kann sodann an DNA binden. CONSTANS interagiert mit allen Untereinheiten des HAP-Komplexes. Des Weiteren besitzt CONSTANS eine Domäne die der DNA-Bindedomäne von HAP2 ähnelt. Mutationen, die hoch konservierte Aminosäuren in dieser Region in CCT- Domänen Proteinen betreffen, haben einen Funktionsverlust zur Folge. Außerdem zeigen wir, dass *HAP3a* mit *CO* durch *GIGANTEA* (*GI*) koreguliert wird. Überexpression von FLAG:HAP3a, welches möglicherweise dominant-negativ agiert, zeigt einen spätblühenden Phänotyp. Die verspäte Blütenbildung wird durch massive Repression von *FT* bewirkt. MtN19, ist ein Protein unbekannter Funktion das mit CONSTANS interagiert und außerdem an den *FT* Promoter binden kann. Studien des *MtN19* Gens deuten darauf hin, dass es eventuell über natürliche antisense-RNA reguliert wird. Transgene *MtN19*-dsRNAi Pflanzen zeigen zwei

extreme Phänotypen, frühe oder späte Blütenbildung. Die früh blühenden Linien zeigen erhöhte Expressionslevel der *MtN19* mRNA sowie der mRNAs von *CO* und *FT*, die das vorzeitige Blühen erklären. Spätblühende Linien können auch durch Überexpression von *CO* nicht komplementiert werden. *MtN19* und *CO* sind beide am Ende der Photoperiode des Langtages exprimiert und könnten so gemeinsam die Expression von *FT* kontrollieren. *FIDGET* (*FIT*) codiert für einen APETALA2 (AP2) -like Transkriptionsfaktor und wurde durch Yeast-one-Hybridscreens als *FT*-Promoter-Bindeprotein isoliert. Expression von *FIT* im Phloem, dem Ort wo *FT* natürlicherweise exprimiert ist, resultiert in früh blühenden Pflanzen. Des Weiteren konnten wir zeigen, dass *FIT* durch UV-Licht im Phloem induziert wird und dass UV-Licht die florale Transition beschleunigen kann. In einem Large-scale Experiment in welchem 1.000 Arabidopsis Transkriptionsfaktoren ektopisch im Phloem exprimiert wurden, resultierte in der Isolierung von verschiedenen AP2-Transkriptionsfaktoren die den Blütezeitpunkt beeinflussen. Zwei dieser AP2-Transkriptionsfaktoren bewirken eine Verzögerung des Blühzeitpunktes und es konnte gezeigt werden, dass diese beiden Proteine auch an den *FT*-Promoter binden können. Diese Ergebnisse deuten darauf hin, dass der *FT*-Promoter als regulatorische Schaltstelle für aktivierende und reprimierende AP2-Transkriptionsfaktoren fungiert. Zusammengefasst beschreibt diese Arbeit die Isolierung verschiedener Proteine mit denen CONSTANS im Komplex an den *FT* Promoter bindet. Des Weiteren wird von der Isolierung von AP2-like Proteinen berichtet, die wahrscheinlich in einem neuen physiologischen Signalweg fungieren der die Blütenbildung auf Stresssignale beeinflusst.

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List of abbreviations

General abbreviations

°C	degree Celsius
2,4D	2,4-Dichlorophenoxy acetic acid
3', 5'	5-prime, 3-prime
ABA	abscisic acid
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
Amp	ampicillin
ATP	adenosin 5-triphosphate
bp	basepair
BSA	bovine serum albumin
CaMV	Cauliflower mosaic virus
cDNA	complementary DNA
Col	Columbia
C-terminal	carboxy terminal
C-terminus	carboxy terminus
DEPC	diethylpyrocarbonate
DMSO	Dimethylsulfoxid
DNA	desoxyribonucleic acid
dNTP	deoxyribonucleic triphosphate
dsRNAi	doublestranded-RNA-interference
DTT	Dithiotreitol
EDTA	ethylendiaminetetraacetic acid
EST	expressed sequence tag
EtBR	ethidium bromide
EtOH	ethanol
G	guanine
GA	gibberellic acid
GST	glutathione S-transferase
h	hour
IAA	indole-3-acetic acid
IPTG	isopropyl-beta-thiogalactoside

kb	kilobase
kDA	kilodalton
kV	kilovolt
l	liter
LB	Luria broth
LD	long day
LDP	long day plant
Ler	Landsberg erecta
M	Mol
MES	2-(N-morpholino)ethane sulfonic acid
mg	milligram
mg	microgram
min	minute
miR	microRNA
ml	milliliter
ml	microliter
mM	millimolar
mRNA	messenger RNA
Ni	nickel
nt	nucleotide
N-terminal	amino terminal
N-terminus	amino terminus
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylenglycol
pH	negative logarithm of the proton concentration
Rif	rifampicine
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
RT-PCR	reverse transcription PCR
SAM	shoot apical meristem

SD	short day
SDP	short day plant
SDS	sodium dodecylsulfate
siRNA	small interfering RNA
ta-siRNA	transacting-siRNA
TE	Tris-EDTA
TEMED	N,N,N',N' tetramethylenethylendiamine
Tet	tetracycline
Tris	Trishydroxymethylaminomethane
UTR	untranslated region
UV	ultraviolet light
V	Volt
Vol.	volume
wt	wildtype
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid

Amino acids

Alanine	Ala	A
Cysteine	Cys	C
Aspartic acid	Asp	D
Glutamic acid	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysin	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R

Serine	Ser	S
Threonine	Thr	T
Valin	Val	V
Tryptophane	Trp	W
Tyrosine	Tyr	Y

Abbreviations of gene and protein names

ACF	ALBUMIN CCAAT-FACTOR
AG	AGAMOUS
AGO	AGONAUTE
AP1	APETALA1
AP2	APETALA2
ASML2	ACTIVATOR OF SPO(MIN)::LUC2
bHLH	basic helix loop helix
CAL	CAULIFLOWER
CBF	CCAAT-BINDING FACTOR
CCA1	CIRCADIAN CLOCK ASSOCIATED1
CDF1	CYCLIC DOF FACTOR1
CK2	CASEIN KINASE
CLF	CURLY LEAF
CO	CONSTANS
COL	CO-LIKE
COP1	CONSTITUTIVE PHOTOMORPHOGENESIS1
CRY1	CRYPTOCHROME 1
CRY2	CRYPTOCHROME 2
CRY3	CRYPTOCHROME 3
DCL	DICLER-LIKE
DET	DEETIOLATED1
DREB	BDROUGHT RESPONSIVE ELEMENT BINDING PROTEIN
EFS	EARLY FLOWERING IN SHORT DAYS
ELF4	EARLY FLOWERING4
ELF7	EARLY FLOWERING7
ELF8	EARLY FLOWERING8
EMF2	EMBRYONIC FLOWER2

EREBP	ETHYLEN RESPONSIVE BINDING PROTEIN
ESD4	EARLY IN SHORT DAYS4
FCA	*
FD	*
FIE	FERTLIZATION INDEPENDENT ENDOSPERM
FIS	FERTLIZATION INDEPENDENT SEED
FIT	FIDGET
FKF1	FLAVIN-BINDING, KELCH REPEAT, F-BOX1
FLC	FLOWERING LOCUS C
FLD	FLOWERING LOCUS D
FLK	FLOWERING LOCUS K
FPA	*
FPF1	FLOWERING PROMOTING FACTOR1
FRI	FRIGIDA
FT	FLOWERING LOCUS T
FUS	FUSCA5
FVE	*
FY	*
FyPP	PHYTOCHROME ASSOCIATE PROTEINPHOSPHATASE 2A
GI	GIGANTEA
HAP	HEME ASSOCIATED PROTEIN
HEN1	HUA ENHANCER1
HFR1	LONG HYPOCOTYL IN FAR RED LIGHT
HY5	ELONGATED HYPOCOTYL 5
HYL1	HYPONASTIC LEAVES
L1L	LEC1-LIKE
LD	LUMINIDEPENDENS
LEC1	LEAFY COTYLEDON
LFY	LEAFY
LHY	LONG ELONGATED HYPOCOTYL
MEA	MEDEA
miR156	microRNA156
miR159	microRNA159
miR172	microRNA172

NF-Y	NUCLEAR FACTOR-Y
OsPIPK1	rice PHOSPHATIDYL INOSITOL MONOPHOSPHATE KINASE1
PAF1	RNA POLII ASSOCIATED FACTOR1
PcG	Polycomb group gene
PHYA	PHYTOCHROME A
PHYB	PHYTOCHROME B
PML	Promyelotic leukemia onco-protein
PNH	PINHEAD
PolI	RNA-polymerase I
PolII	RNA-polymerase II
PolIII	RNA-polymerase III
PolIV	RNA-polymerase IV
RAV1	RELATED TO ABI3/VP1
RDR	RNA-DEPENDENT RNA POLYMERASE
SGS	SUPPRESSOR OF GENE SILENCING
SMZ	SCHLAFMUETZE
SNZ	SCHNARCHZAPFEN
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1
SPL	SQUAMOSA PROMOTER BINDING PROTEIN LIKE
SRP	SIGNAL RECOGNITION PARTICLE
SUMO	SMALL UBIQUITIN RELATED MODIFIER
TFL1	TERMINAL FLOWER1
TIR1	TRANSPORT INHIBITOR RESPONSE1
TOC1	TIMING OF CAB EXPRESSION1
TOE1	TARGET OF EAT1
TOE2	TARGET OF EAT2
TOE3	TARGET OF EAT3
trxG	trithorax group genes
ULI3	UV-B LIGHT INSENSITIVE3
VIN3	VERNALIZATION INSENSITIVE3
VIP4	VERNALIZATION INDEPENDENT4
VRN2	VERNALIZATION2
Xnf7	Xenopus nuclear factor 7
*	Traditionally these genes/proteins have not been given a full name

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1. Introduction

1.1. The control of flowering-time

During plant development three major growth phases can be discriminated, the juvenile-vegetative, the adult-vegetative and the reproductive growth phase (Poethig, 2003). A combination of external and internal factors regulates fate changes during development. The transition to flowering is the most dramatic phase change in the life of a plant and is initiated by both endogenous and exogenous cues. In nature, environmental changes are mostly associated with the changes of the seasons. Exogenous cues such as photoperiod, light quality, temperature and the availability of nutrients are measured by the plant, allowing the floral transition to occur under optimal conditions (Coupland, 1995). Exogenous stimuli are perceived and transformed into a complex pattern of gene and protein activity that regulates this fate change. Based on the different habitats of plants these phase changes are adapted to the local environment. Different species do not respond similarly to these exogenous cues. Based on the plants requirements of the duration of the photoperiod they can be divided into short-day, long-day or day-neutral plants (Garner and Allard, 1919; Bernier, 1988). For some plants, the perception of low temperatures during winter is crucial to initiate flowering in the following spring, a phenomenon called perannialism.

The transition to flowering is not only a major phase change in plant development it is also of economic importance since reproductive success is directly coupled to the yield. Engineering fruit size, the timing of availability of fruits as well as the cultivation of crop plants under unfavourable conditions such as drought are economically important tasks.

1.1.1. The control of floral evocation and the establishment of *Arabidopsis thaliana* as a model organism to study developmental processes

The floral transition in plants is controlled both by exogenous factors such as sunlight, photoperiod, temperature and by endogenous factors such as the internal circadian clock and hormones. The decision to flower is in many plants controlled by the length of the photoperiod. Short-day plants (SDP) and long-day plants (LDP) can be again subdivided into obligate and facultative responses. Obligate long-day plants only flower in long-days and never in short days. In comparison to facultative long-day plants that flower in short-days, but later. However, several environmental factors can influence the flowering behavior. *Pharbitis nil*, a facultative SDP, flowers early in LD when plants are exposed to high irradiance, poor nutrition, low temperatures, cytokinin application or when the root is removed (Ogawa and King, 1980; Shillo, 1985; Swe et al., 1985; Bernier, 1988). Apart from photoperiod, some plants are also able to measure the outside temperature and will only flower when a critical threshold is reached. *Chamelaucium* is an obligate SDP in 24°C/16°C day/night cycles. However, in 20°C/10°C day/night cycles *Chamelaucium* converts to a facultative SDP, which will start flowering also in response to long-days (Shillo, 1985; Bernier, 1988). Leaves of plants perceive light which leads to the synthesis of a so far unknown compound, named florigen, that is transported to the shoot apical meristem to trigger flower development (Knott, 1934; Zeevaart, 1976). Several grafting experiments confirmed the existence of florigen and its transport from leaves to the shoot apex. Leaves from *Perilla crispa*, a SDP that was exposed to inductive short-days, were grafted onto plants growing in long-days. These induced leaves were able to cause flowering in non-inductive conditions (Zeevaart, 1985). It was confirmed that the floral stimulus is transmitted from photosynthetic leaf tissue through phloem sieve elements (King et al., 1968; King and Zeevaart, 1973). All these physiological experiments were done with a variety of different plant species (Corbesier and Coupland, 2005). Analysis of genetic pathways controlling the floral transition required the establishment of a model species appropriate for this approach. Researchers agreed on Arabidopsis and the history of the establishment as a model organism is reviewed here.
One of the first descriptions of *Arabidopsis thaliana* was published by Friedrich Laibach in 1907, when he compared the numbers of chromosomes of different plant species (Laibach, 1907). Laibach later proposed Arabidopsis as a suitable model organism, exhibiting various positive features such as short generation time, ease of crosses and the possibility to do

mutagenesis (Laibach, 1943). In the following decades scientist started to use Arabidopsis for physiological experiments and started to create and describe mutants (Redei, 1975). Redei's article influenced many researchers in the early 1980s when the era of molecular characterization of cellular functions started. One of the most important discoveries was the gene transfer into plants via *Agrobacterium tumefaciens* transformation (Chilton et al., 1977). This was followed by the publication of the first genetic linkage map of Arabidopsis genes on the five chromosomes (Koornneef et al., 1983). The first restriction fragment length polymorphism maps (Chang et al., 1988) resulted in the first map-based cloning of genes in 1992 (Arondel et al., 1992; Giraudat et al., 1992). In 2000 an international consortium of scientists published the sequence of the genome of *Arabidopsis thaliana* (AGI, 2000). Large populations of plants carrying T-DNA insertions at defined positions have enabled reverse genetic approaches to study gene function (Alonso et al., 2003). The history of Arabidopsis as a model species was recently reviewed (Somerville and Koornneef, 2002).

1.1.2. The molecular control of flowering time in Arabidopsis

The initiation of flowering is a crucial process for plants, which in many species takes place only once during the life cycle. Timing of the transition to flowering is important ensuring that flowering occurs in optimal conditions for seed maturation and reproductive success. One of these cues is day length. *Arabidopsis thaliana* is a facultative long-day plant, which means that the plant flowers earlier under long days (LD) (e.g. 16 hours light and 8 hours dark) than under short days (e.g. 8 hours light and 16 hours dark). Other important cues besides day length are temperature and nutrient availability. Perception of these cues results in differential gene expression patterns that are finally responsible for the switch from vegetative to reproductive growth. The mechanisms controlling flowering are highly regulated processes and consist of at least four different genetic pathways that mediate distinct environmental responses. The plant integrates information from all of these pathways to ensure flowering under most optimal conditions. Figure 1 shows the four flowering-time pathways that have been identified and the names of the most important proteins or growth regulators involved in the process. These pathways are described in more detail in the following sections and here for clarity only the most important factors are mentioned.

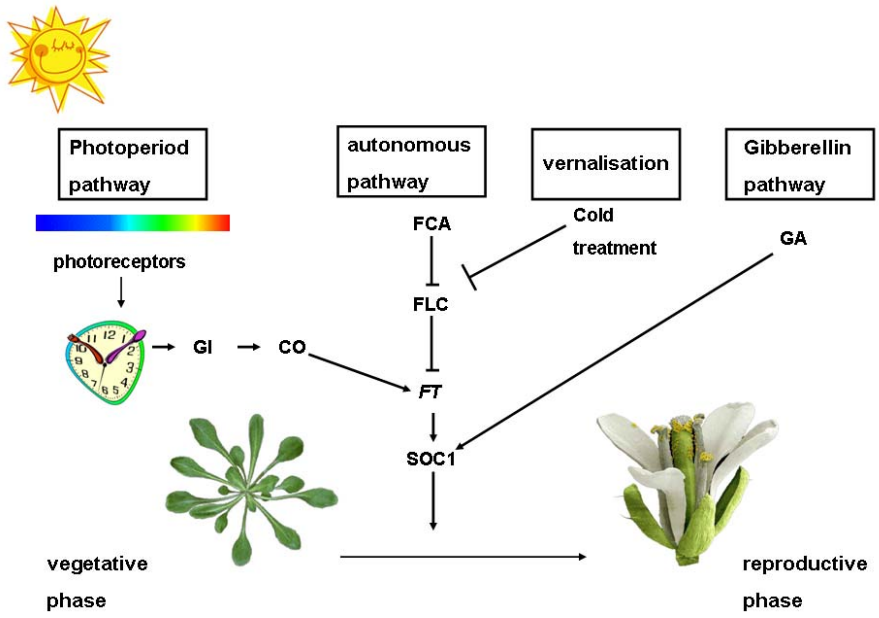

Figure 1. Four different pathways involved in mediating the floral transition in Arabidopsis.

1.1.2.1. Gibberellic acid pathway

One of the first observations indicating that hormones are involved in the floral transition was that exogenous application of gibberellic acid (GA_3) on rosettes of long-day plants resulted in early-flowering under short-day conditions (Lang, 1957; Langridge, 1957). Genetic studies showed that overexpression of GA20-oxidase, an enzyme in the GA-biosynthetic pathway, resulted in early-flowering under both short and long days. (Huang et al., 1998; Coles et al., 1999). FLOWERING PROMOTING FACTOR1 (FPF1) was also proposed to be involved in GA-signal transduction and overexpression of *FPF1* triggers the floral transition (Kania et al., 1997).

Consistent with these hormone treatments and transgenic plant studies, *gibberellic acid insensitive* mutants (*gai*) and impaired GA biosynthetic mutants are late-flowering particularly in short-days (Wilson et al., 1992; Putterill et al., 1995). Strong alleles of *ga-1* prevent flowering in SDs, indicating that under these conditions GA is essential for flowering. In *ga1-3* mutants, *LFY* expression is reduced, in contrast to application of GA which results in upregulation of *LFY* mRNA both in wild-type and *ga1-3* (Blazquez et al., 1998). *At*MYB33 is a MYB-transcription factor that binds to a GA-response element in the *LEAFY* (*LFY*) promoter *in vitro* (Gocal et al., 2001). Recently it was shown that *MYB33* mRNA is targeted

for degradation by miR159. Overexpression of miR159 resulted in late-flowering under short days (Achard et al., 2004). Furthermore miR159 is transcriptionally regulated by GA (Achard et al., 2004). Apart from *LFY*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) mRNA is also increased by GA (Moon et al., 2003).

1.1.2.2. Autonomous pathway

Mutations in genes belonging to the autonomous pathway cause late-flowering phenotypes both in long and short days. The late-flowering phenotypes can be overcome by vernalization (Koornneef et al., 1991). Seven loss-of-function mutants were isolated so far, namely *fca, fpa, flk, fy, ld, fve* and *fld*. The corresponding genes encode RNA processing factors (FCA, FPA, FLK and FY), proteins involved in histone deacetylation (FVE and FLD) and a homeodomain protein (LD) (Lee et al., 1994; Macknight et al., 1997; Chou and Yang, 1998; Schomburg et al., 2001; Simpson et al., 2003a; Ausin et al., 2004; Kim et al., 2004; Lim et al., 2004). The main function of autonomous pathway genes seems to be down regulation of *FLC* mRNA levels because mutations in these genes cause an increase in *FLC* mRNA and mutations in *FLC* suppress the effect of mutations in the autonomous pathway. FLC is a MADS box transcription factor that delays flowering, consistent with it causing a delay in flowering in autonomous pathway mutants (Michaels and Amasino, 2001).
FCA, one of the strongest effectors in this pathway, encodes an RNA-binding protein and controls its own pre-mRNA processing dependent on the developmental status of the plant (Quesada et al., 2003). FCA interacts with FY, another component of the autonomous pathway, which belongs to the class of 3´end-processing factors (Simpson et al., 2003a). The FCA/FY interaction finally results in repression of *FLC* (Simpson et al., 2003b). Another level of regulation is the acetylation status of histones in the *FLC* containing chromatin. FLOWERING LOCUS D (FLD) and FVE, proteins homologous to a member of a human histone deacetylase complex, deacetylate the histones of *FLC*-chromatin and thereby prevent *FLC* transcription, resulting in earlier flowering (He et al., 2003; Ausin et al., 2004; Kim et al., 2004).
Thus, regulation of mRNA stability and epigenetic control of gene expression seem to be the major mechanism by which components of the autonomous pathway regulate the floral transition by modulating *FLC* levels (Boss et al., 2004).

1.1.2.3. Vernalization pathway

Vernalization is the exposure of plants to low temperatures over an extended time period of several weeks. It results in the reduction of *FLC* mRNA levels and thereby triggers the floral transition (Michaels and Amasino, 1999; Sheldon et al., 1999). FLC, a MADS box transcription factor acts as a floral repressor and is the main target of the vernalization process (Michaels and Amasino, 1999). Differences in the response to vernalization exist between different Arabidopsis accessions. Many accessions carry an active allele for *FRIGIDA* (*FRI*), a gene required for high level expression of *FLC* (Johanson et al., 2000). Exposure to low temperatures reduces the FRI-mediated increase in *FLC* mRNA and the plant can then respond to other floral inductive signals (Michaels and Amasino, 1999; Sheldon et al., 1999). However, the Arabidopsis accessions Landsberg *erecta* and Columbia that are commonly used in the laboratory carry loss-of-function *FRI* alleles. They are therefore early-flowering and do not require vernalization to flower (Johanson et al., 2000).
Negative regulators of *FLC* were isolated in genetic screens designed to identify mutants defective for vernalization responses (Chandler et al., 1996; Sung and Amasino, 2004). Among these, VRN1 and VRN2 act to maintain *FLC* repression after vernalization. Wild-type plants show a stable repression of *FLC* after vernalization, whereas *vrn1* and *vrn2* mutants show an increase in *FLC* mRNA levels after shifting plants from cold to warm temperatures, resulting in a delay of the floral transition (Gendall et al., 2001; Levy et al., 2002). *VRN2* encodes a Polycomb group protein, which is likely to act in a chromatin silencing complex (Gendall et al., 2001). VRN1 is a putative B3-domain protein that is thought to interact with DNA in a non-sequence-specific manner (Levy et al., 2002). VIN3 is a homeodomain protein involved in the onset of the establishment of floral repression. A mutation in *VIN3* results in failure to establish repression at the *FLC* locus in response to cold temperatures (Sung and Amasino, 2004). During vernalization histone tails of *FLC* chromatin become deacetylated followed by an increase in H3-K27 and H3-K9 methylation (Bastow et al., 2004; Sung and Amasino, 2004). None of these epigenetic marks in the *FLC* chromatin is detected in *vin3* mutant plants indicating a major role in this process (Sung and Amasino, 2004). VRN1 is required for H3-K9 methylation of *FLC* chromatin and VRN2 is thought to be involved in the H3-K27 methylation process (Bastow et al., 2004; Sung and Amasino, 2004).
Apart from epigenetic marks resulting in repression of *FLC*, acetylated histone tails and H3 trimethylation at lysine 4, an indicator for active chromatin (Santos-Rosa et al., 2002; Ng et al., 2003; Schubeler et al., 2004), were observed prior to vernalization in winter-annual

Arabidopsis (He et al., 2004). Mutations in *EARLY-FLOWERING7* (*ELF7*), EARLY-FLOWERING8 (*ELF8*) and VERNALIZATION INDEPENDENCE4 (*VIP4*) show low *FLC* levels in presence of active *FRI* (Zhang and van Nocker, 2002; He et al., 2004). ELF7, ELF8 and VIP4 are homologous to members of yeast RNA polymerase II associated factor 1 (PAF1), a chromatin modifying protein complex (Betz et al., 2002; Krogan et al., 2002; Squazzo et al., 2002). PAF1 interacts in yeast with SET1, a methylase that mediates H3-K4 trimethylation, an indicator for active chromatin (Ng et al., 2003). Apart from the PAF1 components, Arabidopsis encodes several SET-domain proteins, among them EARLY-FLOWERING IN SHORT-DAYS (EFS) (Soppe et al., 1999). EFS might be involved in H3K4-trimethylation of *FLC*-chromatin by interacting with the PAF1 complex (He and Amasino, 2005).

1.1.2.4. Photoperiod pathway

Changes in photoperiod, such as duration of the light period and spectral composition, are sensed by the photoreceptor system of the plant. In Arabidopsis, the red/far-red light absorbing phytochromes and the UV-A/blue light absorbing cryptochromes are the main photoreceptors involved in sensing light in photoperiod response (Lin, 2002; Quail, 2002). The circadian clock provides the timing mechanism that enables measurement of the duration of the photoperid (Samach and Coupland, 2000). Light inputs are coupled to the circadian timer because light/dark transitions at dawn or dusk reset the clock so that it is entrained to the day/night cycle (Yanovsky and Kay, 2002). The main components of the circadian oscillator, the core circadian clock, are the myb transcription factors LHY and CCA1 and the CCT-domain protein TOC1 (Millar et al., 1995; Schaffer et al., 1998; Wang and Tobin, 1998). *TOC1* expression peaks in the evening and loss-of-function mutations shorten circadian rhythms whereas overexpression causes arrhythmia (Somers et al., 1998; Strayer et al., 2000). *CCA1* and *LHY* peak in the early morning, loss-of-function causes shortened circadian rhythms and overexpression results in arrhythmia (Schaffer et al., 1998; Wang and Tobin, 1998; Green and Tobin, 1999; Mizoguchi et al., 2002). The expression patterns of *TOC1*, *LHY* and *CCA1* as well as genetic data led to the proposal of an autoregulatory negative feedback model based on transcriptional regulation (Alabadi et al., 2001). TOC1 induces the transcription of *LHY* and *CCA1* at the beginning of the light phase. High levels of LHY and CCA1 then repress the transcription of *TOC1*. Repression of *TOC1* causes loss of activation

of *LHY*/*CCA1*. The repressive effect of LHY/CCA1 is removed at the end of the light phase and causes the rise of *TOC1* expression.

GIGANTEA (GI) acts in the circadian clock and loss-of-function of *GI* causes shortening of the period (Huq et al., 2000; Staiger et al., 2003). Furthermore *gi* mutants are late-flowering and have elongated hypocotyls when grown under red light, suggesting it is also involved in phytochrome signaling (Fowler et al., 1999; Park et al., 1999; Huq et al., 2000). *GI* expression is circadian clock regulated with a peak in activity around ten hours after dawn and acts both in the circadian clock and in a clock output pathway where it promotes flowering by upregulating *CO* mRNA levels (Fowler et al., 1999; Park et al., 1999; Huq et al., 2000; Suarez-Lopez et al., 2001; Mizoguchi et al., 2005).

CO was originally isolated from a late-flowering mutant whose flowering was delayed specifically under long days (Putterill et al., 1995). CO protein acts only in the clock output pathway and not in the clock mechanism since neither overexpression, nor loss-of-function affects the expression of clock genes (Ledger et al., 2001). *CO* mRNA itself shows a circadian expression pattern peaking both in long-days and in short-days 16 hours after dawn. Overexpression of *CO* causes extreme early-flowering (Onouchi et al., 2000) and expression analysis indicated that the function of *CO* is to mediate between the circadian clock and the floral transition (Suarez-Lopez et al., 2001). FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1) degrades CYCLIC DOF FACTOR1 (CDF1), a repressor of *CO* transcription (Imaizumi et al., 2003; Imaizumi et al., 2005). Degradation of CDF1 allows *CO* mRNA to accumulate at the end of a long-day around 16 hours after dawn (Imaizumi et al., 2005). In the *fkf1* mutant this peak of *CO* is abolished and the plants display a late-flowering phenotype (Nelson et al., 2000).

Photoreceptors are involved in the floral transition since loss-of-function mutants of *PHYA* and *CRY2* cause late-flowering (Johnson et al., 1994; Guo et al., 1998; Yanovsky and Kay, 2002) and mutations in *PHYB* cause early-flowering (Goto et al., 1991). CO protein abundance also exhibits a diurnal pattern (Valverde et al., 2004). CO accumulates in the presence of light and is degraded by the proteasome in darkness. Involvement of the proteasome is illustrated by accumulation of CO in darkness in the presence of proteasome inhibitors (Valverde et al., 2004). Moreover, PHYA and CRY2 are involved in stabilization of CO whereas PHYB is involved in red-light dependent degradation of CO (Valverde et al., 2004). This illustrates that CO protein stabilization occurs only at the end of a LD when exposure to light coincides with the presence of the *CO* mRNA.

1.1.2.5. Floral integration

CO promotes flowering in LD by direct activation of a set of genes called floral integrators comprising *LEAFY* (*LFY*), *SUPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) and *FLOWERING LOCUS T* (*FT*) (Hayama and Coupland, 2003). LFY is a plant specific transcription factor with no homology to other proteins (Parcy 1998; Weigel 1992). The protein localizes both to the nucleus and the cytoplasm (Parcy et al., 1998; Wagner et al., 1999; Wu et al., 2003) and can move between cells (Sessions et al., 2000). *SOC1* encodes a MADS box transcription factor and *soc1* mutants are late-flowering whereas overexpression of *SOC1* accelerates the floral transition (Borner et al., 2000; Lee et al., 2000; Onouchi et al., 2000; Samach et al., 2000). *FT* encodes a protein with similarities to phosphatidylethanolamine binding proteins and Raf kinase inhibitors in animals (Kardailsky et al., 1999; Kobayashi et al., 1999). Overexpression of *FT* causes extreme early-flowering and the formation of a terminal flower whereas *ft* loss-of-function mutations cause late-flowering (Kardailsky et al., 1999; Kobayashi et al., 1999). Latest results indicate that *SOC1* is not directly induced by CO but rather indirectly through FT (Yoo et al., 2005). The *ft* mutation strongly enhances the *lfy* mutation and *35S::FT* enhances the effect of *35S::LFY* (Ruiz-Garcia et al., 1997; Kardailsky et al., 1999; Kobayashi et al., 1999), indicating that both *LFY* and *FT* act independently in promoting flowering. After activation of the floral integrators, floral meristem identity genes are activated which initiate the fate change from a vegetative to a floral meristem. The floral meristem identity genes encompass *LFY*, *APETALA1* (*AP1*) and *CAULIFLOWER* (*CAL*) (Kieffer and Davies, 2001; Lohmann and Weigel, 2002). While LFY is able to directly activate *AP1* and *CAL* by binding to their promoters (Parcy et al., 1998; Wagner et al., 1999; William et al., 2004), FT can also induce *AP1* independently of LFY since a *lfy* mutant is able to form floral structures in contrast to *lfy ap1* double mutants (Huala and Sussex, 1992; Weigel et al., 1992). However, *TFL1* which encodes a homolog of FT is able to repress both *FT* and *LFY* (Bradley 1998; Ratcliffe 1998, 1999). Recently it was shown that one amino acid change in FT changes this activator of flowering into a repressor and the opposite change in TFL1 converts a repressor into an activator of flowering (Hanzawa et al., 2005).

Another aspect of the floral transition is spatial perception and signal transduction of the floral stimulus. Grafting experiments showed that photoperiod is perceived in the leaf and in response to this treatment a signal, the floral stimulus, is transduced to the shoot apex where the floral transition is induced (Knott, 1934; Zeevaart, 1976). Interestingly, CO is expressed in

the vascular system of Arabidopsis leaves where it induces the transcription of *FT* (Takada and Goto, 2003; An et al., 2004; Ayre and Turgeon, 2004). Moreover, misexpression of *CO* using various tissue specific promoters revealed that *CO* can induce flowering when expressed in the vasculature of the leaf but not when ectopically expressed in the shoot apical meristem (An et al., 2004). This indicates that CO regulates the floral signal in the leaf since it is the last protein in the hierarchy acting only in the leaf. *FT* can trigger early-flowering when expressed both in the leaf and in the shoot apical meristem, indicating that FT itself might be the movable signal or that it may control it (An et al., 2004). Huang et al. claim that *FT* mRNA is the movable signal moving from leaves to the shoot apical meristem (Huang et al., 2005). Using a heat-shock inducible promoter they expressed *FT* at high levels in leaves and were able to detect the mRNA in the shoot apex (Huang et al., 2005). However, they fail to demonstrate that movement of the mRNA triggers flowering and exclude that the FT protein might also move to the shoot apex. FT triggers flowering in the shoot apical meristem by interacting with FD, a bZIP transcription factor that is exclusively expressed in the shoot apical meristem (Abe et al., 2005; Wigge et al., 2005). FD and FT interact and activate *AP1* by interacting with its promoter, thereby inducing floral meristem identity in the primordium (Abe et al., 2005; Wigge et al., 2005).

1.2. Molecular mechanisms regulating gene expression

Flowering-time is a quantitative trait that is regulated by a complex network of genes organized in different regulatory pathways. Understanding the relationships between different genes involved in this process requires an understanding of the mechanisms that control their expression. Such regulatory pathways can be controlled at different levels. A major layer of regulation is the initiation of transcription, which can be influenced by changes in chromatin structure by for example histone modification. Once the chromatin is in an active state, the rate of transcription can be influenced by specific transcription factors that bind to the gene and act as repressors or activators of transcription. Stability of the messenger RNA is another layer of regulation that can be influenced by RNA-binding proteins or small RNA species such as microRNAs. The rate of translation which determines how much protein is made from an mRNA molecule can also be controlled by regulatory proteins or small RNAs. Finally protein turn-over and activity is regulated by post-translational modifications such as

phosphorylation, ubiquitination or sumoylation. These different levels of regulation form a complex and flexible network of gene and protein activity and are discussed in more detail in the following sections. All of these layers of regulation have been adopted at different positions in the regulatory network that controls the floral transition in response to exogenous and endogenous cues. The following sections describe these different layers of regulation and show examples how these processes affect the transition to flowering.

1.2.1. Regulation of transcription in higher eukaryotes

Transcription is the term used for the process of synthesizing RNA from DNA templates and can be regulated at various stages. DNA contains the genetic information and in higher eukaryotes is organized into chromosomes to which proteins such as histones and histone-associated proteins are attached. The protein-coding genes can be divided into different regions. The transcribed region of genes is divided into exons and introns and the latter are spliced out after transcription. Promoters are DNA sequences typically upstream of the transcription initiation sites that regulate the rate of transcription. The whole promoter of a gene is characterized by regulatory elements which can be located in sequences up- and downstream of the transcription initiation site for example in introns. Promoters contain functional motifs where different proteins bind and regulate the rate of transcription often in a precise temporal or tissue specific manner.

In higher eukaryotes RNA is synthesized by three different types of RNA-polymerase. RNA-polymerase I transcribes 18S, 5.8S- and 28S-ribosomal RNAs (rRNA), RNA-polymerase II transcribes mRNA and small nuclear RNA (snRNA) and RNA-polymerase III synthesizes transfer RNAs (tRNA) and 5S-rRNA (Lodish et al., 2000). In contrast to animals, plants have a fourth RNA-polymerase (PolIV) that is involved in synthesis of small interfering RNAs (siRNA) and DNA methylation-dependent heterochromatin formation (Herr et al., 2005; Onodera et al., 2005).

Transcription can be subdivided into three phases. During initiation, the transcription complex binds to the promoter of a gene. In the elongation phase RNA-Polymerase synthesizes and extends the new RNA molecule. Transcription ends with the termination process, where the nascent RNA is released from the transcription complex (Lodish et al., 2000).

The proximal promoters of protein-coding genes, which are transcribed by RNA-polymerase II, are sequences 5' of the transcriptional start site. Almost all proximal promoters contain a TATA box, a motif found around -25 base pairs (bp) from the transcriptional start. RNA-polymerase II can not bind to DNA itself, therefore a transcription factor complex is needed to facilitate binding. This complex was named transcription factor II (TFII, referring to RNA-polymerase II). Transcription initiation starts with the binding of TFIID to the TATA-box. TFIID is a large protein complex and binds to the TATA-box via the TATA-binding protein (TBP) a component of the complex. TFIID binding is followed by association of the subunits TFIIA and TFIIB. Finally, RNA-polymerase II and TFIIE join the complex (Guarente and Bermingham-McDonogh, 1992). TFII and RNA-polymerase II represent the basic transcription machinery, but the rate of transcription can be regulated by association of other transcription factors. These factors can associate with DNA up- and downstream of the transcriptional start site and modify the rate of expression. Factors acting positively on the rate of transcription are called activators, whereas repressors slow down the rate of transcription.

1.2.2. Role of transcription factors controlling developmental processes

Developmental processes in higher organisms are often controlled by transcription through the action of specific transcription factors (Lodish et al., 2000). Mutations causing developmental defects, isolated from a variety of model organisms such as *Drosophila melanogaster*, *Caenorhabditis elegans* or *Arabidopsis thaliana*, often affect genes encoding transcription factors. In many cases these transcription factors are expressed in a stage and tissue specific manner, and particularly in plants many are also expressed in response to environmental signals such as light, heat or drought and confer adaptation to changing environmental conditions (Riechmann et al., 2000; Riechmann and Ratcliffe, 2000). Around 5% of the Arabidopsis genome encodes transcription factors (AGI, 2000). Eleven major families of plant transcription factors can be distinguished. The largest group is the MYB-transcription factor family that comprises around 180 members; these are involved in a variety of processes ranging from morphogenesis to stress signaling. APETALA2 (AP2)/Ethylene responsive element binding proteins (EREBP) are the second largest group also controlling many different processes (Riechmann and Meyerowitz, 1998). Basic helix-loop-helix (bHLH) transcription factors form the third group. Several bHLH transcription

factors act in light signaling (Duek and Fankhauser, 2005), but also in a variety of processes such as cell elongation, germination and trichome development (Kirik et al., 2004; Kim et al., 2005). These three groups together represent around 30% of all Arabidopsis transcription factors (Ratcliffe and Riechmann, 2002). Transcription factors from a variety of different families have been shown to be involved in the regulation of the floral transition. Some act as floral repressors whereas others are activators (Ratcliffe and Riechmann, 2002).

1.2.3. Epigenetic control of gene expression

Initial changes in gene expression can be stabilized through epigenetic effects and inherited through many cell divisions. Epigenetic effects do not change the DNA sequence but alter the chromatin status by for example histone modifications such as methylation or acetylation (Lodish et al., 2000) or modify DNA through methylation. Histones form a scaffold for chromosomal DNA. Based on the density of the packing of DNA, different chromosomal regions can be distinguished (Lodish et al., 2000). Loose packing of DNA regions, named euchromatin, represents areas with high gene activity. Heterochromatic regions represent inactive chromatin.

Imprinting is a process of silencing allelic variants of genes derived from the maternal or paternal lineage and the imprinted loci are inherited differently. One of the two parents inherits a silent allele, whereas the other parent contributes a fully active allele, resulting in a functional non-equivalency of the parental genomes (Autran et al., 2005). In plants normal seed development is regulated by the maternal genes *MEDEA* (*MEA*), *FERTILIZATION-INDEPENDENT SEED* (*FIS*) and *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*). Mutations in these genes result in abnormal seed development regardless of the parental contribution (Chaudhury et al., 1997; Grossniklaus et al., 1998). These results are consistent with findings in mammalian systems, where failure to remember imprinting can result in uncontrolled cell divisions giving rise to developmental defects and cancer (Francis and Kingston, 2001). In *Drosophila melanogaster*, factors leading to stable repression of homeotic gene expression patterns were identified. These factors are encoded by the *Polycomb* (*PcG*) and *trithorax* (*trxG*) group genes. PcG proteins act as repressors at the level of chromatin and *trxG* genes were identified as encoding suppressors of PcG (Kennison and Tamkun, 1988; Brock and van Lohuizen, 2001). Also in Arabidopsis, PcG proteins have a fundamental role in controlling developmental processes. They act by methylating and acetylating histones

generating a PcG-specific histone code that establishes together with other factors heritable epigenetic marks that lead to alterations in transcription through binding of proteins that recognize these marks (Hsieh et al., 2003). The previously described Arabidopsis genes *MEA*, *FIS* and *FIE* all encode PcG proteins (Chaudhury et al., 1997; Grossniklaus et al., 1998; Kiyosue et al., 1999).

The transition to flowering is also regulated by PcG proteins. The first PcG protein identified in this process was CURLY LEAF (CLF). The *clf* mutant displays an early-flowering phenotype caused by ectopic expression of *AGAMOUS* (*AG*) (Goodrich et al., 1997). Seedlings homozygous for a mutation in the *FIE* gene do not form rosette leaves, they flower soon after germination. The early-flowering phenotype seems to be due to de-repression of several floral meristem and floral homeotic genes such as *LEAFY* (*LFY*) and *AGAMOUS* (*AG*) (Kinoshita et al., 2001). Mutations affecting another PcG protein, EMBRYONIC FLOWER2 (EMF2), exhibit an extreme early-flowering phenotype and EMF2 is thought to act in a complex with FIE (Yoshida et al., 2001). The function of EMF2 seems to be to repress the floral transition, since analysis of *emf2* mutant plants indicate that it is epistatic to *CONSTANS* (*CO*), *FLOWERING LOCUS T* (*FT*) AND *APETALA1* (*AP1*) (Chen et al., 1997; Haung and Yang, 1998).

1.2.4. The role of RNA in gene silencing and epigenetic control of gene expression

An early observation that lead to the discovery that RNA can interfere with gene expression resulting in gene silencing was made by Jorgensen and co-workers when they attempted to enhance the colour of the petals of petunia flowers by overexpressing the *chalcone-synthase* gene (Napoli et al., 1990). Instead of achieving more deeply coloured petals they created reduced or even unpigmented flowers. Analysis of transgenic plants revealed that both the endogenous gene and the transgene were silenced (van der Krol et al., 1990). Gene silencing can occur post-transcriptionally such as in the case of petunia flowers (van Blokland et al., 1994), or at the transcriptional level as was found in tobacco plants when researchers aimed to introduce two different transgene complexes regulated by the same promoter. The promoters of these two transgenes carried methylation marks, a modification often related to silencing (Matzke et al., 1989; Park et al., 1996). Andrew Fire et al. demonstrated in 1998 that the expression of double-stranded RNA (dsRNA) triggers the degradation of target messenger

RNA in the nematode worm *Caenorhabditis elegans* (Fire et al., 1998), a process later named RNA-interference (RNAi). A further milestone in the history of gene silencing was the discovery by David Baulcombe and co-workers in 1999, of small RNAs homologous to the silenced mRNA during the process of post-transcriptional gene silencing (Hamilton and Baulcombe, 1999).

A requirement for effective gene-silencing is the existence of a double-stranded RNA-precursor molecule that can be processed to generate small RNA species by an RNaseIII enzyme called Dicer (Carmell and Hannon, 2004). In the case of small interfering RNAs (siRNAs), the double-stranded RNA precursor can originate from transposable elements (Lippman et al., 2004) or viruses (Hamilton et al., 2002). SiRNAs anneal to target RNAs and the RNA-RNA duplex can be extended by an RNA-dependent RNA-polymerase. Several studies have shown that siRNAs are involved in histone methylation and thereby promote the formation of heterochromatin at DNA regions homologous to the RNA (Xie et al., 2003; Zilberman et al., 2003). Components required for the formation of siRNA-mediated heterochromatin are Dicer-like3 (DCL3) and the RNA-dependent RNA-polymerase2 (RDR2).

MicroRNAs (miRNA; miR) are small RNA-species that are transcribed like messenger RNAs by RNA polymerase II from a miRNA-locus termed pri-miRNA which does not encode a functional protein. The pre-miRNA primary transcript is approximately 1kb in length, is both capped and polyadenylated (Aukerman and Sakai, 2003a) and contains an internal region that forms a foldback loop. The RNA duplex is targeted by a protein complex, the Dicer complex, that cleaves the dsRNA and generates small 15 to 25 basepair dsRNAs (Grishok et al., 2001; Park et al., 2002). Four DICER-like proteins are encoded in the *Arabidopsis thaliana* genome (DCL1-4). Each of these proteins contains a PIWI-PAZ domain, two RNase III domains and at least one dsRNA-binding domain (Kidner and Martienssen, 2005). DCL1, DCL2 and DCL3 are involved in biogenesis of miRNAs as well as in the processing of viral and transposon siRNAs (Xie et al., 2004). DCL1 is the only protein of the group that harbours a nuclear localisation signal. This makes it more likely that DCL1 acts in processing miRNAs together with the protein HUA ENHANCER1 (HEN1). DCL1 and HEN1 are both required for post-transcriptional gene silencing (PTGS) (Park et al., 2002). In summary, miRNA-mediated mRNA destruction is believed to require the activities of ARGONAUTE1 (AGO1), DCL1, HEN1 and HYPONASTIC LEAVES1 (HYL1) whereas siRNA-mediated heterochromatin formation requires DCL3 and RNA-DEPENDENT RNA POLYMERASE2 (RDR2) (Kidner and Martienssen, 2005).

Contrary to animals plants have a fourth RNA-polymerase (PolIV) that is involved in siRNA production and DNA methylation-dependent heterochromatin formation (Herr et al., 2005; Onodera Y, 2005). Recently, Peragine et al. reported, that another subspecies of siRNA exists, that were named trans-acting siRNAs (ta-siRNA). Ta-siRNAs direct the destruction of target messenger RNA *in trans* (Peragine et al., 2004). Ta-siRNAs require a plant specific protein of unknown function called SUPPRESSOR OF GENE SILENCING3 (SGS3) and RNA-DEPENDENT RNA-POLYMERASE6 (RDR6) for the synthesis of 21nt siRNAs (Peragine et al., 2004). For proper gene-silencing the ta-siRNA-pathway requires the activities of the miRNA-pathway (AGO1, DCL1, HEN1 and HYL1) plus RDR6 and SGS3 (Vazquez et al., 2004). Finally, Allen et al. demonstrated that miRNAs can anneal to pre-ta-siRNA and serve as primer for the RDR6-catalyzed formation of the RNA duplex to yield a phased ta-siRNA that negatively regulates target messenger RNAs (Allen et al., 2005).

Small RNA species are recognized by another protein complex containing a PAZ/PIWI-domain protein called ARGONAUTE. The PAZ domain of the ARGONAUTE protein binds the 3' end of the small RNA (Bartel, 2004) and is kept inside a fold in the PAZ domain (Song et al., 2003). The small RNA can then bind to substrate mRNAs in which the mRNA adheres to the PIWI domain which shows structural similarities to RNase H (Song et al., 2004). After RNA basepairing the doublestrand is cleaved, resulting in destruction of target mRNA. The cleaved mRNA is released from the ARGONAUTE complex and another mRNA molecule can be processed as before (Tang and Zamore, 2004). Since the completion of sequencing the *Arabidopsis thaliana* genome (AGI, 2000) ten ARGONAUTE protein encoding genes were identified. From some of these proteins, loss-of-function mutants are known, which provide insights into the physiological processes that these proteins are involved in. AGO1 is involved in post-transcriptional gene silencing and therefore accumulates miRNAs. *Ago1* mutants are not only impaired in PTGS, but also lose resistance to viruses (Morel et al., 2002). AGO4 is involved in siRNA-silencing of transposons and repeats (Zilberman et al., 2003). *ago4-1* was identified in a screen for mutants that suppress silencing of the SUPERMAN gene. The identified mutant exhibited loss of DNA and histone methylation and displayed accumulation of siRNAs (Zilberman et al., 2003).

A gene closely related to *AGO1* is *PINHEAD* (*PNH*) that is expressed in provascular tissue, shoot apical meristem and the adaxial side of lateral organ primordia. PNH regulates axis determinacy in Arabidopsis (Lynn et al., 1999). PNH seems not to be involved in PTGS, thus it could be redundant with AGO1. Since AGO1 and PNH are closely related and are both involved in establishing organ polarity in Arabidopsis they might have redundant functions. In

support of this pnh/ago1 double mutants are lethal. ZIPPY/AGO7 is involved in the regulation of developmental timing, since loss-of-function mutations exhibit the premature expression of adult vegetative traits (Hunter et al., 2003). Based on the composition of the ARGONAUTE complex, the complex is called RISC (RNA-induced silencing complex) or RITS (RNA-induced initiation of transcriptional gene silencing), meaning that RISC is involved in RNA destruction and RITS is involved in genome modification (Verdel et al., 2004). The dsRNAs against promoter regions lead to methylation of target promoters that often result in transcriptional gene silencing (Mette et al., 2000). *De novo* methylation of cytosines is restricted to the region of RNA-DNA complementarity (Aufsatz et al., 2000). So far, it has been shown that DNA-methylation is naturally regulated by siRNA which has evolved to protect plants from viral attacks (Chan et al., 2005). However, Bao et al. demonstrated that microRNAs are also involved in DNA-methylation (Bao et al., 2004). Unravelling this mechanism is a challenge for future investigations to understand the relationship and interplay between microRNA-mediated gene silencing by mRNA destruction, translational inhibition and epigenetic modification of chromosomal DNA.

A computational approach undertaken by David Bartel and co-workers led to the identification of Arabidopsis miRNAs (Jones-Rhoades and Bartel, 2004). Their study revealed that miRNAs often target transcription factors that are involved in various developmental processes such as meristem determination, organ polarity and vascular development as well as floral patterning and hormone response (Kidner and Martienssen, 2005). In plants, microRNAs target genes can be regulated by mRNA destruction (Llave et al., 2002; Palatnik et al., 2003) or translational inhibition (Aukerman and Sakai, 2003a; Chen, 2004). The importance of microRNAs for normal development is emphasized by their spatial and temporal expression patterns (Ambros et al., 2003; Aravin et al., 2003; Bartel and Bartel, 2003; Aravin et al., 2004; Juarez et al., 2004b; Juarez et al., 2004a; Kidner and Martienssen, 2004).

Recent studies revealed that the floral transition is also controlled by small RNAs. The *FLOWERING LOCUS C* (*FLC)* allele present in the Arabidopsis accession Landsberg *erecta* (L*er*) is relatively weak due to the insertion of a transposon in the first exon (Gazzani et al., 2003; Michaels et al., 2003). SiRNAs corresponding to the transposon were identified in L*er* and it has been shown that these siRNAs mediate H3-K9 dimethylation resulting in silencing of the *FLC* locus (Liu et al., 2004). Additionally, HEN1, which is involved in siRNA metabolism, is required for siRNA mediated H3-K9 dimethylation and silencing of the *FLC* locus (Liu et al., 2004). Apart from siRNAs, miRNAs also regulate flowering-time.

SQUAMOSA PROMOTER BINDING PROTEIN LIKE3, SPL3 which has been shown to accelerate flowering upon overexpression (Cardon et al., 1997) is targeted for degradation by miR156 (Rhoades et al., 2002). In addition to *SPL3*, also *SPL4* and *SPL5* are targeted by miR156 (Rhoades et al., 2002). Overexpression of *miR156b* by the *35S* promoter caused late-flowering (Schwab et al., 2005) suggesting a role of *SPL*-genes in flowering-time control.
MicroRNA159 targets *MYB33*, a regulator of the floral pathway integrator *LFY*. Overexpression of miR159 resulted in a delay of flowering in short-days due to a decrease in MYB33 mRNA (Achard et al., 2004). Finally, several *APETALA2-like* genes were described that are involved in floral repression. Among those, *TARGET of EAT1* (*TOE1*), *TOE2*, *SCHLAFMUETZE* (*SMZ*), *SCHNARCHZAPFEN* (*SNZ*) and At5g67190 are repressors of flowering and regulated by miR172 (Aukerman and Sakai, 2003b; Schmid et al., 2003). Overexpression of these genes delays flowering in contrast to overexpression of miR172 that accelerates flowering by translational inhibition of AP2-like target genes (Aukerman and Sakai, 2003b; Kasschau et al., 2003; Schmid et al., 2003; Schwab et al., 2005).

1.2.5. Post-translational modifications and regulation of protein activity

Regulatory processes can also be controlled at the post-translational level by protein modification. Proteins can be modified by cleavage such as removal of signal peptides by the proteolytic signal recognition particle (SRP), allowing transport of proteins into the endoplasmatic reticulum (Rapoport, 1990). Another prominent example of proteolytic cleavage is the maturation of the insulin protein. Insulin is secreted from the pancreas as pre-proinsulin. Cleavage of a 24 amino acid signal peptide yields proinsulin which, after a second cleavage, folds to active insulin (Hutton, 1994).
Secreted membrane bound proteins are often modified by attachment of sugar moieties, a process termed glycosylation (Kent, 1967). Defects in degrading glycoproteins often results in neurological diseases such as mental retardation (Cantz and Ulrich-Bott, 1990). Protein-glycosylation is also of importance in plant development. For example, Arabidopsis contains an extracellular glycosyl phosphatidylinositol anchored glycoprotein that is involved in mediating directional root growth (Sedbrook et al., 2002).
Prenylation is a process where compounds from the isoprenoid pathway, such as farnesyl and geranylgeranyl, are covalently bound to the carboxy terminal ends of proteins by forming a thioester linkage. Prenylation participates in growth regulation and signal transduction as well

as in membrane trafficking and cell cycle regulation (Anderegg et al., 1988; Hancock et al., 1989; Vorburger et al., 1989; Anant et al., 1992). Ras, an important oncogene in mammals, belongs to the superfamily of small GTPases. Ras is farnesylated and inhibition of farnesylation results in loss of Ras function (Hill et al., 2000). This makes the farnesylation process an important target for the development of anti-cancer drugs. Farnesylation is also involved in regulating the floral transition. APETALA1 (AP1), a MADS box transcription factor involved in mediating the transition from inflorescence shoots to floral meristems, is farnesylated *in vitro* and *in vivo* (Yalovsky et al., 2000). Overexpression of AP1 causes formation of a compound terminal flower, which is absent in plants overexpressing a mutant form of AP1 that lacks the farnesyl-acceptor. Furthermore, ectopic expression of AP1 in *era1-2* mutants, in which the gene for the farnesyltransferase β-subunit (*At*FTB) is deleted, shows the same phenotype as overexpression of the AP1 farnesyl acceptor mutant (Yalovsky et al., 2000). This indicates that function and specificity of AP1 involves post-translational regulation by farnesylation. Other post-translational modifications such as ubiquitination, sumoylation and phosphorylation and their contributions in controlling the floral transition are discussed in the three following sections.

1.2.5.1. Ubiquitination

Failures in regulated proteolysis result in uncontrolled cell divisions and can have dramatic effects for the normal development of an organism. Many proteins that stimulate the progression of the cell cycle, such as proto-oncogenes, are under strict proteolytic control ensuring their degradation and cell cycle arrest. In many types of cancer this proteolytic control is disrupted resulting in accumulation of cell-cycle stimulating proteins (Hochstrasser, 1995; Tanaka, 1995). Ubiquitin is a small, globular protein of 76 amino acids. It can be attached to target proteins involving a complex machinery of different enzymes. The ubiquitin-activating enzyme (E1) attaches ubiquitin via the glycine residue at position 76 forming a thioester linkage. Subsequently, the ubiquitin-moiety is transferred from the E1-enzyme to the ubiquitin-conjugating enzyme (E2). Prior to approaching target proteins, ubiquitin is transferred from the E2 to the ubiquitin-ligase (E3) that can then ligate ubiquitin to the target protein. After attachment of a mono-ubiquitin to the target protein, several ubiquitin-moieties can be attached by the E3-ligase resulting in the formation of a polyubiquitin chain. Polyubiquitinated proteins are targeted for degradation by a multiprotein

complex, the 26S-proteasome (Sullivan et al., 2003). Some proteins are not targeted for the attachment of a polyubiquitin chain after mono-ubiquitination. Mono-ubiquitination has been shown to influence several processes, such as protein trafficking, regulation of transcription and translation (Weissman, 2001; Conaway et al., 2002; Aguilar and Wendland, 2003). In Arabidopsis around 5% of the genes in the genome encode proteins involved in the degradation pathway (Vierstra, 2003).

Forward genetic screens carried out in Arabidopsis have identified a variety of components of the proteolysis pathway that are involved in regulating developmental processes. The identified genes encode mostly F-box proteins or RING finger proteins that act as E3-ubiquitin ligases. FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1) mediates the cyclic degradation of CYCLING DOF FACTOR1 (CDF1) a Dof transcription factor involved in repression of the flowering-time gene *CONSTANS* (Imaizumi et al., 2003; Imaizumi et al., 2005). TIR1, another F-box protein acts as receptor for the plant hormone auxin and mediates degradation of Aux/IAA proteins (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). Also proteins closely related to TIR1 interact with auxin and mediate degradation of substrates (Dharmasiri et al., 2005b).

Downstream components of the plant photoreceptor signaling pathways that are involved in repressing photomorphogenesis in darkness were identified by mutant screens in Arabidopsis. These are the *COP*/*DET*/*FUS* genes, which have pleiotropic effects on the development of the plant (Pepper et al., 1994; Wei et al., 1994; Wei and Deng, 1996; Suzuki et al., 2002). COP1, a RING finger protein, mediates the degradation of HY5, a promoter of photomorphogenesis, in response to darkness (Deng et al., 1992); (Osterlund et al., 2000). Also for the flower promoting protein CONSTANS (CO) COP1 and CO co-localize in sub-nuclear speckles (chapter 9.1) and interact with each other *in vivo* (chapter 9.2). Since *cop1* mutants exhibit an early-flowering phenotype both under long and short-day conditions (Dieterle et al., 2003), it is likely that COP1 is the E3-ligase regulating CO protein abundance in response to photoperiod.

1.2.5.2. Sumoylation

SMALL UBIQUITIN RELATED MODIFIER (SUMO) proteins are structurally and mechanistically similar to ubiquitin. Although they only show around 18% identity in their amino acid sequences, they show a high degree of structural similarity (Bayer et al., 1998); (Mossessova and Lima, 2000). The cyclic process of SUMO attachment to substrate proteins is highly similar to the ubiquitin cycle. The enzymes involved in this process are also termed E1 (SUMO-activating enzymes), E2 (SUMO-conjugating enzymes) and E3 (SUMO ligases) (Dohmen, 2004). In yeast SUMO is encoded by a single copy gene. In mammals four genes code for SUMO and in Arabidopsis eight SUMO-genes were identified (Dohmen, 2004). SUMO can be attached as a single entity as well as a poly-SUMO chain. In 1996 SUMO was identified as a modifier of the shuttling protein complex RanGAP. Sumoylation targets RanGAP to the nuclear pore complex and stimulates an interaction with RanBP2 (Matunis et al., 1996; Mahajan et al., 1997). Apart from protein trafficking, SUMO is involved in a variety of processes, such as chromosome segregation, cell division, DNA replication and repair (Dohmen, 2004). Also transcription factors can be sumoylated and sumoylation is involved in regulating transcription factor activity (Ross et al., 2002; Sapetschnig et al., 2002). Another interesting finding was that the polycomb group protein PC2 acts as a SUMO E3 ligase (Kagey et al., 2003) and that in *Caenorhabditis elegans* SUMO modification is required for the regulation of homeotic genes via the polycomb group protein SOP2 (Zhang et al., 2004).

In Arabidopsis, recent studies indicate a broad function for sumoylation in a variety of processes. For example, heat stress induces the conjugation of SUMO1 and SUMO2 (Kurepa et al., 2003). Also the floral transition seems to be controlled by sumoylation. Forward genetics led to the isolation of *EARLY-FLOWERING IN SHORT-DAYS4* (*ESD4*). Loss-of-function *esd4* mutant plants flower early under short-days. The early-flowering phenotype is due to low levels of the floral repressor *FLC* and to an *FLC*-independent mechanism (Reeves et al., 2002). Cloning the *ESD4* gene revealed that it shows high similarity to SUMO proteases of yeast and mammals that are involved in removing SUMO from target proteins (Murtas et al., 2003). A mutation in *ESD4* results in accumulation of sumoylated substrates and a decrease in free SUMO. Overexpression of mature SUMO in *esd4* mutants enhances the phenotype, which supports its role in removing SUMO from target proteins (Murtas et al., 2003). Since sumoylation can affect transcription factors as well as epigenetic modifications it

could interfere with the flowering process at various stages of transcriptional and post-translational regulation.

1.2.5.3. Phosphorylation

Phosphorylation is the transfer of a phosphate group (H_2PO_3), mostly from an energy-rich donor such as ATP, onto a target protein. This transfer is often mediated by protein kinases (Lodish et al., 2000). Phosphorylation of transcription factors by CASEIN KINASE2 (CK2) modulates their ability to interact with other proteins (Bek and Kemler, 2002). Examples from Arabidopsis are CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LONG ELONGATED HYPOCOTYL (LHY), two myb transcription factors that are considered to form part of the central oscillator of the circadian clock (Schaffer et al., 1998; Wang and Tobin, 1998; Alabadi et al., 2001; Mizoguchi et al., 2002). CCA1 is phosphorylated by CK2 and phosphorylation is required for the formation of protein-DNA complexes containing CCA1 (Daniel et al., 2004). Overexpression of *CCA1* abolishes expression of the circadian oscillator and of output genes. A mutant form of *CCA1* can not be phosphorylated and does not show this reduction in clock gene expression, indicating that phosphorylation is crucial for the activity of the CCA1 protein (Daniel et al., 2004).
During the floral transition, phosphorylation also appears to have a regulatory function. In rice, a phosphatidyl inositol monophosphate kinase (*Os*PIPK1) negatively regulates floral initiation (Ma et al., 2004). In tobacco, a calcium/calmodulin-binding protein kinase acts to repress flowering (Hua et al., 2004). Beside kinases, phosphatases that are involved in removing phosphate groups from target proteins, influence the floral transition. Overexpression of *PHYTOCHROME-ASSOCIATED PROTEIN PHOSPHATASE 2A* (*FyPP*) causes a delay of flowering whereas knock outs of *FyPP* cause acceleration of flowering. This indicates that FyPP modulates phytochrome signals in the control of flowering (Kim et al., 2002). Abundance of HY5, a bZIP transcription factor involved in photomorphogenesis and root development (Oyama et al., 1997) is regulated by phosphorylation. COP1, an E3 ubiquitin ligase, is involved in repressing photomorphogenesis by degrading HY5 (Ang and Deng, 1994; Ang et al., 1998). HY5 exists in two isoforms, resulting from phosphorylation of the COP1-binding domain. It was shown that the dephosphorylated isoform is physiologically more active and a target for degradation by COP1 (Hardtke et al., 2000). LONG HYPOCOTYL IN FAR-RED LIGHT1 (HFR1), a putative bHLH transcription factor is also degraded by COP1 (Duek et al., 2004). In contrast to HY5 the phosphorylated form of HFR1

is physiologically active and targeted for degradation by COP1 (Duek et al., 2004). Apart from being a putative target for COP1, CONSTANS also exists in phosphorylated and non-phosphorylated isoforms (Wim Soppe, unpublished). Furthermore, preliminary results indicate that the phosphorylated form is biologically active and targeted for degradation by COP1 (Wim Soppe, unpublished). A PP2C phosphatase was isolated that interacts with CONSTANS in yeast and *in vivo* (chapters 9.3 and 9.4). Overexpression of *PP2C* does not show alterations in the floral transition but *35S::PP2C 35S::CO* F1 plants seem to flower later compared to *35S::CO* plants (chapter 9.5).

1.3. Aims of the thesis

Regulation of CO protein activity plays a major role in controlling the response of the photoperiod pathway to daylength, by activation of *FT* transcription specifically under LDs. CO acts as a transcription factor regulating *FT* expression, but does not contain a known DNA-binding motif. In order to understand the biochemical function of CO, how the protein is modified and targeted for degradation in the dark, extensive yeast-two-hybrid screening was carried out to isolate proteins that interact with CO. In particular DNA-binding proteins that could mediate an interaction between CO and DNA were of interest.
Several flowering-time pathways converge on *FT* promoting or repressing the floral transition. Since *FT* transcription integrates signals from these various pathways identifying novel regulators of *FT* transcription was also an aim of this work as this could identify components of other flowering pathways. To identify transcription factors that bind to *FT*, yeast-one-hybrid screening was carried out with a fragment of the *FT* promoter. Combining yeast-one- and yeast-two-hybrid screening technologies was intended to identify new regulators of flowering that could then be studied by reverse genetics and biochemical approaches.

2. Materials and Methods

2.1. Materials

2.1.1. General molecular biological techniques, enzymes and chemicals

Standard molecular biological techniques such as working with DNA (PCR-amplification, separation on agarose gels, restriction digests, etc.), protein (SDS-PAGE, western blot etc.) and preparation of buffers and media were carried out as described by Sambrook and Russell (Sambrook and Russell, 2001).
Chemicals used were purchased from Amersham, Sigma, Merck, Roth, Boehringer, GIBCO unless otherwise stated.

2.1.2. Kits

ECL western blotting system	Amersham, Piscataway, USA
TA cloning kit	Invitrogen, Karlsruhe, Germany
Qiaex agarose gel extraction kit	Qiagen, Hilden, Germany
Qiagen plasmid preparation kits	Qiagen, Hilden, Germany
PCR purification kit	Qiagen, Hilden, Germany
TNT Quick coupled system	Promega, Mannheim, Germany

2.1.3. Enzymes

All enzymes were used following the manufacturers instructions unless otherwise stated.

Expand High Fidelity Taq polymerase	Roche, Mannheim, Germany
T4 ligase	New England Biolabs, Frankfurt am Main
Shrimp alkaline phosphatase	Roche, Mannheim, Germany
DNaseI	Roche, Mannheim, Germany
SuperscriptII Reverse Transcriptase	Invitrogen, Karlsruhe, Germany
Gateway BP clonase	Invitrogen, Karlsruhe, Germany
Gateway LR clonase	Invitrogen, Karlsruhe, Germany
Restriction enzymes	New England Biolabs, Frankfurt am Man
Quick change in vitro mutagenesis kit	Stratagene, La Jolla, USA
Lysozyme	Roche, Mannheim, Germany

2.1.4. Vectors

Yeast-vectors

pAS2.1	Gateway compatible bait vector containing the DNA-binding domain of GAL4
pACT2	prey vector containing the GAL4-activation domain (Clontech)
pHISi	yeast-one-hybrid bait vector (Clontech)
pLacZi	yeast-one-hybrid LacZ vector (Clontech)
pGADT7	prey vector containing the GAL4-activation domain (Clontech)
pDEST22	prey vector containing the GAL4-activation domain (Invitrogen)
pDEST32	bait vector containing the GAL4-binding domain (Invitrogen)

TA and Gateway cloning vectors

pCR2.1 TOPO	Invitrogen
pDONR201	entry clone (Invitrogen), kanamycine-resistant
pDONR207	entry clone (Invitrogen), gentamycine-resistant

Vectors for protein expression

pDEST14	Invitrogen
pDEST17	Invitrogen, N-terminal HIS-tag
pASK-IBA43plus	IBA, Goettingen, N-terminal HIS-tag, C-terminal StrepII-tag
pGEX-6P2	Promega, N-terminal GST-fusion
pTNT-GW	Promega, modified containing Gateway® attB sites
pTNTGAD-GW	Promega, modified containing GAL4-AD and Gateway® attB sites

Vectors for plant expression

pLeela	35S-promoter, Gateway® attB sites
pAligator2	double 35S-promoter triple HA-tag, Gateway® attB sites
pSUC2	SUC2-promoter, Gateway® attB sites
pGPTV-BAR	Gateway® attB sites, GUS gene
pJawohl8	Gateway compatible for dsRNAi creating a stem-loop structure

Vectors for FRET

pENSG-YFPN	35S-promoter, Gateway® attB sites, YFP
pENSG-CFPN	35S-promoter, Gateway® attB sites, CFP

2.1.5. Oligonucleotides

Yeast one-/two-hybrid screen

300FTs	GAATTCTTTCTCTATAAACTTGGCGGTAC
300FTas	CTCGAGACGTACATCACACATTGTCGT
3AD	GTGAACTTGCGGGGTTTTTCAGTATCTACGATT
5AD	CTATTCGATGATGAAGATACCCCACCAAACC
T7	GTAATACGACTCACTATAGGGC

Gateway entry clones

phd-s	(GWF)GCATGGACGCTGATTCCAAGAGATT
phd-as	(GWR)CTTAGACTTCATCGAAAATGG
hap5a-s	(GWF)GCATGGATACCAACAACCAGCAACC
hap5a-as	(GWR)CTTAACCTTGGCCGTCGAGAT
ap2-s	(GWF)GCATGGAGAGACGAACGAGACGAG
ap2-as	(GWR)CTTAATCGAAAGAGTGATGATGATGG
mtn19f	(GWF)GCATGATGGCTCGTTACCACAG
mtn19r	(GWR)CTTAAGTACTAAGTGATTGGTAACCAT
HAP2aF	(GWF)GCATGCAATCAAAACCGGGAAGAG
HAP2aR	(GWR)CTTATGGTGCACCAGAAGAATTCA
OsMtN19f	(GWF)GCATGAGAAGGGCGACCATTCTTG
OsMtN19r	(GWR)CTCAGAGATTGCTCGTCAGCCAT
pPHD-s	(GWF)GCTCTGCTTAGAAAGCATTTCCTTCAT
pPHD-as	(GWR)CATCGATTTCTCAGAAATGGTGA
pHAP5a-s	(GWF)GCGGTCGGTTTTGGATTTTGATTT
pHAP5a-as	(GWR)CATTCAACAAGGCCCAAAATGAG
pAP2-s	(GWF)GCTGATAGTAGTATCAACGTGTCGGG
pAP2-as	(GWR)CGTTTCTCAGGAACATCCTTCTTAAA
pMtN19f	(GWF)GCAGGAAAGAGATTGATTCAGCTAATC
pMtN10r	(GWR)CCCCTCAATCTTCGTTTATAGTTTAA
pHAP3aF	(GWF)GCTAATGTAATCATATATGTT
pHAP3aR	(GWR) CTGGGTTTATACTCTACAGAAACA

Protein expression in E.coli

HISfitSTRf	CCCCCGAATTCATGGAGAGACGAACGAGACG
HISfitSTRr	CCCCCCTGCAGCATCATCATCACTCTTTCGATTAA
pGEXHAP2f	CTCGAGCTATGCAATCAAAACCGGGAAGA
pGEXHAP2r	CTCGAGTTATGGTGCACCAGAAGAATTC
pGEXHAP3f	GAATTCGCATGGCGGATACGCCTTCGAG
pGEXHAP3r	CTCGAGTTACCAGCTCGGCATTTCTTC
pGEXHAP5f	GAATTCGAATGGATACCAACAACCAGCAAC
pGEXHAP5r	GTCGACTTAACCTTGGCCGTCGAGAT
GSTFITf	GAATTCGCATGGAGAGACGAACGAGACGA
GSTFITr	CTCGAGTTAATCGAAAGAGTGATGATGATG
COCTf	GGATCCTCTTTCAGCTCCATGACCACTACT
COCTr	GAATTCTCAGAATGAAGGAACAATCCCAT

Real-time quantitative PCR

tubF	GAGCCTTACAACGCTACTCTGTCTGTC
tubR	ACACCAGACATAGTAGCAGAAATCAAG
COQF	CATGGAAACTGGTGTTGTGC
COQR	TATCTCAGGACCCTGGCTTC
FITQF	AAGCGCTCACGAATTTCCTA
FITQR	CGGTATCCCAAAAACATTCG
HAP5jQF	ACGATATTGCTGCTGCTGTG
HAP5jQR	CCATTCCCGGATTTCCTATT
HAP5cQF	CAACGCCATGACCACTACAC
HAP5cQR	TCTTGCCAATGGAAGGCTAT
MtN5UTRf	TTCCGTTTTATCCGATTCGT
MtN5UTRr	TCTGTCCCTAGGAAGCCTTG
RTMtNF	CTGCACAACCCTCCATTTTT
RTMtNR	TGCTGAAGTGGTTGACGAAG
RTHAP2aF	ACGTGCTTTTCTTCGCCTTA
RTHAP2aR	AAATGACCCAGCGCTCTCTA
RTHAP3aF	ACCCTCCAACTCCCTGTACC
RTHAP3aR	GCGTTGCCTCCTAATGGTAA

RTHAP5aF	CGAACATCTTCATCGGCTTT
RTHAP5aR	TGGATACCAACAACCAGCAA
RTPHDf	CGGTATGCCCTGTTTGTTCT
RTPHDr	CGACAGGAGAGGATCAGGAG
PHDSf	GGA CGC TGA TTC CAA GAG ATT TC
PHDSr	CTT TCT CTT TCG CTG CAA CTT AA
actinF	GGT GAT GGT GTG TCT
actinR	ACT GAG CAC AAT GTT AC

In vitro mutagenesis

HAP3gE90RF	ATACAGTTCAGAGATGCGTCTCTGAGTT
HAP3gE90RR	AACTCAGAGACGCATCTCTGAACTGTAT
co9F	TCGAGGAAGGCATATACAGAGATAAGACCGC
co9R	GCGGTCTTATCTCTGTATATGCCTTCCTCGA
co7F	ATGCAGAGATAAGACCGCAGGTCAATGGC
co7R	GCCATTGACCTGCGGTCTTATCTCTGCAT

Chromatin-Immunoprecipitation

ChIPFT1F	TTG GCG GTA CCC TAC TTT TT
ChIPFT1R	TCT CCC ACT TGG TAG CCA CT
ChIPFT2F	TTT CCA GTT TGG ACA GTA GAA CC
ChIPFT2R	GCA CGA CCA GGA TAA TTG GT
ChIPFT3F	CAA AGG GCA CTC ATG AGG AT
ChIPFT3R	AGA TTG GCA AGT GGA TGA GG
ChIPFT4F	TCT GAT ATT CAA GCC AGC CTT T
ChIPFT4R	TGA GGG TTG CTA GGA CTT GG
ChIPFT5F	TTA GTG TGG TGG GTT TGG AA
ChIPFT5R	CAG GTG GTT TCT CTG TGT TGA
ChIPFT6F	TGC GTA TTT GAG TTC GGA CA
ChIPFT6R	TCG AAA GCG AAA ACG TTC TAA

FT promoter oligonucleotides used for *in vitro* gelshifts

FT-3fwd	GGG CCA CAA ACA GAA ATA AAA AGA AAG AAA AAT ATG AAA TAA GAC GAC AAT GTG TGA TGT ACG TAG AAT CAG TTT TAG AT
FT-3rev	GGG ATC TAA AAC TGA TTC TAC GTA CAT CAC ACA TTG TCG TCT TAT TTC ATA TTT TTC TTT CTT TTT ATT TCT GTT TGT GG

FIT knock-out screening/promoter-isolation

FITf	GCG GCG AGG AAA GGT AAG CA
FITr	CTC TGT TTC CTC GTT GAC GTT G
FK1f	ATT CTG CCA AGG TGA ATC TTC TTC
FK1r	GAA TCT TAG AAC AGA GGT CGG TGA
P1f	(GWF)GCT GAT AGT AGT ATC AAC GTG TCG GG
P1r	(GWR)CGT TTC TCA GGA ACA TCC TTC TTA AA

2.2. Methods

2.2.1. Yeast-one hybrid screen

Construct preparation

For yeast one hybrid screening a 300-bp fragment of the *FT* promoter was amplified using the 300FTs/as primers and High Fidelity Taq polymerase. The PCR product was digested with EcoRI/XbaI and ligated into the pHISi1 plasmid using T4 DNA-ligase.

Transformation of *Saccharomyces cerevisiae*

A single colony of PJ69-4A was inoculated in 5ml YPAD and grown overnight. 1ml of overnight culture was then transferred to 50ml YPAD and grown for four hours at 30°C. After incubation the mixture was centrifuged at 2000g for five minutes and resuspended in 1ml of 100mM Lithium acetate pH 7.5. After transfer to an Eppendorf tube, cells were pelleted by centrifugation and resuspended in 500µl Lithium acetate pH 7.5. Aliquots of 50µl were used for transformation. These 50µl aliquots were transferred to Eppendorf tubes and cells were pelleted by centrifugation. After removing the supernatant, 240µl 50% PEG, 36µl 1M Lithium

acetate pH 7.5, 25µl 2mg/ml ssDNA and 50µl water containing 0.5µg of plasmid DNA were added. Cells were resuspended by pipetting, incubated for 30 minutes at 30°C followed by incubation for 30 minutes at 42°C. Finally, cells were pelleted by centrifugation, resuspended in 100µl 1M sorbitol and spread on selective medium.

Integration of the fragment into the yeast genome

In order to integrate the *FT*-promoter fragment into the yeast genome, the pHISi-1-construct was digested with XhoI and transformed into the YM4271 yeast strain. The promoter fragment integrates by homologous recombination in front of the HIS-locus. For screening, the HIS-marker is used for selection.

Prescreening

In order to identify a suitable strain for screening, different transformants were isolated. The transformants were plated on SD-His plates containing increasing concentrations of 3-aminotriazole. The strain used for screening was inhibited by low amounts of 3-AT, which indicates a single insertion event.

Library transformation

For one-hybrid screening two different Arabidopsis cDNA libraries were used, one prepared from shoot apex material and one from whole plants. Both libraries were constructed by Dr. Hans Sommer, MPIZ Cologne. The library transformation was carried out as described in section 2.2.2.

2.2.2. Yeast-two hybrid screen

Constructs used for screening

For the CONSTANS yeast-two-hybrid screen two constructs were used, the B-Boxes of CO, COBB (comprising the amino acids 20-105) and a construct encoding for the CCT domain of CO (CCTend, amino acids 306-373). Both constructs (in pAS2.1) were made by Dr. José Gentilhomme-Le Gourrierec.

Prescreening

In order to identify a suitable yeast strain for two-hybrid screening several transformants were selected and streaked on SD-Trp plates containing varying amounts of 3-aminotriazole, a

competitive inhibitor of the HIS-gene product. For the screenings, the selected strains were inhibited in growth by low concentrations of 3-aminotriazole.

Library transformation and screening

For the screenings with the CO B-Boxes and the CO CCT-domain two different cDNA libraries were used. A library containing *Arabidopsis thaliana* cDNAs from whole plants and a library containing cDNAs from the *Arabidopsis* shoot apex. Both libraries (constructed by Dr. Hans Sommer, MPIZ Cologne) were cloned into the pGADT7 vector and transformed into the *Saccharomyces cerevisiae* strain Y187. The libraries were introduced into the bait-containing yeast strains by mating.

Mating procedure

A 50ml bait-culture (COBB, COCCTend in PJ69-4A) was grown in synthetic drop-out medium (SD-medium) supplemented with 4% glucose and all necessary amino acids except tryptophan (Trp). Cells were counted in a hemocytometer and the volume of 3×10^8 cells was calculated. This volume was then transferred to a 50ml Falcon tube. The library containing cells were thawn at 42°C and 5ml of the library were transferred to the bait-containing falcon tube (bait/library ratio = 2.5). The mixture was centrifuged for five minutes at 4000rpm and the pellet was resuspended in 6ml YCM pH3.5. After 100 minutes incubation at 30°C with constant shaking, 5ml were transferred to 500ml sterile water and mixed well. 236ml were transferred onto a 47mm membrane filter (450nm; PALL Gelman Lab.) and incubated for five hours at 30°C on YCM pH 4.5 plates. The filter was then transferred on SD-Trp-Leu-His plates and incubated over night. The filter was overlaid in a 50ml falcon with 1M sorbitol and vortexed. The filter was removed and the tube centrifuged at 4000rpm for five minutes. The pellet was resuspended in 10ml 1M sorbitol. Cells were counted and 1µl / 0.1µl / 0.01µl / 0.001µl were plated on SD-Trp-Leu plates to calculate mating efficiency (cells on SD-Trp-Leu / all cells). Cells were spread on large SD-Trp-Leu-His plates and the amount of 3-Aminotriazole determined in the pre-screening was added. Plates were incubated for seven to ten days at 30°C. Single colonies emerging on the plates were isolated and transferred to 96-well plates containing 20µl 1M sorbitol. Cells were spread on SD-Trp-Leu-His plates using a hedgehog and transferred to 30°C. After the appearance of colonies, these plates were kept at 4°C as a masterplates. To identify interactors 2µl from the 96-well plates were used in a 25µl colony PCR reaction using the 3AD/5AD primer pair. After purification of the PCR products using the QIAGEN PCR-purification kit, PCR fragments were sequenced using T7-primer.

2.2.3. In vitro co-immunoprecipitation

In vitro transcription/translation

All proteins were produced with the TNT® Quick Coupled Transcription/Translation System from Promega. cDNAs encoding proteins of interest were recombined into pTNT-GW when used as prey and into the pTNTGAD-GW, providing an in-frame fusion of the gal4-activation (GAD) domain, when used as bait.

For a single immunoprecipitation 12.5 µl of protein was produced mixing the following components:

TNT Quick master mix	10.0µl
35S-methionine (1000Ci/mmol at 10mCi/ml)	0.5µl
Plasmid DNA (1µg/µl)	0.5µl
T7-TNT enhancer	0.5µl
Water	1.0µl

The reaction mix was incubated at 30°C for 60 minutes.

Co-Immunoprecipitation

In order to test an interaction between two or more proteins, 10µl of GAD-tagged protein was mixed with 10µl of every prey protein in 250µl 1x binding buffer (1xBB) containing 20mM Tris pH 7.5, 150mM NaCl, 1mM DTT, 0.1% Tween-20, two tablets of Complete Protease Inhibitor Cocktail (Roche) per 50ml. The mixture was incubated in an Eppendorf tube for three hours at 4°C on a rotating wheel. After three hours, 5µl of anti-GAD monoclonal antibody (Santa Cruz Biotechnology) was added and the mixture was incubated for another hour under the same conditions. Finally 20µl of protein A-coated magnetic beads (Dynal) were added and incubated for another hour. Prior to addition, the beads were washed once with 1ml 1xBB.

The tubes containing the reaction mixes were placed in a rack that contained magnets to pull the beads to the wall of the tube. The supernatant was removed, mixed with an equal volume of 2xLaemmli buffer and kept as the supernatant fraction of the immunoprecipitation. The beads were washed three times using 1ml cold 1xBB. After the final wash 30µl of 1xLaemmli was added to the beads. Beads and the supernatant fraction were heated to 80°C for five minutes. Beads were again placed in the magnetic rack and the pellet fraction was recovered.

The immunoprecipitations were analyzed by SDS-PAGE loading 1µl of the prepared proteins (input), 5µl of each supernatant fraction and 10µl of each pellet fraction. After electrophoresis the gels were dried and exposed overnight to a phosphorimager screen.

2.2.4. Gel retardation assay

Purification of recombinant protein from *E. coli*

E. coli BL21ai harboring the pDEST17-FIT plasmid were grown overnight to saturation. The next day, a fresh one liter culture was started and after two hours protein expression was induced by addition of 1ml 10% arabinose. The culture was grown until an O.D. of around 0.8-1.0 was reached. Bacterial cells were harvested by centrifugation for ten minutes at 4°C. The pellet was resuspended in 5ml buffer B (100mM NaH_2PO_4, 10mM Tris, 8M urea, pH 8.0) per gram wet weight and lysed for 30 minutes at room temperature. The lysate was centrifuged for 30 minutes at 4,000 rpm to pellet cellular debris and the supernatant. The lysate was mixed with 1ml 50% Ni-NTA slurry (Qiagen) for 60 minutes at room temperature. The mixture was transferred to an empty column with bottom cap attached. After settlement of the resin, bottom cap was removed to let out the supernatant. The resin was washed twice with buffer B and twice with buffer C (100mM NaH_2PO_4, 10mM Tris, 8M urea, pH 6.3). Recombinant protein was eluted four times with 0,5ml buffer D (100mM NaH_2PO_4, 10mM Tris, 8M urea, pH 5.9) and four times with buffer E (100mM NaH_2PO_4, 10mM Tris, 8M urea, pH 4.5). Protein concentration was determined by SDS-PAGE and Coomassie staining.
Due to insolubility the protein was purified under denaturing conditions. To use it for DNA-binding assays, a refolding was carried out by dialysis. For dialysis the fractions containing most protein were pooled and transferred into a dialysis tube. The tube was incubated at 4°C for one hour in 500 ml dialysis buffer I (100mM NaH_2PO_4, 10mM Tris, 6M urea, pH 5.0), followed by one hour in 500 ml dialysis buffer II (100mM NaH_2PO_4, 10mM Tris, 4M urea, pH 6.0) and two hours in 500 ml dialysis buffer III (100mM NaH_2PO_4, 10mM Tris, 2M urea, 10% glycerol, pH 6.5). The final dialysis was done over night at 4°C in 500ml dialysis buffer IV (200mM NaH_2PO_4, 20mM Tris, 10%glycerol, 1mM PMSF, pH 7.5). To concentrate the protein content, the dialysis mixture was applied on an Amicon Centrifuge Column with a size exclusion of 5kD. After concentrating, the protein mixture was diluted in an equal volume of 50% glycerol and frozen at -20°C.

Retardation assay

The DNA probe was prepared by PCR followed by restriction digestion. Digested and gel-purified DNA-fragments were labeled by end filling using ^{32}P-labeled dCTP and Klenow-enzyme (Roche). The probes were then purified using the Qiagen PCR purification kit according to the manufacturers instructions. The 100ng of DNA which were initially used for labeling were finally diluted in 100µl elution buffer. For retardation assays 5µl of recombinant protein was mixed with 5µl TA buffer (20% glycerol, 25mM HEPES-KOH pH 7.9, 50mM KCl, 1mM EDTA, 1mM DTT). After that 8µl of TM buffer (10mM Tris-HCl pH 7.9, 25mM $MgCl_2$) was added with 1µl poly dI/dC and the mixture was incubated on ice for 10 minutes to block unspecific DNA binding. The radiolabeled probe was added and the mixtures were incubated on ice for another 30 minutes. The binding reactions were loaded on large (15 x 20cm) gels containing 4% polyacryl amide in 0.5xTBE and separated at 100V for two hours. Gels were dried and exposed to a phosphorimager screen.

2.2.5. Transformation of Arabidopsis leaves by particle bombardment

For each bombardment experiment 5µg of plasmid DNA was used per construct. The final volume of DNA should not exceed 5µl. For ten bombardments 30µg of gold (size 1.0 micron) was washed with 1ml of 70% ethanol for 15 minutes while shaking. The gold-ethanol mixture was spun down for a few seconds in a microcentrifuge and washed three times with sterile water. Finally the gold particles were resuspended in 500µl of sterile 50% glycerol. To each DNA-mix of each bombardment experiment 50µl of the gold-glycerol mix was added under constant shaking, followed by the addition of 50µl of 2.5M $CaCl_2$ and 20µl 0.1M spermidine. The mixtures were incubated for another three minutes shaking and spun down in a microcentrifuge. After two washes, first with 140µl of 70% ethanol, second with 140µl of 100% ethanol, the DNA-gold mixture was resuspended in 50µl of 100% ethanol. For each bombardment using the BIORAD biolistic system, 25µl of the DNA-gold mixture was used.

2.2.6. Confocal microscopy and *in vivo* analysis of protein-protein interactions using Foerster resonance energy transfer (FRET)

To generate fluorescent proteins we used pENSG-YFP:GW and pENSG-CFP:GW which are Gateway destination vectors yielding N-terminal fusions of YFP/CFP driven by the 35S-promoter (kind gift from Dr. Nieves Medina-Escobar, MPIZ Cologne). Plasmids were

transformed by particle bombardment on Arabidopsis leaves. Co-localization studies and FRET analysis were performed 14-16 hours after bombardment.
Colocalization of YFP, CFP and dsRED was performed using a Leica TCS SP2 AOBS confocal microscope allowing a flexible selection of emission bandwidths and simultaneous multicolor-imaging. For FRET analysis transformed Arabidopsis epidermal cells expressing equal levels of CFP and YFP were selected. Analysis of FRET was performed using a Zeiss LCS510 META confocal microscope equipped with argon ion and He-Ne lasers. FRET was quantified using the acceptor-photobleaching (APB) technology and FRET-efficiencies were determined according to Karpova and Bhat (Karpova et al., 2003; Bhat et al., 2004).

2.2.7. Promoter-Luciferase assay

Arabidopsis leaves were transformed by particle bombardment. Gold beads were coated with pFT:LUC, 35S::GFP and 35S::effector plasmids. For negative controls, an empty vector was used instead of the 35S-effector plasmid. After incubation over night in a long day chamber (Percival), transformed leaves were sprayed with 1mM Luciferin (in 0,1% SDS) and Luciferase was detected using a Hamamatsu photon counting system. Luciferase expression of single leaves was quantified using the Hamamatsu photonics device control program HD-LIZ. After Luciferase measurements, the GFP signals were recorded using a Leica MZ-FLIII binocular system. GFP images were processed and integrated using Adobe Photoshop and Scion Image software.

2.2.8. GUS assay

To visualize the spatial expression pattern of genes, their promoters were amplified by PCR, recombined into the pDONR207 plasmid and finally introduced into the pGPTV-BAR plasmid in front of the β-glucuronidase gene. Transgenic GUS-expressing plants, or parts of them, were harvested and incubated for 10 minutes in heptane to remove cuticular waxes and dried at room temperature for about 5 minutes. The tissue was then submerged in GUS-solution (for 400ml: 15.6ml 1M NaH_2PO_4, 24.4ml 1M Na_2HPO_4, 360ml H_2O, 263mg $K_3Fe(CN)_6$, 200mg x-Gluc, 400µl triton-X100) overnight at 37°C. The solution was removed and 70% ethanol was added to remove the chlorophyll, followed by washes of 100% ethanol until the green color was completely removed and the blue GUS-pattern became visible. For long time storage, GUS solution was kept at -20°C, after thawing at 4°C.

2.2.9. Isolation of genomic DNA from plant tissue

DNA from plants was isolated using the method described by Edwards et al. (Edwards et al., 1991). For genotyping, a single Arabidopsis leaf was harvested per sample and frozen in liquid nitrogen. Samples were macerated using disposable grinders or an electric drill. Immediately after maceration 400µl of extraction buffer (200mM Tris pH 7.5, 250mM NaCl, 25mM EDTA, 0.5% SDS) was added and the sample was vortexed for five seconds. Samples were kept after this step at room temperature until the last sample was extracted. The extracts were centrifuged for 5 minutes and 300µl of supernatant was transferred to a new tube containing 300µl of isopropanol and vortexed. After a two minute rest, samples were centrifuged at 14,000 rpm. The supernatants were discarded and pellets dried at room temperature. After dissolving the pellets in 50µl TE, 1µl was used for PCR. DNA was stored at -20°C.

2.2.10. RNA isolation from plant tissue

To analyze expression levels of genes after hormone treatments or in time-course experiments, half a 1.5ml Eppendorf tube was filled with seedlings grown on plates. Total RNA was extracted using the RNeasy kit from Qiagen according to the manufacturer's instructions.

DNase treatment

To remove contaminating DNA, which would negatively interfere in quantitative PCR analysis, a DNase digestion was carried out. To 43µl of total RNA 1µl RNase inhibitors, 1µl DNaseI (10 units) and 5µl 10xbuffer (200mM Tris HCl pH 7.5, 10mM EDTA, 75mM $MgCl_2$ in DEPC-H_2O) were added and the mix was incubated for 30 minutes at 37°C.

Phenol-chloroform extraction

To remove DNase from the RNA, a phenol-chloroform extraction was carried out.
50µl DNase digest was mixed with 150µl DEPC-H_2O and 200µl Phenol. The mix was vortexed and centrifuged for 30 seconds at 20,000xg. Aqueous layer was transferred in a new Eppendorf tube and 200µl Phenol-chloroform-isoamylalcohol (25:24:1) was added. The mix was vortexed and centrifuged for 30 seconds at 20,000xg. The aqueous layer (approximately 180µl) was transferred to a new tube and 120µl isopropanol was added. The mix was vortexed, incubated at -20°C for 30 minutes and centrifuged (4°C, five minutes at 20,000xg).

The pellet was washed with 1ml 70% ethanol and centrifuged again for five minutes at 20,000xg. After drying, RNA was dissolved in 25µl DEPC-H_2O. RNA was quantified in a spectrometer and stored at -20°C.

2.2.11. Reverse transcription

Equal amounts of RNA were used for reverse transcription (usually between 1 and 5µg). Reverse transcription was carried out in 20µl final volume using the SuperscriptII reverse transcriptase from Invitrogen according to the manufacturers instructions. Independent PCR reactions were performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, California, USA). Data analysis was done by the comparative $\Delta\Delta C_T$ method (ABI PRISM 7700 user bulletin) and represent means and standard deviations of independent amplifications with three to five replicates each. Alternatively, real-time quantitative PCR reactions were performed on an iQ5 real-time PCR detection system (Biorad Laboratories Inc., Hercules, CA, USA) and data analysis was performed using the Biorad iQ5 software.

2.2.12. Quantitative RT-PCR

For real-time quantitative PCR, 2µl of a ten-fold dilution of the cDNA produced by reverse transcription was used. The amplification mix contained the following: 2µl PCR buffer containing 0.5µl/ml SYBR-green, 1µl 10µmM of each primer, 0.5µl 10mM dNTPs, 0.25µl Taq polymerase and 13.25µl water.

2.2.13. Plant Chromatin Immunoprecipitation (ChIP)

This protocol is modified from Gendrel et al. (Gendrel et al., 2002). Seedlings from each genotype (e.g. wild-type and mutant) were grown for 12-14 days on two large square plates containing MS-agar. After harvesting seedlings in 50ml Falcon tubes they were rinsed twice with deionized water followed by 20 minutes incubation in 50ml 1% formaldehyde under vacuum. The crosslinking was quenched by adding 2M glycine to a final concentration of 0.125M. Seedlings were rinsed twice with deionized water and frozen in liquid nitrogen. Samples were then ground in liquid nitrogen to a fine powder. 30ml of extraction buffer 1 (0.4M sucrose, 10mM Tris-HCl pH 8.0, 5mM β-mercaptoethanol, 1mM PMSF, one mini-tablet of protease inhibitors cocktail (Roche) per 10ml) were added to the powder in a 50ml

Falcon tube, vortexed and kept on ice. The mixture was filtered through two layers of Miracloth into a fresh 50ml Falcon and centrifuged at 4,000 rpm for 20 minutes. The supernatant was removed and the pellet was resuspended in 1ml extraction buffer 2 (0.25M sucrose, 10mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 5mM β-mercaptoethanol, 1mM PMSF, 1% Triton-X 100, 1 mini-tablet of protease inhibitors cocktail (Roche) per 10ml) and transferred into a 2ml Eppendorf tube. The mixture was centrifuged in a bench-top centrifuge at 14,000 rpm for 10 minutes. The supernatant was remove and the pellet was resuspended in 300µl extraction buffer 3 (1.7M sucrose, 10mM Tris-HCl pH 8.0, 2mM $MgCl_2$, 5mM ß-mercaptoethanol, 1mM PMSF, 0,15% Triton-X 100, 1 mini-tablet of protease inhibitors cocktail (Roche) per 10ml). 300µl of extraction buffer 3 were added in a clean 1,5ml Eppendorf tube and the resuspended pellet was layered on top. This solution was then centrifuged for one hour at 14,000rpm at 4°C. The supernatant was removed and the pellet was resuspended in 500µl nuclei lysis buffer (50mM Tris-HCl pH 8.0, 10mM EDTA, 1% SDS, 1mM PMSF, protease inhibitor cocktail). Chromatin was sonicated on ice for four times avoiding foaming and then centrifuged for 10 minutes at 14,000 rpm at 4°C. The supernatant was transferred to a new tube and centrifuged again. Finally, the supernatant was transferred to a new tube and stored at -80°C. 60µl chromatin-aliquots were transferred into new tubes and diluted with 540µl ChIP-dilution buffer (1.1% Triton-X 100, 1.2mM EDTA, 16.7mM Tris-HCl pH 8.0, 167mM NaCl). 10µl of equilibrated Protein-A-coated Dynabeads (equilibrated by washing three times in ChIP-dilution buffer) were added to each sample and mixed by rotating for one hour at 4°C. This results in pre-cleared chromatin. Samples were concentrated by using a magnetic rack and the supernatant was transferred to a new Eppendorf tube. After the addition of 5µl antibody (α-rat as negative control and α-HA for chromatin IP) to each tube, the mixture was incubated overnight at 4°C on a rotating wheel. 40µl of equilibrated Protein-A Dynabeads were added and the incubation was allowed to proceed for another hour to collect immune complexes. After removing the supernatant using a magnetic concentrator, precipitated chromatin was washed at 4°C for five minutes with 1ml low salt buffer (150mM NaCl, 0.2% SDS, 0.5% Triton, 2mM EDTA, 20mM Tris pH 8.0) followed by washing at 4°C for five minutes with 1ml high salt buffer (500mM NaCl, 0.2% SDS, 0.5% Triton, 2mM EDTA, 20mM Tris-HCl pH 8.0). Chromatin was then washed at 4°C for 5 minutes with 1ml $LiCl_2$ buffer (0.25M $LiCl_2$, 0.5% NP-40, 0.5% sodiumdeoxycholate, 1mM EDTA, 10mM Tris-HCl pH 8.0) followed by washing at 4°C for 5 minutes with 1ml TE (10mM Tris-HCl pH 8.0, 1mM EDTA). Immune complexes were eluted by the addition of 250µl elution buffer (1% SDS, 0.1M $NaHCO_3$) to pelleted beads and vortexed vigorously

followed by incubation at 65°C for 15 minutes with shaking. Elution was done twice and eluates were combined. To reverse protein-DNA crosslinks 20µl 5M NaCl was added followed by incubation at 65°C overnight. Then, 10µl 0.5M EDTA, 20µl Tris-HCl pH 6.5 and 1µl proteinase K were added for a one hour incubation at 45°C. DNA was cleaned by the addition of 500µl Phenol:chloroform, vortexing and transfer of the aqueous phase to a new tube. Then, 2µl 15mg/ml glycogen, 50µl 3M sodium acetate and 1ml 100% ethanol were added and the mixture was vortexed and incubated at -80°C for at least one hour. After a ten minute centrifugation at 14,000 rpm the supernatant was discarded and the dry pellet was dissolved in 50µl TE. Finally, 2µl of this mixture was used for PCR including a 1:20 dilution series from the chromatin of each immunoprecipitation experiment was used as input control.

2.2.14. Plant growth conditions and flowering time experiments

Plant material used for gene-expression and ChIP analysis was grown on GM plates containing 1% sucrose. After ethanol sterilization seeds were spread on plates, stratified for 3 days at 4°C and finally transferred to growth chambers with LD (16 hours/8 hours dark) or SD (8 hours light/ 16 hours dark) regimes at 22°C. For flowering time experiments seeds were sawn on soil, cold-treated for 3 days at 4°C, transferred to Percival growth chambers and grown in the desired light regime (SD or LD) at 20°C. Flowering time was determined by counting the number of rosette leaves at bolting and the number of cauline leaves after bolting.

2.2.15. Agrobacterium-mediated transformation of Arabidopsis plants

Transformation of *A. tumefaciens*

An aliquot (50µl) of an electrocompetent agrobacteria (strains GV3101 pMP90 or pMP90RK) was thawn on ice. 1µl of plasmid DNA was added, cell and DNA were mixed by flicking the tube and than transferred to an electroporation cuvette. An electric pulse was applied (2.5kV/cm, 25µF, 400Ω for 8-12ms), cells were resuspended with 1ml LB medium and transferred to a 15ml Falcon tube. After incubation at 28°C with constant shaking, 100µl were plated on LB plates containing appropriate antibiotics.

Transformation of Arabidopsis plants

Plasmid carrying Agrobacterium strains were grown overnight in one liter LB medium and appropriate antibiotics. Cells were harvested by a 30 minute centrifugation at 4.000rpm and pellets were resuspended in one liter transformation buffer (2.2g MS salts, 50g sucrose, 0.6g MES, 300µl silvet, pH 5.7). Arabidopsis plants (nine plants per pot) were dipped in the transformation mixture for two minutes and than bagged with plastic for 24 hours. After this 24 hour incubation, plastic bags were removed. After seed set plants were bagged for seed collection.

3. Identification of proteins interacting with the *FT* promoter and the CONSTANS protein

3.1. Proteins interacting with the promoter of *FLOWERING LOCUS T* (*FT*)

In order to identify proteins involved in regulating the expression of flowering-time gene *FT*, a yeast-one hybrid screen was performed. A second aim was to identify proteins that mediate an interaction between the flowering-time protein CONSTANS (CO) and the *FT* promoter. For the screen a 300-basepair (bp) fragment of the *FT* promoter that has been shown by Dr. Aidyn Mouradov to contain most of the regulatory elements required for *FT* activation by CO was used (Dr. A. Mouradov, unpublished). More recent studies show that in order to obtain a strong pattern of expression by promoter-GUS analysis a 10kb fragment of the *FT* promoter is necessary.

The 300-bp fragment was integrated into the yeast genome of the Y187 strain and tested for background expression on selective media containing increasing concentrations of 3-aminotriazole, the competitive inhibitor of the yeast *HIS* gene product (Figure 2).

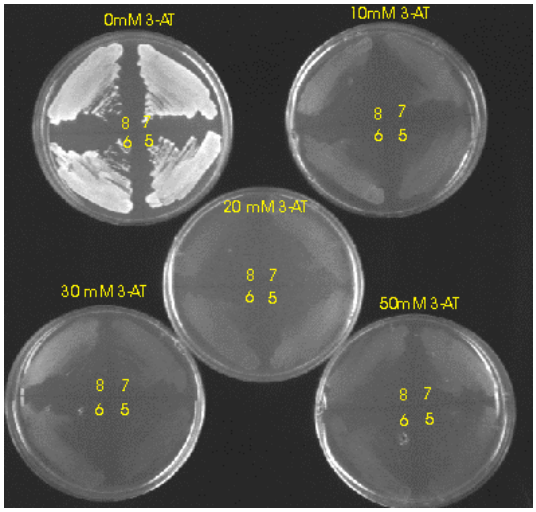

Figure 2. Analysis of the bait (300FT) containing yeast strain Y187 for growth on various 3-AT concentrations. For the screen colony #5 was selected. The screen was carried out using 15mM 3-AT.

After screening of around 200 individual yeast colonies two proteins were identified that were considered for further investigation. The first protein was an APETALA2-like protein and the second was AtMtN19, a protein of unknown function.

3.2. Proteins interacting with CONSTANS

In order to gain more insights into the biochemical function of CO, its mode of binding to DNA and possible post-translational modifications that could occur after interacting with regulatory proteins, a yeast-two-hybrid screen was performed.
CO is a B-box type transcription factor containing two conserved domains, the amino terminal B-boxes and a carboxy terminal CCT-domain (Figure 3.)

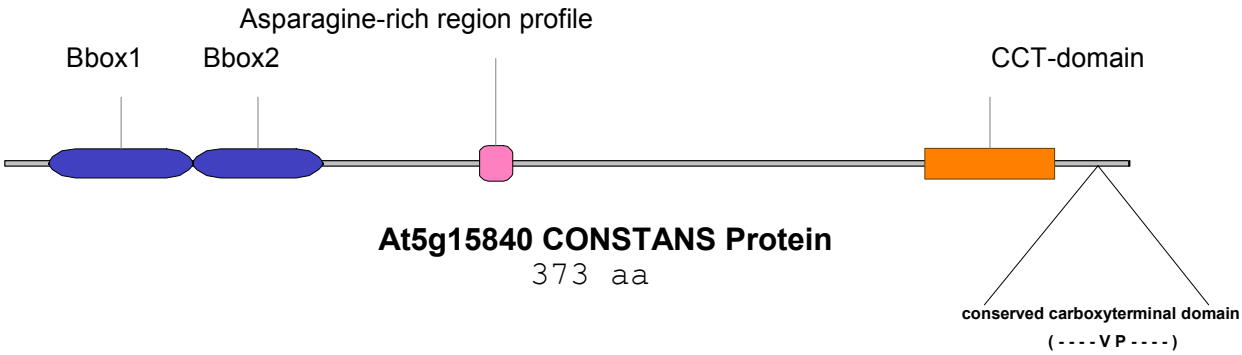

Figure 3. Conserved domains in the CONSTANS protein. The carboxyterminal end contains a VP-domain, which might be involved in interacting with the E3-ligase COP1.

The screen was carried out using two baits, the B-boxes and the CCT-domain. We screened two Arabidopsis libraries which were kindly provided by Dr. Hans Sommer. One library was made from shoot apical tissue the other was a total plant library.

After screening around 300 individual colonies by PCR (Figure 4) 20 putative interactors were selected for further investigation (Table 1).

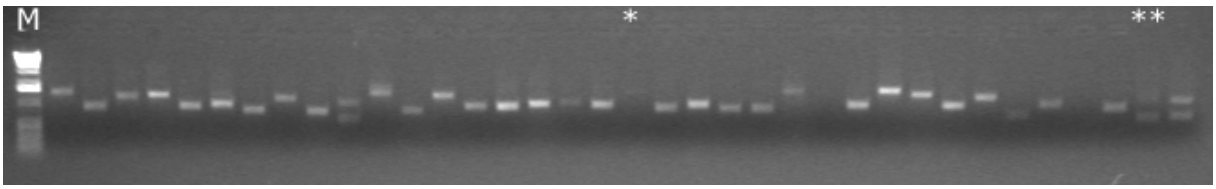

Figure 4. Amplification of prey-inserts by colony-PCR. Annealing temperature 55°C, extension time 1 minute. The gel shows the PCR fragments after purification. * No fragment visible, a second PCR reaction was carried out with 2 minutes extension. ** Two fragments visible, plasmids were extracted from yeast, transformed in E. coli, extracted and sequenced.

CCT-interactors	AGI code	B-Box interactors	AGI code
C2H2 zinc finger	At5g26610	expressed protein	At2g20890
receptor kinase	At5g16000	PHD-finger	At1g56280
armadillo protein	At1g44120	Protein kinase C receptor	At1g05100
cation transporter	At2g28180	INNER NO OUTER	At1g23420
histone-like TF	At1g56170	LIM-domain protein	At5g59990
PP2C phosphatase	At3g12620	hypothetical protein	At1g48630
kinesin-related protein TBK5	At3g16630		
LIM7-protein	At5g62850		
heme-activated protein	At1g54830		
HAP5a	At3g48590		
AtLIN10	At3g51130		
AtMtN19	At5g61820		

Table 1. Interactors selected for further investigation after yeast-two-hybrid screening.

The plasmids were extracted from yeast and transformed into *E. coli* for amplification. After DNA-extraction and sequencing of the prey-plasmids isolated from *E. coli*, the plasmids were retransformed into the bait containing yeast-strain and a strain without the bait. After screening a second time on selective medium, several candidates were discarded because of auto activation. The following candidates were selected for further investigation and the full-length cDNAs were isolated by RT-PCR: *zinc finger*, *receptor kinase*, *HAP5a*, *PP2C* phosphatase, *AtLIN10*, *MtN19*, *expressed protein*, *PHD-finger*. A third screen was carried out using the full length cDNAs of the interacting proteins in a two-hybrid screen and the interaction with the CO protein retested. The receptor kinase (At5g16000) and the expressed protein (At2g20890) showed no interaction using the full length proteins and were discarded from further analysis.

In order to identify true interacting proteins, protein interactions were tested in two other independent systems. The methods used were *in vitro* co-immunoprecipitations and *in vivo* FRET analysis. As a result we decided to further investigate the zinc finger (At5g26610), AtLIN10 (At3g51130), HAP5a (At3g48590), MtN19 (At5g61820), PP2C (At3g12620) and the PHD-finger (At1g56280) (Table 2).

interactor	AGI code	Y2H	Co-IP	FRET
zinc finger	At5g26610	+	**nd**	**-**
AtLIN10	At3g51130	+	**nd**	**-**
PHD-finger	At1g56280	+	**nd**	+
AtMtN19	At5g61820	+	+	+
PP2C	At3g12620	+	+	+
HAP5a	At3g48590	+	+	+

Table 2. Interactions observed in three independent systems. + = positive interaction observed; - = no interaction observed; n.d. not determined.

The following chapters show results obtained from studies on HAP5a (chapter 4), MtN19 (chapter 5) and FIDGET, the AP2-like protein identified by yeast-one hybrid screening (chapter 6). In the appendix (chapter 9) results from studies on the PP2C phosphatase and the PHD-finger protein are shown.

4. Physical interaction and sequence-similarities between CONSTANS and the CCAAT-box-binding factor complex

4.1. Introduction

In Arabidopsis *CO* is a member of a gene family comprising 17 members (Putterill et al., 1995; Robson et al., 2001). *COL1* and *COL2*, two very close homologues, were isolated based on sequence homology (Ledger et al., 2001). Overexpression of *COL1* and *COL2* had only very little effect on flowering-time, but transgenic plants overexpressing *COL1* sustain a shortened circadian period, which seems to be fluence rate dependent (Ledger et al., 2001). Recently it was shown that COL9 acts as a floral repressor that downregulates both the expression of *CO* and *FT* (Cheng and Wang, 2005). Additionally, several *CO* homologues were isolated from a variety of organisms including different *Brassica* species (Robert et al., 1998), rice (Yano et al., 2000), *Pharbitis nil* (Liu et al., 2001), barley (Griffiths et al., 2003), wheat (Nemoto et al., 2003), ryegrass (Martin et al., 2004) and the moss *Physcomitrella patens* (Zobell et al., 2005). The Chlamydomonas genome also encodes a single *CO-like* gene (Dr. Federico Valverde, personal communication). Therefore, although *CO-like* genes do not occur in animals they arose early during the evolution of the plant lineage.
CO homologues are involved in photoperiodic responses in species other than Arabidopsis. In rice, the *CO*-orthologue *Hd1* is also involved in the flowering response to photoperiod and has a dual function. It represses flowering in response to LD and induces flowering in SD (Hayama et al., 2003). Overexpression of Arabidopsis *CO* in potato represses tuberization in response to short days, suggesting that an endogenous *CO*-like gene acts as a repressor of tuberization in response to photoperiod (Martinez-Garcia et al., 2002).

CO encodes a protein with two amino terminal B-boxes, each containing a zinc-finger motif, and a carboxy terminal plant-specific CCT (CO, CO-like, TIMING OF CAB EXPRESSION1)-domain (Putterill et al., 1995; Strayer et al., 2000; Robson et al., 2001). In animals, a variety of B-box zinc-finger proteins exist. Most of these contain a RING-finger domain, one or two B-boxes and a coiled-coiled domain (Torok and Etkin, 2001). The first described B-box zinc finger protein was *Xenopus* nuclear factor 7 (Xnf7) (Miller et al., 1991; Reddy et al., 1992), which is involved in dorso-ventral patterning in *Xenopus* (El-Hodiri and Etkin, 1998), and probably acts as a transcriptional regulator. Structural analysis of the

promyelocytic leukemia onco-protein (PML) revealed that B-box zinc-finger proteins can bind one zinc atom per B-box (Borden et al., 1995; Borden et al., 1996). PML exists in the cell in a multimeric complex that localizes to nuclear bodies (Cao et al., 1998), a feature also observed for Arabidopsis CO (see section 9.1). The CCT-domain is plant specific and a common characteristic of CO and CO-like proteins. One of the functions of the CCT-domain is localizing the protein to the nucleus. All point mutations that strongly influence the activity of CO were found in the B-boxes or the CCT-domain, indicating the functional importance of these domains (Robson et al., 2001). Furthermore, the *co-7* mutation in the CCT-domain causes a severe late-flowering phenotype but still correctly localizes the protein to the nucleus (Robson et al., 2001). VRN2, a CCT-domain protein lacking B-boxes was isolated from wheat by positional cloning (Yan et al., 2004). VRN2 is a floral repressor whose mRNA levels are downregulated in response to vernalization. A loss-of-function mutation of *VRN2* that affects the same residue mutated as in *co-7* converts winter wheat to spring wheat, which does not require vernalization to flower (Yan et al., 2004).

TIMING OF CAB EXPRESSION1 (TOC1) of Arabidopsis is one of the most prominent CCT-domain proteins that has no B-boxes but contains an atypical response regulator receiver domain (Strayer et al., 2000). The *toc1-1* mutant is impaired in the circadian system and produces shortened circadian rhythms (Somers et al., 1998). Flowering is controlled through the function of TOC1 on the circadian clock where it feeds back on its own expression in a positive feedback loop (Strayer et al., 2000; Alabadi et al., 2001; Mizoguchi et al., 2002; Yanovsky and Kay, 2002). Recently, an activation tagging approach led to the identification of ASML2, a novel CCT-domain protein that is involved in activating the expression of a subset of sugar-inducible genes (Masaki et al., 2005).

Since CO acts upstream of *FT* and *SOC1* and the expression of *FT* and *SOC1* is down-regulated in *co* mutant plants we considered the possibility that CO acts as a transcription factor. Extensive sequence homology searches indicate that the CCT-domain contains a high degree of sequence similarity to the DNA-binding domain of the heterotrimeric CCAAT-box-binding factor subunit HAP2 (see section 4.7.). CCAAT-boxes are cis-acting elements present in approximately 25% of all eukaryotic promoters (Gelinas et al., 1985; Bucher, 1990) and are usually found 80-300 basepairs upstream of the transcriptional start site (Chodosh et al., 1988a; Chodosh et al., 1988b; Hatamochi et al., 1988). A protein complex interacting with the CCAAT-motif was identified and named CCAAT-box factor (CBF), also known as Heme Activated Protein complex (HAP) or nuclear factor Y (NF-Y) (Guarente, 1984; Guarente and Hoar, 1984; Pinkham and Guarente, 1985; Hahn and Guarente, 1988; Hahn et al., 1988;

McNabb et al., 1995). In yeast, this complex is required for the transcription of genes involved in mitochondrial respiration (Guarente, 1984; Keng and Guarente, 1987; Trueblood et al., 1988; Schneider and Guarente, 1991). The complex consists of three subunits. HAP3 (NF-YB; CBF-A) heterodimerizes with HAP5 (NF-YC; CBF-C) to create a complex surface for the interaction with HAP2 (NF-YA; CBF-B), the DNA-binding subunit (Maity and Decrombrugghe, 1992a; Kim et al., 1996; Sinha et al., 1996). After trimer assembly the complex binds with high affinity and specificity to DNA (Kim and Sheffery, 1990; Bi et al., 1997). In yeast, the trimeric HAP-complex can not activate gene expression on its own, a fourth subunit termed HAP4, containing an acidic activator domain, must join the complex (Forsburg and Guarente, 1989). In mammals, the transcriptional activator domain of HAP4 is incorporated into the HAP2 and HAP5 subunits (Coustry et al., 1995; Coustry et al., 1996). In higher eukaryotes, the HAP-complex controls a variety of genes, ranging from stage- and tissue-specific genes (Berry et al., 1992) to ubiquitously expressed housekeeping genes (Roy and Lee, 1995; Marziali et al., 1997). In addition to the HAP-complex, other proteins are also able to interact with the CCAAT-motif. For instance, Albumin CCAAT factor (ACF), a protein unrelated to the HAP-family, binds to the CCAAT-box of the albumin gene in liver and spleen (Raymondjean et al., 1988).

In yeast and mammals, each of the HAP subunits is represented as a single copy gene in the genome (Mantovani, 1999). In Arabidopsis all of the subunits are encoded by multigene families and multiple and distinct genes were identified (Edwards et al., 1998; Gusmaroli et al., 2001, 2002). The Arabidopsis genome encodes ten HAP2, ten HAP3 and nine HAP5-isoforms (Gusmaroli et al., 2002). Unlike mammals and yeast, the HAP-complex has not been shown to be necessary for gene transcription in plants. However, the first plant orthologues were identified by functional complementation of yeast *hap3* mutants (Edwards et al., 1998), indicating that plant HAP proteins are functional. The observation that all subunits are encoded by many genes in plants suggests a high degree of genetic redundancy. However, mutations in genes encoding HAP proteins were identified and found to be associated with defects in development. Silencing of a *Brassica napus HAP2* gene by antisense expression resulted in reduced male fertility due to precocious degradation of the tapetal cell layer (Levesque-Lemay et al., 2003). Recently overexpression of Arabidopsis *HAP2a* was found to cause a severe dwarf phenotype (Dr. Franziska Turck and S. Wenkel, unpublished). In Arabidopsis eight out of ten *HAP2* genes are regulated by microRNA miR169 (Rhoades et al., 2002). The microRNA binding site is located in the 3'UTR and was not present in the overexpression construct that caused the dwarf phenotype mentioned above. *LEAFY*

COTYLEDON1 (*LEC1*), which encodes a HAP3 protein, is a regulator of embryogenesis (Lotan et al., 1998). Ectopic expression of *LEC1* induces embryonic development in differentiated leaf tissue (Lotan et al., 1998; Stone et al., 2001). *LEAFY COTYLEDON1-LIKE* (*L1L*), a *LEC1* homologue, also plays a role in embryo development (Kwong et al., 2003). Furthermore, three HAP3 subunits from rice were recently isolated and named *OsHAP3A, B* and *C* (Miyoshi et al., 2003). Plants expressing *OsHAP3A* antisense or RNAi-constructs have reduced mRNA levels of *OsHAP3A, B* and *C* and this correlated with pale green leaves with degenerated chloroplasts (Miyoshi et al., 2003). This indicates that HAP3 subunits are likely to be important factors in plant development. Since genes encoding HAP4 subunits are not present in the Arabidopsis genome it is likely that another transcription factor is required to interact with the trimeric HAP-complex to activate transcription. In rice OsMADS18, which is a MADS-box transcription factor, interacts with a rice HAP3 (Masiero et al., 2002). OsMADS18 also binds the HAP3/5 dimer *in vitro* but the complex could neither interact with the DNA-binding HAP2 subunit nor bind to a CCAAT box motif *in vitro*. The authors further hypothesize that the OsMADS18/HAP3/HAP5-complex probably interacts with CArG-boxes, the target sequence for MADS-box proteins. Apart from an interaction with the CCAAT-motif, another function of a HAP3/5 dimer could be stabilizing protein-DNA-interactions with other transcription factors. From studies of the HAP-complex in mammals it is known that the HAP3/5 dimer is able to interact with TFIID (Frontini et al., 2002) and the TATA-binding protein (TBP) (Bellorini et al., 1997).
Since CO acts as a transcription factor without a known DNA-binding motif we assume that CO requires DNA-binding proteins to be recruited to DNA. Here, we present evidence that suggests that the HAP-complex interacts with CO to control the floral transition.

4.2. AtHAP5a interacts with the CCT-domain of CO in yeast

Yeast-two-hybrid screening (as described in chapter 3) resulted in the identification of three HAP5-isoforms (At3g48590, At1g54830 and At1g56170) that interacted with the CCT-domain. The interaction with HAP5a (At3g48590) was analyzed in more detail (Figure 5).

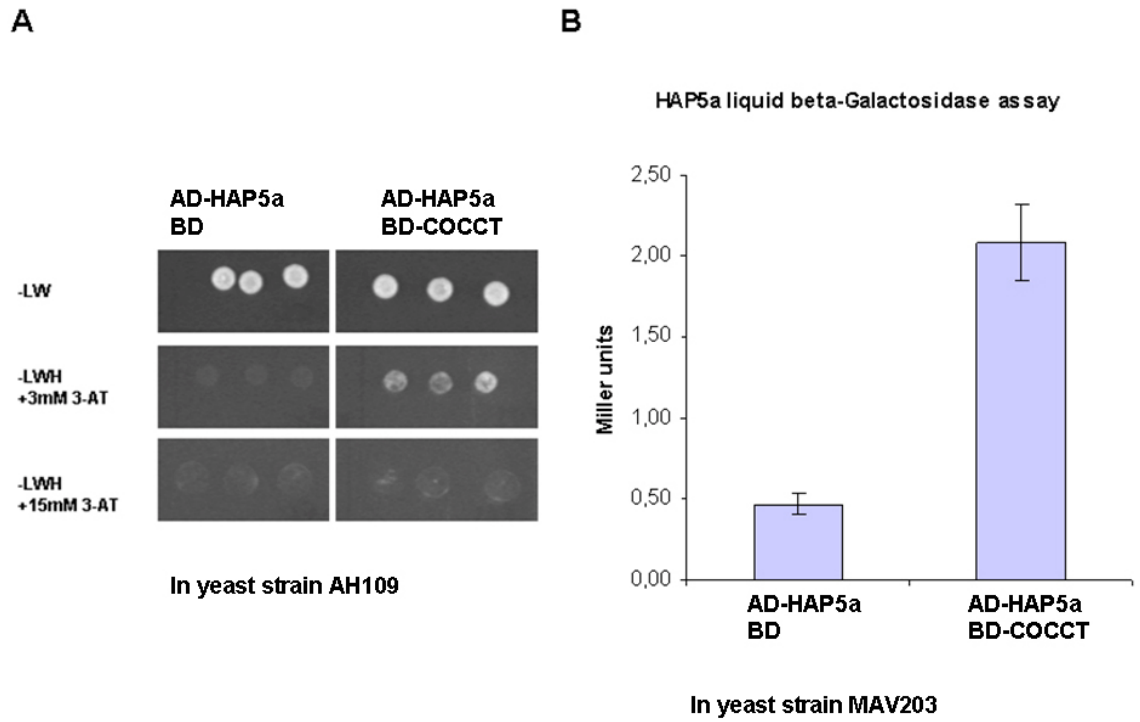

Figure 5. HAP5a reconfirmation test in yeast. A) On the left three colonies are shown that express a fusion of HAP5a to the activation domain (AD) of GAL4 and the DNA-binding domain of GAL4 (BD). These colonies can only grow on non-selective SD-medium lacking tryptophan and leucine compared to three colonies expressing AD-HAP5a and the CCT-domain of CO fused to BD which can grow on selective SD-medium lacking tryptophan, leucine, histidine supplemented with 15mM 3-AT. The screen was done in the yeast strain AH109. B) Quantitative liquid β-galactosidase assay (in yeast strain MAV 203) testing the same constructs as in A).

Reconfirmation tests in yeast analyzing the interaction between HAP5a and the CCT-domain of CO verified the interaction initially identified in the yeast-two-hybrid screen. Yeast expressing both HAP5a and the CCT-domain of CO can grow on selective medium (Figure 5a) and in liquid β-galactosidase assays a significant difference between the negative control (BD alone) was observed (Figure 5b).

The **Re**gulatory **G**ene **I**nitiative in **A**rabidopsis (REGIA) constructed a library containing 1.200 open reading frames of Arabidopsis transcription factors which were used in a large-scale yeast-two-hybrid screens (Javier Paz-Ares, 2002). One of the transcription factors isolated in this large scale screening was HAP3a (At2g38880) that was found to interact with the CCT-domain of CO.

4.3. CO interacts with HAP-proteins *in vitro*

Since CO interacts with HAP5 and HAP3 proteins in yeast, we tested the interactions between all three subunits *in vitro* by co-immunoprecipitation to confirm the binding to CO and to test whether the interactions observed in yeast are direct or indirect. CO was expressed as a fusion protein with the Gal4-activation domain (GAD) *in vitro* using the TNT Quick Coupled *in vitro* Transcription/Translation System (Promega), ^{35}S-methionine was included to label newly synthesized proteins. The HAP-proteins were produced similarly but without the GAD tag.

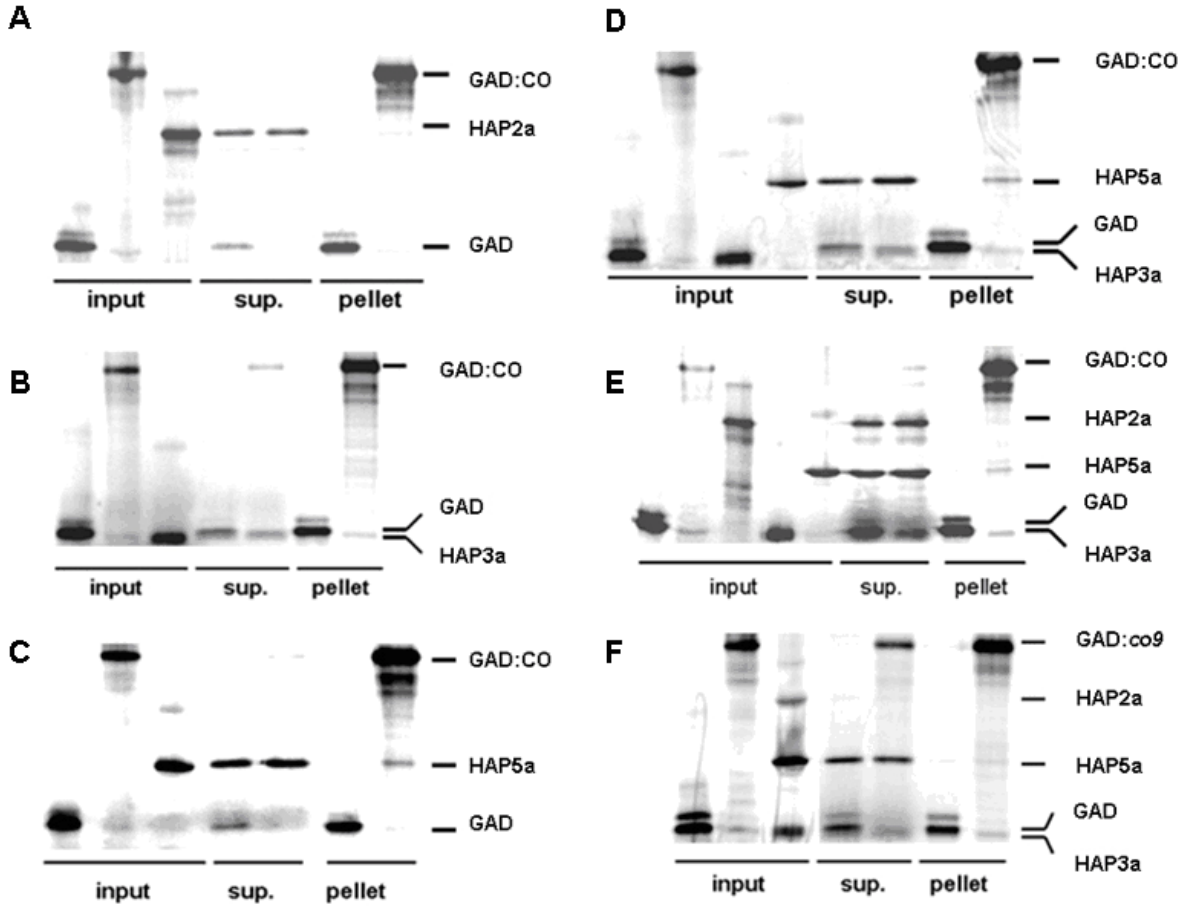

Figure 6. *In vitro*-analysis of the protein-protein interactions observed in yeast. Co-immunoprecipitation of A) HAP2a, B) HAP3a, C) HAP5a, D) HAP5a/HAP3a, E) HAP2a/HAP3a/HAP5a using GAD:CO. F) Co-immunoprecipitation of HAP2a/HAP3a/HAP5a using GAD:co9. Input: aliquots of *in vitro* produced proteins that was used for co-immunoprecipitations; sup.: supernatant fraction containing unbound proteins; pellet: fraction containing bait proteins (GAD, GAD:CO or GAD:co9) and the proteins bound to them.

Using GAD:CO and a GAD-specific antibody, immunoprecipitation of HAP3a (Figure 6b), HAP5a (Figure 6c) and the HAP3a/HAP5a dimer was observed *in vitro* (Figure 6d). HAP2a was not immunoprecipitated (Figure 6a). However, inclusion of all HAP-subunits to form the full HAP complex (HAP2a/HAP3a/HAP5a) resulted in precipitation of a faint band for HAP2 and stronger bands for the HAP3a/5a dimer (Figure 6e). Whether the co-9 mutation has an effect on the interaction with HAP2a can not be determined in these assays since the band precipitated using wild-type CO is too faint (Figure 6f). However, immunoprecipitation of HAP3a and HAP5a using GAD:co-9 was not affected.

4.4. All three HAP-complex subunits co-localize with CO in the nucleus

Transient expression of YFP-labeled CO protein and CFP-labeled HAP proteins after bombardment of Arabidopsis leaves with gold particles coated with DNA revealed that CO and HAP-proteins co-localize in the nuclei of Arabidopsis epidermal cells (Figure 7). Co-localisation is a prerequisite for an interaction *in planta*.

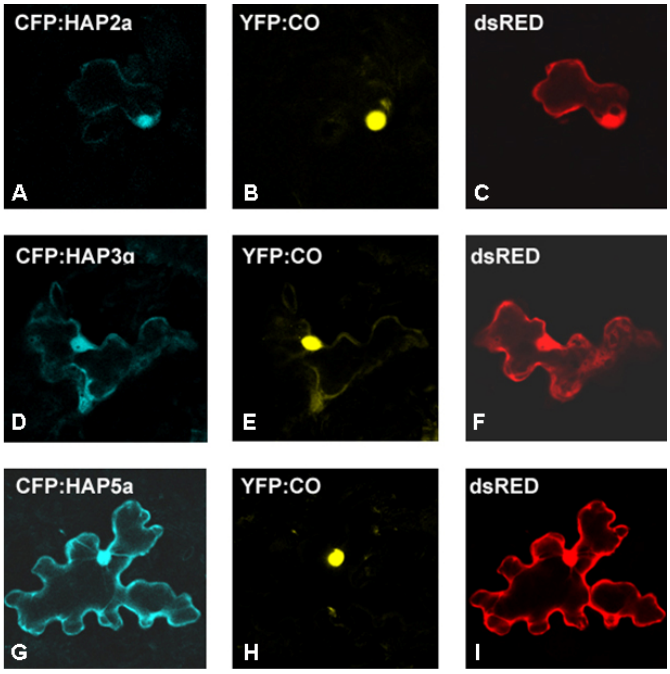

Figure 7. Transient co-expression of *35S::YFP:CO* and *35S::CFP:HAP* constructs. A *35S::dsRED* construct was co-transformed to highlight the whole cell. A-C) Co-localization of A) CFP:HAP2a B)YFP:CO C) dsRED D-F) Co-localization of D) CFP:HAP3a E)YFP:CO F) dsRED G-I) Co-localization of G) CFP:HAP5a H)YFP:CO I) dsRED.

Co-localization studies revealed that all HAP isoforms co-localize with CO in the nucleus (Figure 7). Furthermore, it was observed that HAP5a also localizes to the cytoplasm (Figure 7g).

4.5. All three tested HAP-complex subunits interact with CO *in vivo* and mutations in the CCT-domain of CO disrupt the interaction with the HAP2-subunit

Transient expression by particle bombardment of Arabidopsis leaves shows that all HAP subunits co-localize with CO in the nucleus of transfected cells. To find out whether the interactions observed in yeast and *in vitro* also occur *in vivo* we used fluorescence resonance energy transfer (FRET). Positive FRET-interactions exhibit an increase in the CFP-spectrum after YFP-photobleaching.

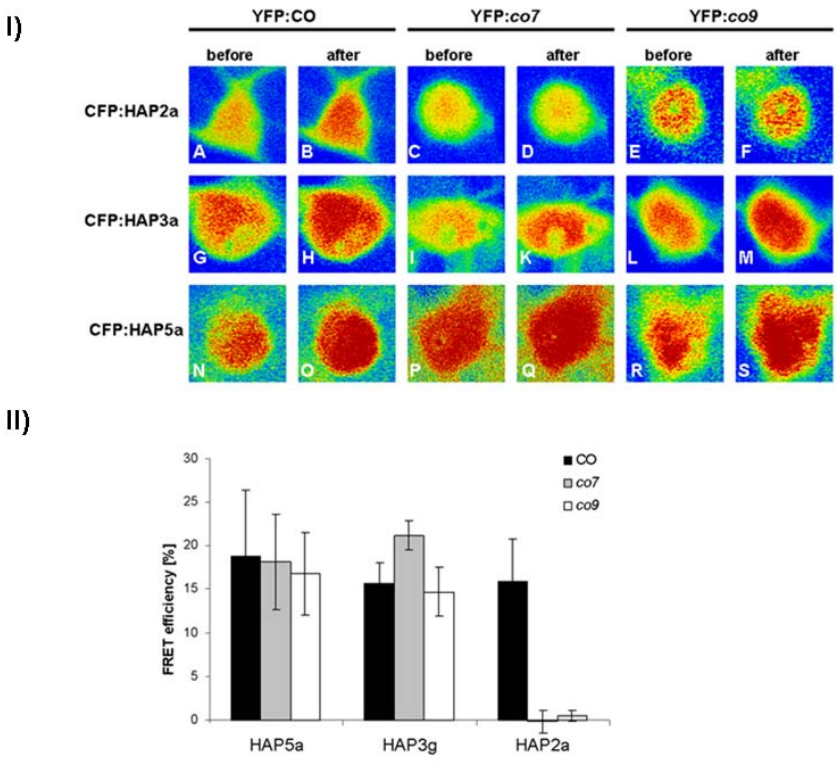

Figure 8. I) *In vivo* FRET analysis showing that CO interacts with all HAP-subunits. co-7 and co-9 do not interact with HAP2, since no increase in intensity was observed after photobleaching. YFP:CO tested with HAP2a (A,B), HAP3a (G,H), HAP5a (N,O); YFP:co7 tested with HAP2a (C,D), HAP3a (I,K), HAP5a (P,Q); and YFP:co9 tested with HAP2a (E,F), HAP3a (L,M), HAP5a (R,S) is shown. Pictures display the CFP-channel in false-colors before and after photobleaching. II) Quantification of FRET efficiencies after acceptor photobleaching.

FRET was detected between YFP:CO and CFP:HAP2a (Figure 8a,b), YFP:CO and CFP:HAP3a (Figure 8g,h) and YFP:CO and CFP:HAP5a (Figure 8n,o) *in vivo*. Since all tested interactions were positive, we analyzed whether mutations in the CCT-domain that cause late-flowering (co-7 and a recently isolated allele co-9) cause a decrease in FRET-efficiency. YFP:co-7 and YFP:co-9 both exhibited good FRET signals with CFP:HAP3a (Figure 8i-m) and CFP:HAP5a (Figure 8p-s). However, the interaction with HAP2a was abolished (Figure 8c-f). This result confirms the *in vitro* result where immunoprecipitation of HAP3a and HAP5a was not impaired using GAD:co-9. Furthermore, it demonstrates the mutations resulting in the co-7 and co-9 mutant forms affect the interaction with the HAP2 subunit *in vivo*.

4.6. Systematic interaction analysis of Arabidopsis HAP proteins with the CCT domains of CO and COL15

To test whether HAP-proteins can interact with the CCT-domains of other COL proteins and whether this is restricted to particular HAP isoforms six out of ten HAP2, nine out of ten HAP3 and nine out of ten HAP5 isoforms were tested for whether they interact with the CCT-domains of CO and COL15 (Figure 9). In these yeast-two-hybrid experiments CO and COL15 interacted with a variety of HAP proteins, implying that one feature of the CCT-domains of COL proteins could be the interaction with the HAP-complex (Figure 10, Table 3).

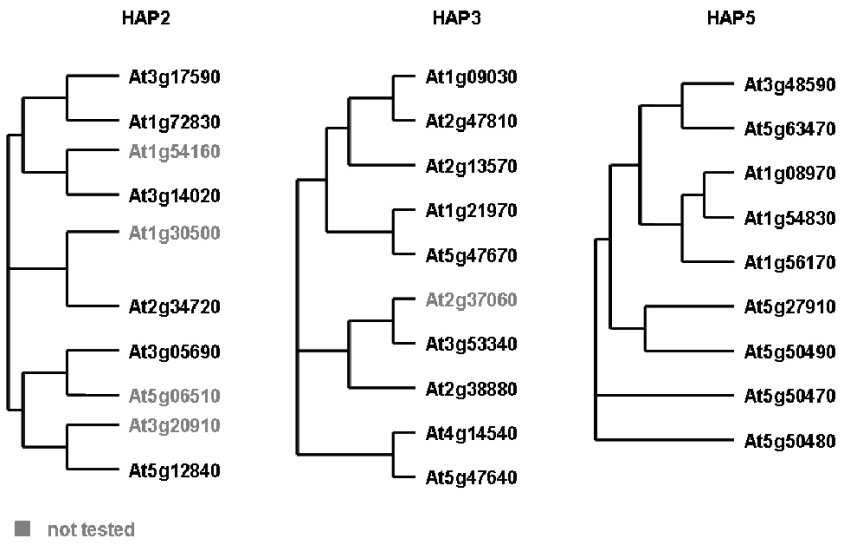

Figure 9. Phylogenetic analysis showing all Arabidopsis HAP proteins. Proteins in grey were not included in the systematic interaction analysis.

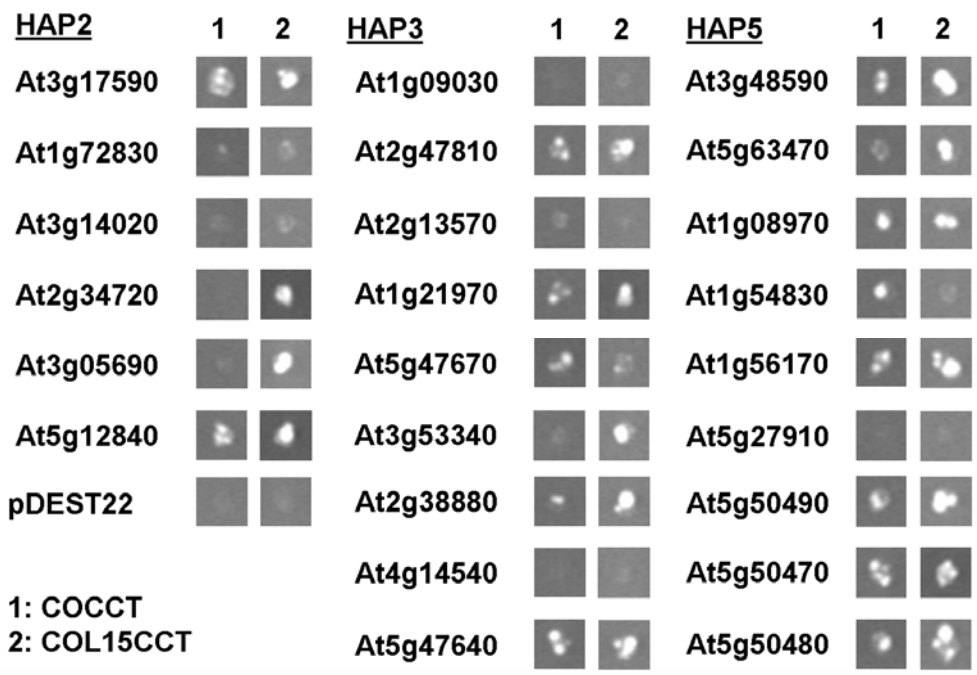

Figure 10. Yeast-two-hybrid analysis of HAP proteins interacting with the CCT-domains of CO and COL15. All prey constructs were in pDEST22 and baits in pDEST32. The screen was carried out in the MAV203 yeast strain on medium lacking histidine, leucine and tryptophan supplemented with 50mM 3-aminotriazole.

HAP2				HAP3				HAP5			
AGI-code	other name	CO	COL15	AGI-code	other name	CO	COL15	AGI-code	other name	CO	COL15
At1g17590		+	+	At1g09030		-	-	At3g48590	HAP5a	+	+
At1g72830	HAP2c	-	-	At2g47810		+	+	At5g63470		-	+
At3g14020		-	-	At2g13570		-	-	At1g08970	HAP5c	+	+
At2g34720		-	+	At1g21970	LEC1	+	+	At1g54830		+	-
At3g05690	HAP2b	-	+	At5g47670	L1L	+	-	At1g56170	HAP5b	+	+
At5g12840	HAP2a	+	+	At3g53340		-	+	At5g27910		-	-
				At2g38880	HAP3a	+	+	At5g50490		+	+
				At4g14540		-	-	At5g50470		+	+
				At5g47640	HAP3b	+	+	At5g50480		+	+

Table 3. Interactions of HAP-proteins with the CCT-domains of CO and COL15 observed in yeast.

4.7. The CCT-domain and HAP2 are related in sequence

An alignment of HAP2 and COL proteins revealed that these proteins share a domain of homology. Romier et al. distinguished two small domains in the HAP2 protein sequence, named NF-YA1 and NF-YA2, which are interconnected by a small linker region (Romier et al., 2003). NF-YA1 interacts with the HAP3/5 dimer, whereas NF-YA2 is the DNA-binding motif. Comparing the amino acid sequences of HAP and COL proteins led to the identification of a NF-YA2-type DNA-binding motif in CCT-domain proteins. Residues in

the CCT-domain that have been reported to be crucial for its function are present in the region of homology (Figure11). For example, the *toc1-1* mutation converts alanine 562 to valine resulting in non-functional TOC1 protein and to shortened circadian rhythms (Strayer et al., 2000). The same mutation in *CO*, named *co-9*, was recently isolated by screening for suppressors of *35S::CO* function in blue light (Valverde and Hayama, unpublished). The R340Q mutation in the CCT domain of CO found in the *co-7* mutant results in an extreme late-flowering phenotype under LD conditions (Robson et al., 2001). An amino acid change in the same position, from arginine to tryptophan, in the wheat VRN2 protein impairs the function of the strong floral suppressor VRN2 and results in the switch of winter wheat, which requires vernalization, to spring wheat which is vernalization insensitive (Yan et al., 2004). These four examples highlight the significance of these conserved residues between HAP2 and the CCT-domain in proteins found in different species.

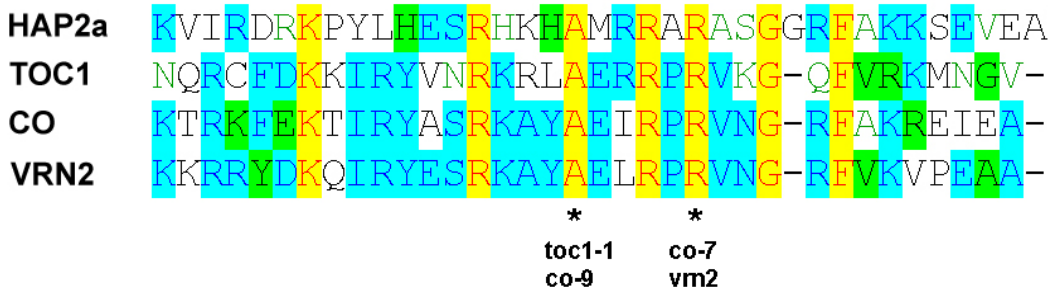

Figure 11. Protein alignment showing HAP2a in comparison to three CCT-domain proteins. The asterisks indicate the mutated residues. Both of the residues show a high degree of conservation. Color code: Yellow: conserved residues; blue: identical residues; green: similar residues.

An amino acid alignment of the NF-YA2 domain of all Arabidopsis HAP2 proteins and the corresponding regions of the CCT-domain of all COL proteins as well as the CCT-domains of TOC1 and VRN2 was performed (Figure 12). This alignment demonstrated that one can distinguish four groups of domains (Figure12). The first group comprises the HAP2 proteins, the second COL4, COL 6, COL 7 and TOC1, the third several COLs and VRN2 and the fourth group containing CO and its homolog from rice Hd1 (Figure 13). This alignment reveals also that the alanine mutated in *toc1-1* and *co-9* and the arginine that is mutated in *co-7* and *vrn2* are highly conserved (Figure 12). Very recently, PPD-H1, a CCT-domain protein controlling photoperiodic flowering in barley was isolated. The *ppd-H1* mutation is a point mutation that converts the highly conserved glycine residue (Figure 12) to tryptophan (Turner et al., 2005), further emphasizing the functional significance of this homology.

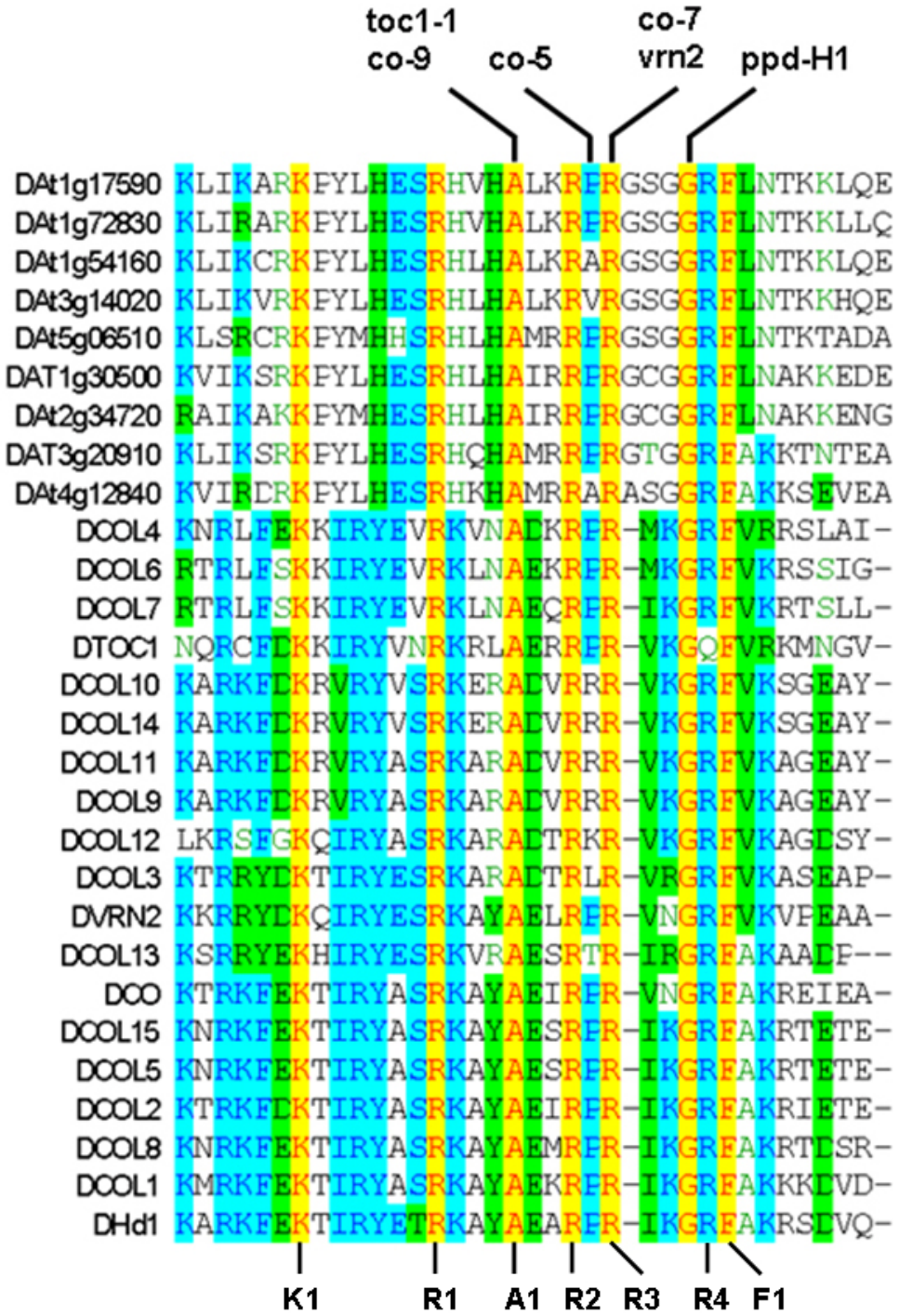

Figure 12. Sequence alignment of the conserved domain of all Arabidopsis COL proteins, rice Hd1, wheat VRN2, Arabidopsis TOC1 and all Arabidopsis HAP2. Indicated are mutations affecting these residues cause loss of function of CO, TOC1, VRN2 and PPD-H1. Color code: Yellow: conserved residues; blue: identical residues; green: similar residues. K1, A1, F1, R1-4: highly conserved residues that are of importance in HAP2 proteins.

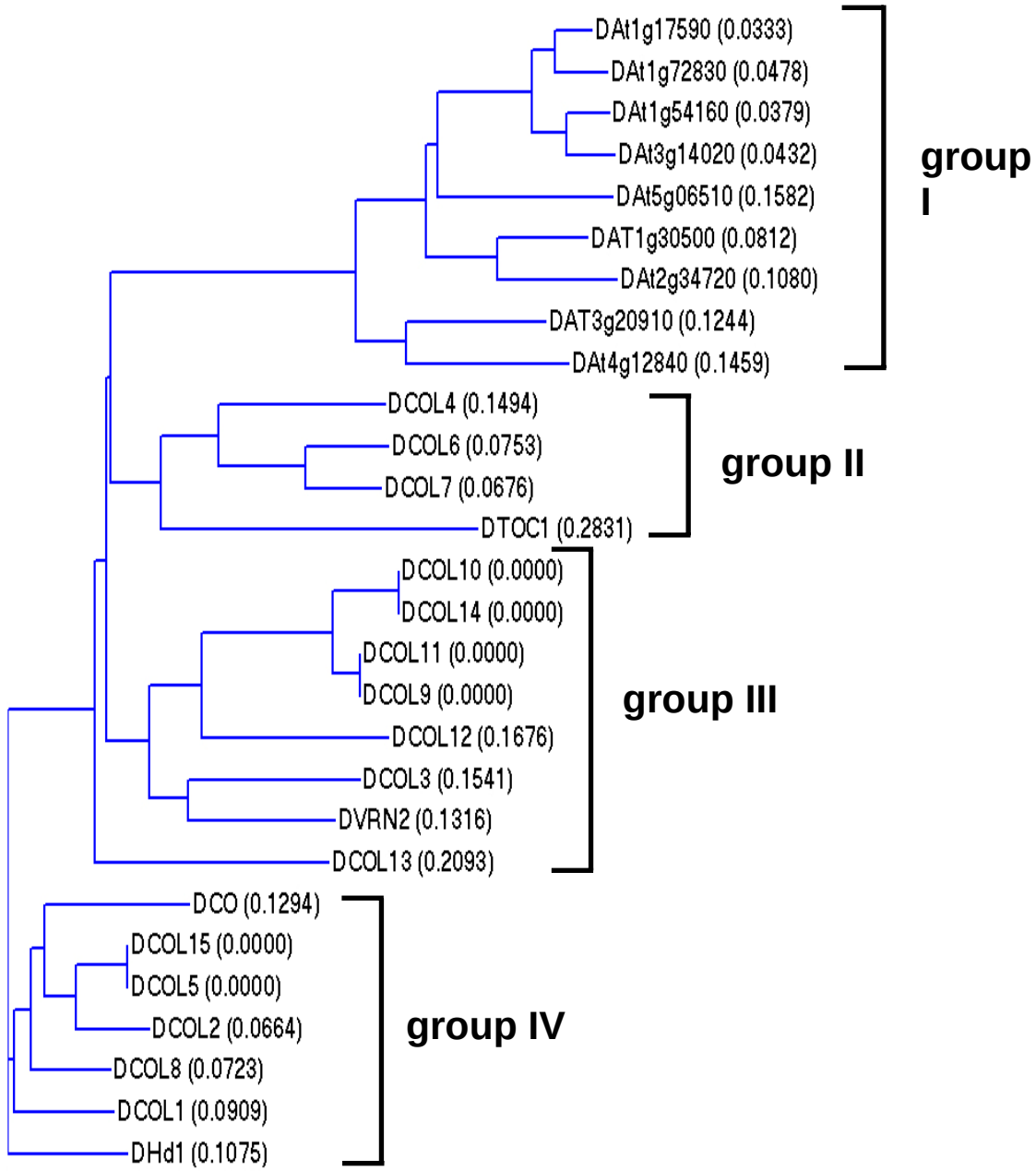

Figure 13. Phylogenetic tree showing that CCT-domain proteins can be subdivided into four different subgroups based on their conserved domain.

Comparing the homologies among HAP2 and CCT-domain proteins shows that similarities among the NF-YA2 domain of HAP2 proteins ranges from 49 to 92%. Similarities of the NF-YA2 domain among all CCT-domain proteins range from 49 to 89%. Comparing the NF-YA2 domains of HAP2 and CCT-domain proteins, the similarities range from 19 to 46% (Figure 14).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	100	92	92	89	68	76	68	65	57	35	30	30	22	30	30	30	30	24	30	32	32	32	32	32	32	32	38	35
2		100	84	81	70	70	65	62	59	35	30	32	22	27	27	27	27	24	32	35	35	30	30	30	30	30	35	30
3			100	92	70	76	65	62	59	30	30	30	19	30	30	30	30	24	30	30	30	30	30	30	30	30	35	32
4				100	68	76	65	62	57	30	30	27	19	30	30	30	30	24	30	30	30	30	30	30	30	30	35	32
5					100	68	62	62	59	30	27	27	24	30	30	30	30	24	32	35	30	30	30	30	30	30	35	27
6						100	81	68	59	30	30	30	24	30	30	32	32	27	32	32	32	35	32	32	32	32	38	32
7							100	57	49	27	32	32	24	30	30	32	32	27	30	30	30	32	30	30	30	30	32	32
8								100	73	30	30	32	30	32	32	32	32	27	32	35	35	38	46	46	43	40	40	38
9									100	30	30	27	24	32	32	32	32	27	40	38	38	32	38	38	38	32	38	38
10										100	76	68	54	54	54	54	54	51	57	54	51	54	59	59	54	59	57	62
11											100	86	54	49	49	49	49	51	54	54	49	57	57	57	59	57	57	62
12												100	54	49	49	49	49	51	51	54	51	57	62	62	62	62	57	62
13													100	51	51	49	49	49	46	51	40	49	49	49	51	49	51	49
14														100	100	92	92	65	65	59	49	54	57	57	59	54	54	54
15															100	92	92	65	65	59	49	54	57	57	59	54	54	54
16																100	100	73	70	62	51	59	62	62	65	59	59	57
17																	100	73	70	62	51	59	62	62	65	59	59	57
18																		100	65	59	54	57	57	57	57	62	59	54
19																			100	73	65	59	57	57	62	54	54	57
20																				100	59	68	62	62	65	59	59	59
21																					100	57	62	62	57	62	62	62
22																						100	78	78	81	78	76	73
23																							100	100	89	89	81	78
24																								100	89	89	81	78
25																									100	81	78	76
26																										100	84	81
27																											100	81
28																												100

1	DAt1g17590	11	DCOL6	21	DCOL13
2	DAt1g72830	12	DCOL7	22	DCO
3	DAt1g54160	13	DTOC1	23	DCOL15
4	DAt3g14020	14	DCOL10	24	DCOL5
5	DAt5g06510	15	DCOL14	25	DCOL2
6	DAT1g30500	16	DCOL11	26	DCOL8
7	DAt2g34720	17	DCOL9	27	DCOL1
8	DAT3g20910	18	DCOL12	28	DHd1
9	DAt4g12840	19	DCOL3		
10	DCOL4	20	DVRN2		

Figure 14. Table comparing similarities between the NF-YA2-domain of HAP and COL proteins (in % identity).

4.8. *GI* regulates the mRNA abundance of *HAP3a*

Microarray analysis comparing *35S::GI* and wild-type plants revealed that *HAP3a* mRNA levels are increased in *35S::GI* plants. The expression levels of *HAP3a* mRNA in wild-type Landsberg *erecta*, *gi-3* and *35S::GI* seedlings were compared over a 24 hour time course.

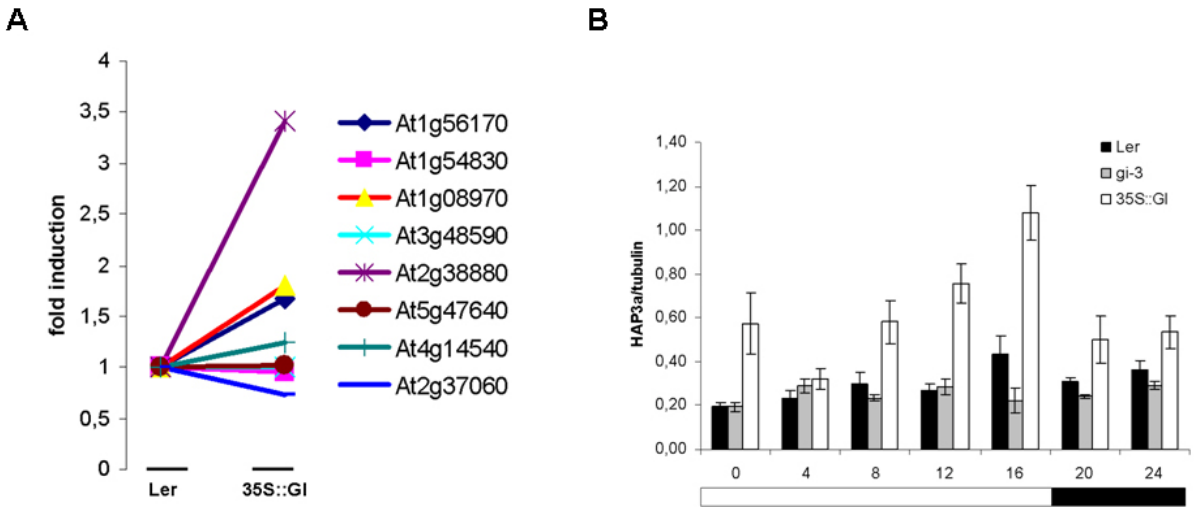

Figure 15. *GI* regulates the mRNA-abundance of *HAP3a*. A) Data extracted from microarray analysis (Affymetrix 8k chip) comparing the expression levels of several *HAP* genes between L*er* and *35S::GI*. B) qRT-PCR analysis of the *HAP3a* expression pattern in a 24 hour timecourse in L*er*, *gi-3* and *35S::GI*. Expression levels were quantified against β-tubulin.

Microarray experiments comparing the expression levels of several *HAP* genes in L*er* and *35S::GI* revealed that *HAP3a* (*At2g38880*) expression levels are significantly higher in *35S::GI* (Figure 15a violet line; microarray experiment was carried out by Dr. Dean Ravenscroft).

In wild-type plants *HAP3a* mRNA levels peak in LD at the end of the day and this peak is absent in the *gi-3* mutant (Figure 15b). In *35S::GI* expression levels of *HAP3a* mRNA are significantly increased at all timepoints of the day. The *HAP3a* expression pattern resembles the *CO* expression pattern, which is also regulated by GI (Suarez-Lopez et al., 2001).

4.9. Discussion

CONSTANS interacts with HAP5a in yeast

By yeast-two-hybrid screening, several HAP5 isoforms were isolated that interact with the CCT-domain of CO. All identified HAP5 proteins appeared independently several times in the screen. In a parallel approach, the REGIA large scale yeast-two-hybrid screen identified HAP3a as a putative interactor with the CCT-domain of CO. However, no other interaction between COL and HAP proteins was observed in the REGIA screen, which might be due to the high concentrations of the competitive inhibitor of the *HIS3* gene product, 3-aminotriazole, in this screen.

Interactions between CO and HAP2, HAP3 or HAP5 were confirmed in three independent systems

In order to confirm the interactions between the CCT-domain of CO and HAP3a and HAP5a, two other methods were applied. Furthermore, whether a HAP2-subunit, the third component of a functional CCAAT-box-binding complex, also interacts with CO was tested.
By co-immunoprecipitation using *in vitro* transcribed and translated proteins the interactions of HAP5a and HAP3a with the CCT-domain of CO were confirmed (Figure 6b, c). However, since HAP3a and the Gal4-activation domain (GAD) are similar in size, it could not be excluded that HAP3 can interact with the GAD domain of GAD:CO. An interaction of HAP2a and CO was not observed (Figure 6a), indicating that other protein factors, most likely a HAP3 or a HAP5 subunit, might be necessary. Since HAP5 and HAP3 proteins dimerize prior to complex formation (Romier et al., 2003) and CO also interacts with the HAP3a/5a dimer *in vitro* (Figure 2d) whether HAP2a was co-immunoprecipitated with the full HAP-complex was tested. A weak band of the size of HAP2a in these immunoprecipitation experiments was detected (Figure 6e). Using the mutated CCT-domain of *co-9* the interaction with HAP3a and HAP5a was not affected (Figure 6f). To confirm the interaction observed in the yeast and *in vitro* screens we used an *in vivo* Fluorescence Resonance Energy Transfer (FRET) approach. CO was fused to yellow fluorescent protein (YFP) and the HAP-subunits were fused to cyan fluorescent protein (CFP). After co-bombardment in Arabidopsis leaves FRET-signals were measured, applying the acceptor photobleaching method, between CO and all HAP-members (Figure 8a, b, g, h, n, o). The interaction of CO and HAP2a which was detected *in planta* but not observed *in vitro* may have been stabilized by other plant HAP-subunits. Also in this screen, whether known mutations in the CCT-domain of CO negatively

interfere with the interactions observed was tested. Mutations corresponding to *co-7* and *co-9* were introduced by *in vitro* mutagenesis and the mutated proteins were fused to YFP. Analysis of the interactions between the co-7 and co-9 proteins and both HAP3a and HAP5a showed that they still interact (Figure 8 l-m, p-s). However, no interaction between HAP2a and co-7 or co-9 was detected (Figure 8 c-f). Establishing a negative interaction is difficult with the FRET-system because even the positive interactions observed do not occur in all cells expressing both fluorescent proteins. In summary, the *in vivo* FRET analysis confirmed that mutations in the CCT-domain affect the interaction with HAP2-proteins. These data also demonstrate that CO interacts with HAP3a and HAP5a in yeast, *in vitro* and *in vivo*.

CONSTANS and CONSTANS-LIKE15 interact with all members of the HAP complex

To analyze how specific the interactions between CO and the HAP proteins are, yeast-two-hybrid tests were performed with six of ten Arabidopsis HAP2 isoforms, nine out of ten HAP3 isoforms and nine out of ten HAP5 isoforms together with the CCT-domains of CO and COL15 (Figure 9 and 10). COL15 was chosen because it is one of the most distant proteins from CO (Griffiths et al., 2003). CO and COL15 were able to interact with multiple isoforms of all subunits (Figure 10). These interactions were only tested in yeast and might not occur *in planta*, but support the idea that one of the functions of the CCT-domain may be to interact with HAP-proteins probably to access DNA and regulate transcription.

CONSTANS and HAP2 share a domain of homology

The CCT-domain and HAP2 proteins share a domain of homology. Mutations in three highly conserved residues in this domain negatively affect the function of known CCT-domain proteins such as CO, TOC1, VRN2 and PPD-H2 (Strayer et al., 2000; Robson et al., 2001; Yan et al., 2004; Turner et al., 2005). Biochemical analysis of the mammalian CBF-B subunit of the heterotrimeric CCAAT-binding factor, which is homologous to the HAP2 subunit revealed that mutations in the highly conserved arginine (R2, Figure 12) affect DNA-binding but not the formation of the trimeric complex (Maity and Decrombrugghe, 1992b). From studies of HAP2 in yeast it is known that amongst others, the residues A1, R2, R4 and F1 (Figure 12) are involved in DNA binding and mutations affecting these residues cause loss of the DNA-binding ability (Xing et al., 1993). The effect of triple amino acid substitutions was analyzed in mammalian NF-YA (HAP2) proteins. Interestingly, a triple substitution including the arginine residue R3 caused loss of DNA-binding but the formation of the complex remained unaffected (Mantovani et al., 1994). These studies demonstrate that mutations in

HAP2 proteins affecting alanine A1 (that is mutated in *co-9* and *toc1-1*) and arginine R3 (mutated in *co-7* and *vrn-2*) result in loss of DNA-binding. This suggests that one of the functions of the NF-YA2 domain in CCT-domain proteins might involve DNA-binding.

Analysis of the NF-YA2 domain of all Arabidopsis HAP2 and COL proteins revealed that based on sequence similarities these protein families can be subdivided into four different groups. This analysis demonstrated that the choice of COL15 in the systematic yeast-two-hybrid approach was not optimal because although it was originally selected as being most distant from CO, comparing the sequences of the NF-YA2 domain showed that CO and COL15 are close homologues sharing 78% of sequence identity (Figure 12 and 14).
The similarities within the HAP2 family are comparable to similarities within the COL family ranging from around 50 to 90% (Figure 14). Comparing both families against each other, the sequence similarities range from 19 to 46%, which appears to be significant because apart from this domain both protein families are very divergent. Comparing sequence homologies of the HAP5 domain, which is unrelated to the CCT-domain, and all COL proteins revealed sequence identities ranging from 9-28% (data not shown).

Composition of a putative CO protein complex binding to DNA

Based on the presented data, four plausible models could explain how a functional CCT-domain complex could access DNA.

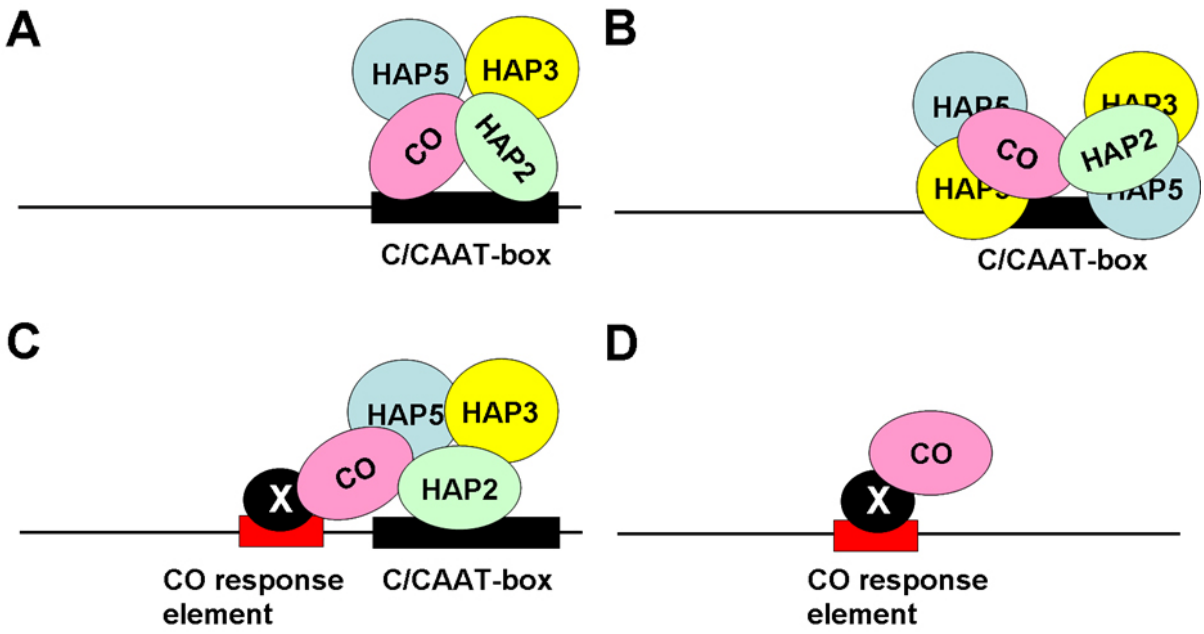

Figure 16. Four possible models of a functional CO protein complex. A) The CCT-domain protein CO and HAP2 both bind to the HAP3/5 dimer. B) One CO-HAP3/5 complex interacts with another HAP2/3/5 complex. C) CO binds to DNA by an unknown mechanism, interacts with a trimeric HAP complex and causes transcriptional activation. D) CO binds to DNA and activates transcription without HAP complex.

One possibility is that both the CCT-domain protein and the HAP2 protein interact with a HAP3/5 dimer. Both NF-YA2 domain of HAP2 and the CCT-domain protein interact transiently with each other to access DNA (Figure 16a). In an alternative model, both the HAP2 and the CCT-domain protein interact with a HAP3/5 dimer. To regulate target genes, the complexes would have to interact via their NF-YA2 domains. Since the HAP2-CCT interaction appears to be weak or transient, both HAP3/5 dimers could interact with each other and stabilize the interaction (Figure16b). Another possibility of transcriptional activation of target genes like *FT* could be that CO interacts with another protein and binds to DNA. After DNA-binding, the CCT-domain of CO interacts with a neighboring HAP complex and activates transcription (Figure 16c). Finally, we can not totally exclude that the mechanism of how CO activates *FT* does not involve the HAP complex (Figure 16d).

HAP3a is regulated by GIGANTEA

A comparison of wild-type plants with plants overexpressing *GI* in a global expression analysis performed on Affymetrix microarrays revealed that *HAP3a* expression is positively regulated by *GI*. A comparison of *HAP3a* expression levels in *gi-3*, wild-type and *35S::GI* confirmed and extended the results of the microarray experiments. In a 24-hour time course *HAP3a* shows higher expression in *35S::GI* than wild-type plants. The difference is less pronounced between *gi-3* and wild-type, however, at the 16 hour timepoint, where also *CO* mRNA shows its highest expression, the expression level of *HAP3a* is reduced by 50% in the *gi-3* mutant compared to wild-type. These data indicate that *HAP3a* and *CO* are both co-regulated by GI. Since they are expressed at the same time of day and physically interact with each other, this implies that they could also function together in a protein complex.

Analysis of transgenic plants

Available SALK and GABI-KAT T-DNA insertion lines in *HAP2a* (SALK_030989), *HAP3a* (GABI_057H09) and *HAP5a* (SALK_086334; GABI_417B12) were analyzed for phenotypic abnormalities and changes in the floral transition (data not shown). No flowering-time phenotypes or extreme morphological abnormalities were observed. Since there is likely to be genetic redundancy among members of the HAP complex, the effect of a single gene knock-out might not be obvious. This is likely, since it was already shown, that most subunits show ubiquitous expression patterns (Gusmaroli et al., 2001, 2002). Overexpression of *HAP3a* and *HAP5a* also did not cause acceleration of the floral transition in our test conditions (data not shown). These results suggest that CO is the rate-limiting component necessary to drive expression of downstream genes since *CO* overexpression causes dramatic early-flowering and increased *FT* expression (Onouchi et al., 2000); (Samach et al., 2000). Overexpression of myc:HAP2a caused a severe dwarf phenotype (results from Dr. Franziska Turck, data not shown), an effect that is probably enhanced due to uncoupling a post-transcriptional regulation by miR169. Also these dwarfed plants did not exhibit a significant slowdown or acceleration in their flowering-time behavior.

Plants overexpressing *FLAG:HAP3a* show a late-flowering phenotype that is due to reduced *FT* mRNA levels

Overexpression of *FLAG:HAP3a* by the *35S*-promoter resulted in late-flowering plants. The late-flowering phenotype correlates with a severe reduction in *FT* transcript levels. Furthermore the late-flowering phenotype is stronger in plants showing higher levels of

FLAG:HAP3a protein (results from Dr. F. Turck, see section 9.11). *FLAG:HAP3a 35S::CO* plants show an intermediate flowering-time phenotype between wild-type and *35S::CO* indicating that overexpression of *CO* partially suppresses the late-flowering phenotype (results from Dr. Franziska Turck, section 9.12). Since transgenic plants overexpressing *HAP3a* without epitope tag do not flower later than wild-type (data not shown) indicates that the FLAG-epitope probably produces a dominant-negative effect suppressing CO activity. However, the fact that overexpression of HAP3a results in late-flowering and reduced *FT* expression levels indicates that CO and HAP3a probably interact *in vivo*.

Is the HAP-complex required for activation of *FT* by CO?

35S::FLAG:HAP3a plants flower late and have low levels of *FT* transcript so if FLAG:HAP3a functions as HAP3a this suggests that the HAP-complex is not involved in the activation of *FT*. Alternatively if HAP3a is a binding partner of CO *in vivo* then HAP3a overexpression could causes its sequestration into non-flowering promoting complexes and therefore *FT*-expression would be low. Overexpression of *CO* in *35S::FLAG:HAP3a* lines can partially suppress the late-flowering phenotype perhaps by restoring the stoichiometry between HAP3a and CO due to an excess of unbound CO protein. However, other CCAAT-box containing CO-target genes should show higher expression levels in *35S::FLAG:HAP3a* plants, which we could not detect (data not shown). It could also be that the FLAG-epitope creates a dominant-negative effect, maybe by destroying the formation of the complex and therefore sequestering CO into non-functional complexes. Based on our current data, we can not conclude whether the HAP-complex is necessary for the activation of *FT*.

Analysis of the *FT* promoter reveals no CCAAT-box in the proximal promoter. However, so far it has not been demonstrated that plant HAP-proteins interact with CCAAT-elements. An interaction of the HAP-complex with CAAT-elements of the A*tp*C gene, a subunit of the chloroplast ATP-synthase, has been demonstrated in tobacco (Kusnetsov et al., 1999). Also the proximal *FT* promoter contains CAAT-elements as shown in Figure 17.

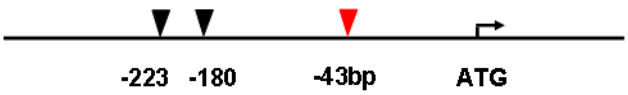

Figure 17. Distribution of CAAT-elements in the proximal *FT*-promoter. The element located close to the translational start (-43bp) is conserved at the same position in the promoter of the *Hd3a* gene from rice.

According to findings from yeast and humans a functional CCAAT-box should be located 80-300 basepairs upstream of the transcriptional start site (Chodosh et al., 1988a; Chodosh et al., 1988b; Hatamochi et al., 1988). This requirement is fulfilled by the two CAAT-elements located at -223 and -180. Interestingly the element located at -43bp in the *FT* promoter is conserved at the same position in the promoter of rice *Hd3a*, the *FT* homolog.

4.10. Conclusion

The data presented here aim to explain how CONSTANS, a transcription factor with no known DNA-binding domain could access DNA via an interaction with the heterotrimeric HAP complex. CO interacts with all members of the complex in various systems and mutations in the CCT-domain, which affect the ability to promote flowering, abolish an interaction with the HAP2 subunit. Furthermore, HAP2 and CCT-domain proteins share a domain of homology and mutations in conserved residues of CCT-domain proteins cause loss-of-function. Moreover, we demonstrated that *HAP3a* is co-regulated with CO by the flowering-time gene *GI*. Overexpression of FLAG:HAP3a results in a strong delay of the floral transition, which might be due to a dominant negative effect created by the FLAG epitope. Even in the case that the HAP-complex is not involved in flowering and CO is sequestered into non-flowering promoting complexes, CO interacts with HAP3a *in vivo* since 35S::FLAG:HAP3a plants are late flowering. Also COL15 interacts with a variety of HAP-proteins. These data indicate that CCT-domain proteins might more generally regulate target genes by interacting with the HAP-complex. These data suggest a function for a plant-specific protein domain whose function was previously unknown.

5. Characterization of *At*MtN19, a protein that provides a potential link between the flowering-time genes *CONSTANS* and *FLOWERING LOCUS T*

5.1. Introduction

The transition to flowering is regulated by different genetic pathways that have been identified (see chapter 1). Among these pathways, the photoperiod pathways controls flowering in response to day length. CO, the major component of this pathway acts by upregulation of the floral integrator gene *FT*. Since CO functions as a transcription factor but does not have a known DNA-binding domain it is likely that it needs to interact with a DNA-binding domain protein to fulfill its function. *At*MtN19 was identified independently using two approaches: as a protein interacting with the promoter of *FT* in a yeast-one-hybrid screen and as a protein interacting with the CCT-domain of CO in a yeast-two-hybrid screen.
MtN19 was first identified in *Medicago truncatula* (Gamas et al., 1996), where it is a single copy gene. However, no function could be assigned to the protein so far. In *Alstroemeria* *MtN19* is induced during post-harvest senescence (Breeze et al., 2004) and microarray analysis from soybean revealed that it is upregulated in leaves compared to roots (Vodkin et al., 2004).
MtN19 homologues respond to a variety of physiological and stress related processes such as auxin, nitric oxide (NO), insect elicitors and high fluence light. Low concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin, induce cell division and elongation. High doses of 2,4-D have inhibiting effects on plant development, making it an attractive herbicide in agriculture (Grossmann, 2000; Zheng and Hall, 2001). Treatment of Arabidopsis with high doses of 2,4-D to investigate the onset of senescence reveals a strong induction of *AtMtN19*, which is in agreement with findings in *Alstroemeria* (Breeze et al., 2004; Raghavan C. et al., 2005). NO plays a role in many physiological processes including photomorphogenesis and the regulation of stomatal closure (Beligni and Lamattina, 2000; Neill et al., 2002). NO is also involved in triggering defense responses involving reactive oxygen species (ROS) leading to the activation of pathogenesis related genes (Delledonne et al., 1998; Durner et al., 1998). The involvement of ROS is particularly intricate, because it can damage the host and the plant (Huang et al., 2002). Recently, NO was proposed to delay

the floral transition in Arabidopsis by downregulation of *CO* and *GI* and induction of the floral repressor *FLC* (He et al., 2004). Treatment of Arabidopsis suspension cells with the NO donor NOR3 in the dark leads to a strong induction of *AtMtN19* (Huang et al., 2002). Treatment of pea pots with the insect elicitor Bruchin B is also able to induce pea *MtN19* giving further evidence for a role in defense response (Doss, 2005). Bruchin B causes stimulation of cell divisions and neoplasm formation (Doss et al., 2000; Oliver et al., 2000). However, there could also be a function for *MtN19* in cell cycle regulation, since Bruchin B and auxin both stimulate cell divisions and induce *MtN19*. Finally, exposing Arabidopsis seedlings to high intensity light conditions also causes a strong induction of *MtN19* (Kimura et al., 2003). High light intensities increase endogenous levels of reactive oxygen species due to leakage of electrons from the overloaded photosynthetic apparatus (Niyogi, 1999). The broad range of information available on expression patterns of *MtN19* does not provide a strong indication of the function of this protein. However, it is striking that many of these processes have one common feature, the production of reactive oxygen species. Treatment with NO and high light elicits the production of ROS (Huang et al., 2002; Kimura et al., 2003). Also auxin-treatment induces ROS and it has been shown that ROS are essential for auxin-induced root gravitropism (Joo et al., 2001; Joo et al., 2005). Treatment of Arabidopsis with catalase-inhibitors, causing oxidative stress by the production of ROS, induces senescence-related genes indicating a role for ROS during senescence (Navabpour et al., 2003).

5.2. Sequence comparison of MtN19-like proteins from Arabidopsis and other plant species and computational analysis of the *MtN19* gene

In Arabidopsis *MtN19* exists as a single copy gene and BLAST search was carried out to identify homologous proteins from other species. The results obtained indicate that MtN19-like proteins are only found in higher plant species and they seem to exist as single copy genes. In order to identify functional domains, the sequences of Arabidopsis, rice, *Medicago* and pea were aligned using ClustalW (Figure 18).

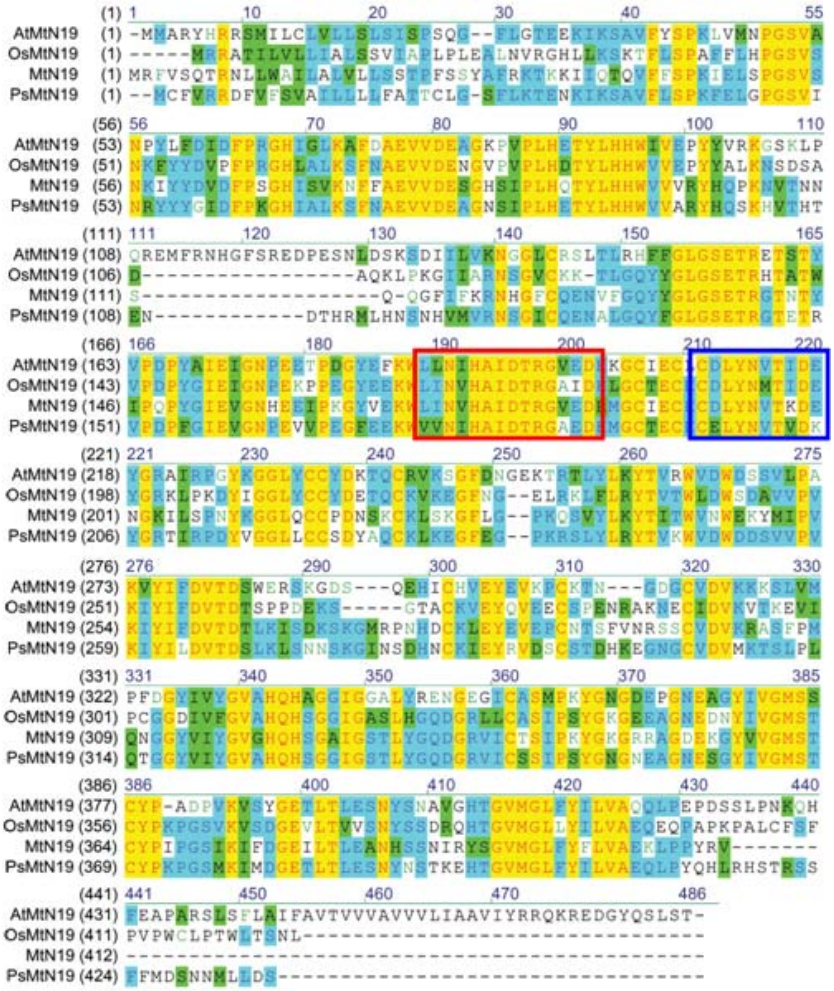

Figure 18. ClustalW alignment of MtN19-like proteins from different plant species. The sequence in the red box resembles a sequence found in a sensory box histidine kinase protein from *Desulfvibrio vulgaris*, the sequence in the blue box resembles the transcriptional regulator dicA/hipB/ansR from *Clostridium acetobutylicum*. Color code: Yellow: conserved residues; blue: identical residues; green: similar residues.

After ClustalW alignment, the sequence patches showing conservation in all analyzed proteins were used to carry out a BLAST search for short nearly exact matches (NCBI). The search revealed homology with two bacterial proteins, a transcriptional regulator and a sensory box histidine kinase (Figure 18). The conservation was only found in conserved residues but since the sequences used for the BLAST search were small (15-20 amino acids) this result might not be informative. All other BLAST searches only referred to MtN19-like proteins.

Analysis of the amino acid sequence using the TMHMM program to predict transmembrane helices (http://www.cbs.dtu.dk/services/TMHMM-2.0) reveals a putative transmembrane region near the carboxy terminus (Figure 19).

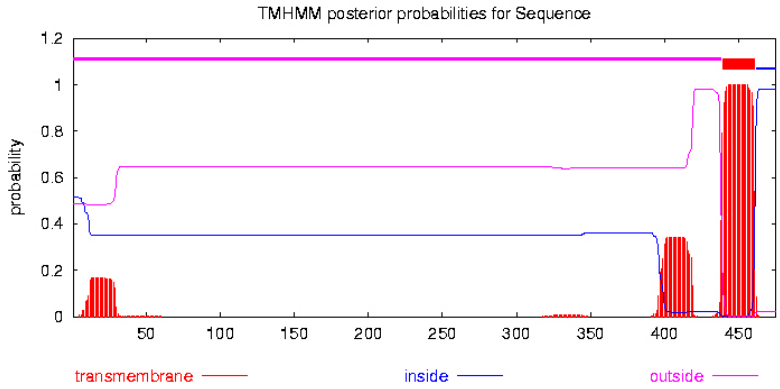

Figure 19. Analysis of transmembrane helices in MtN19 using TMHMM. Near the N-terminus of the MtN19 protein, a putative transmembrane region is predicted with a probability close to 100%.

Analysis of the amino acid sequences of *Os*MtN19, Medicago MtN19 and *Ps*MtN19 the TMHMM program predicts putative transmembrane regions at the amino terminus (data not shown).

To gain more insight into the regulation of the *MtN19* gene itself, publicly available microarray data were analyzed (www.genevestigator.ethz.ch).

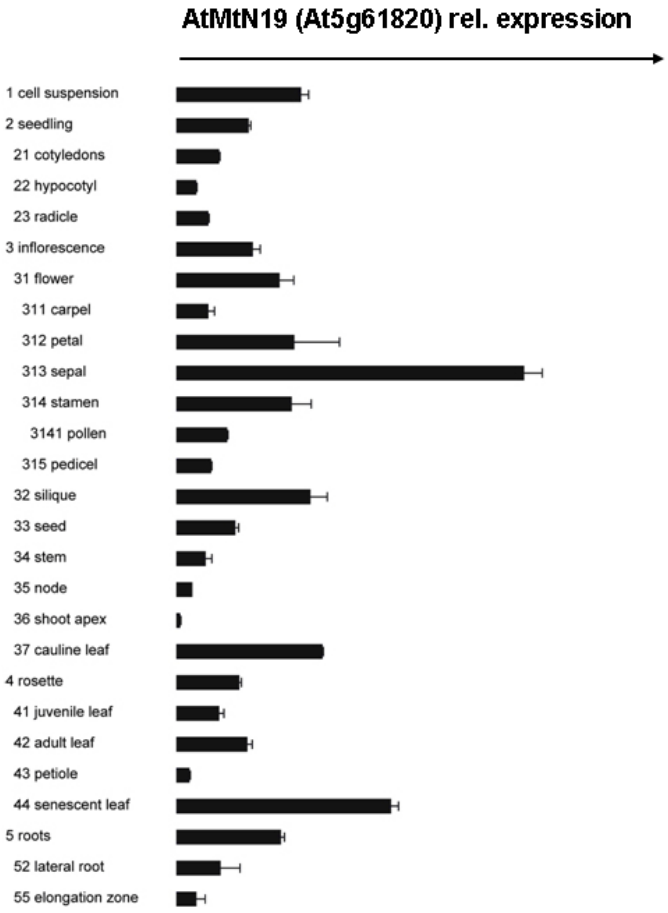

Figure 20. Digital northern analysis of *MtN19* mRNA expression. This figure was extracted from the Genevestigator homepage (www.genevestigator.ethz.ch) using the Gene Atlas tool.

Analysis of the spatial expression pattern of *MtN19* on a digital northern blot revealed that *MtN19* shows highest expression levels in sepals, senescent leaves and cauline leaves (Figure 20).

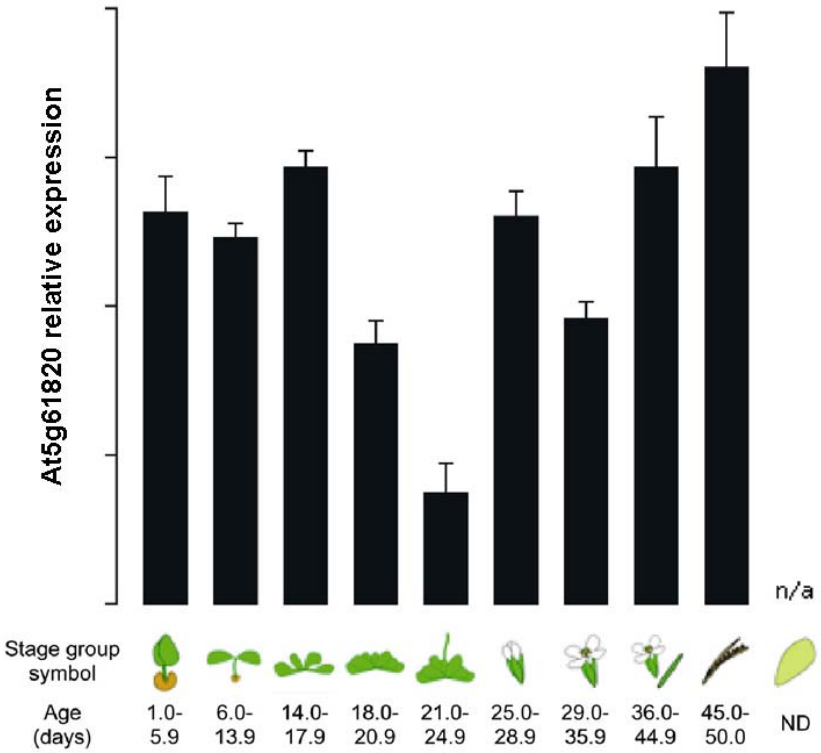

Figure 21. Analysis of the *MtN19* expression pattern during Arabidopsis the development. The graph was extracted from Genevestigator using the Gene Chronologer tool.

Analysis of the expression pattern of *MtN19* in a developmental context indicates that it is highly expressed early in development and late in development, after the transition to flowering lowest expression levels were detected (Figure 21).

Furthermore, the conditions under which *MtN19* is expressed in Arabidopsis were tested (Figure 22).

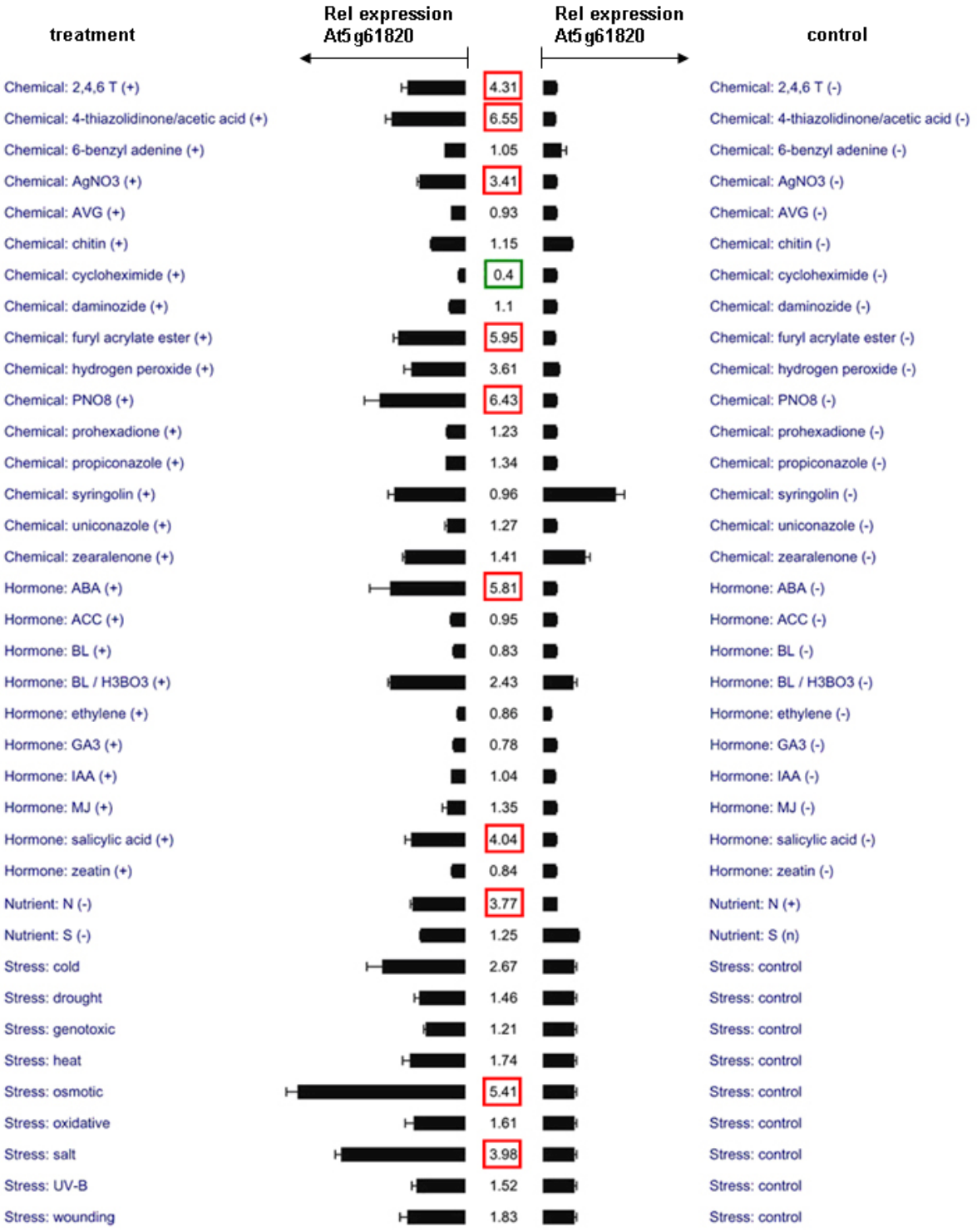

Figure 22. Analysis of *MtN19* expression in response to various stresses. Microarray-data were extracted from Genevestigator using the Response Viewer tool. Boxes in red indicate conditions under which *MtN19* is upregulated, green boxes represent conditions under which *MtN19* is downregulated.

Expression of *MtN19* is strongly induced by treatment with auxin inhibitors (2,4,6 T, thiazolidinone acetic acid, furyl acrylate ester) as well as by treatment with silver nitrate which is an inhibitor of ethylene and in addition causes oxidative stress (Navabpour et al.,

2003). Moreover, *MtN19* upregulation is observed in response to osmotic and oxidative stress as well as by ABA and salicylic acid treatments (Figure 22).
Since MtN19 interacts with CO we analyzed whether both genes are co-regulated in response to certain conditions or developmental stages (Figure 23).

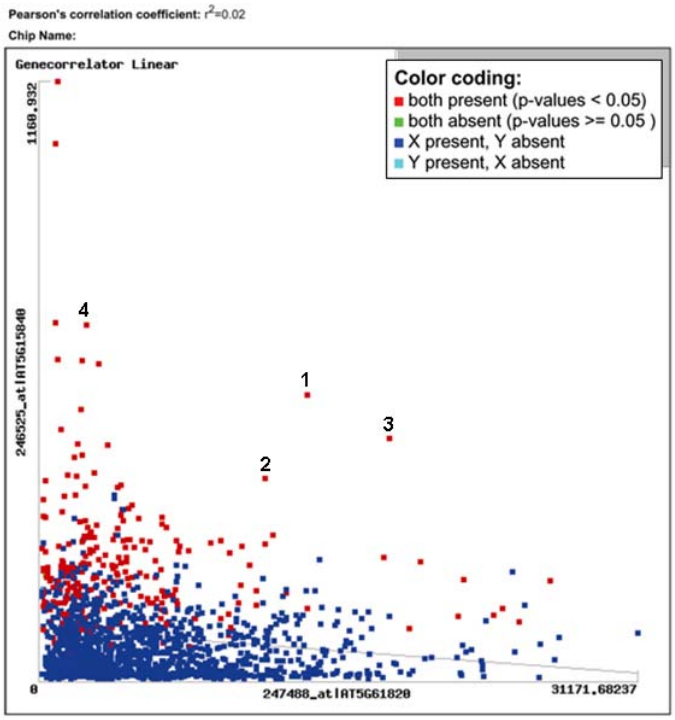

Figure 23. Analysis of co-regulation of *CO* and *MtN19*. Microarray-data were extracted from Genevestigator using the Gene Correlator tool. On the x-axis the expression of *MtN19* (*At5g61820*) is plotted, on the y-axis the expression of *CO* (*At5g15840*) is plotted. Blue dots represent conditions where *MtN19* is present but *CO* is absent. Red dots represent conditions where both genes are expressed. Dots labelled represent: 1: co-regulation in juvenile leaves in the *bountiful* mutant; 2: co-expression in response to salt stress; 3: co-expression in siliques; 4: co-regulation in response to *Phytophtora infestans* spores.

MtN19 and *CO* are co-expressed in a variety of conditions (Figure 23) allowing both proteins to function together. Both genes are expressed strongly in young leaves of the bountiful mutant, in response to salt stress and in siliques.

5.3. MtN19 interacts with the CCT-domain of CO in yeast

By yeast-two-hybrid screening MtN19 was isolated as an interactor of the CCT-domain of CO. Since MtN19 is a single copy gene in Arabidopsis and rice and the amino acid sequences

show a high degree of conservation it was tested whether the MtN19 proteins from both species are able to interact with CO in yeast. Therefore, both full length proteins were tested in the yeast-two-hybrid system.

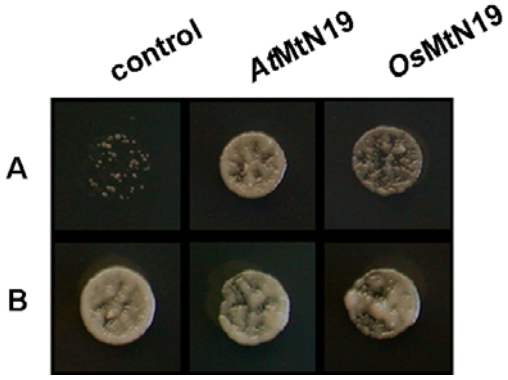

Figure 24. Analysis of interactions between AtMtN19 and OsMtN19 with the CCT-domain of CO. A) SD-medium -Leu,-Trp,-His plus 15mM 3-AT; B) SD-medium -Leu, -Trp. AtMtN19 and OsMtN19 interact with the CCT-domain of CO but CO does not interact with the empty prey plasmid (control).

AtMtN19 and OsMtN19 were both able to interact with the CCT-domain of CO (Figure24) indicating that the domain required for this interaction is conserved between both species.

5.4. MtN19 interacts with CO *in vitro*

In order to verify the interaction observed in yeast, *in vitro* co-immunoprecipitation experiments were carried out to confirm the interaction. Figure 25 shows that immunoprecipitation of GAD:CO allowed weak co-immunoprecipitation of MtN19.

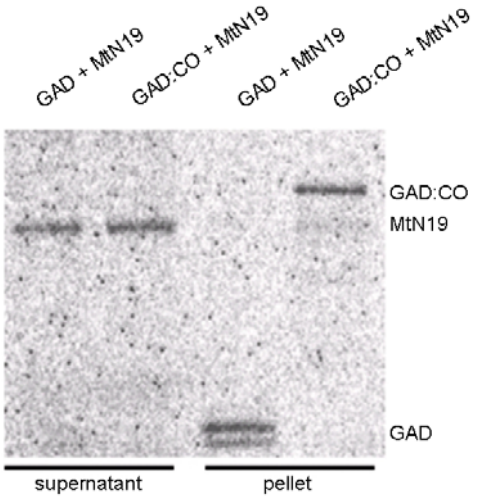

Figure 25. *In vitro* co-immunoprecipitation of MtN19 with GAD:CO. The supernatant fraction contains unbound prey proteins. After successful immunoprecipitation the pellet fractions contain both bait and prey proteins. The precipitated band of MtN19 is weak but was obtained repeatedly.

5.5. MtN19 and CO co-localize in the nucleus and interact *in vivo*

Protein analysis using web-based tools identifies a putative trans-membrane domain at the carboxy terminus. Since CO is a nuclear protein, it was tested whether both proteins are present in the nucleus. Co-localisation studies using fluorescence-labeled proteins showed that both MtN19 and CO are targeted to the nucleus (Figure 26).

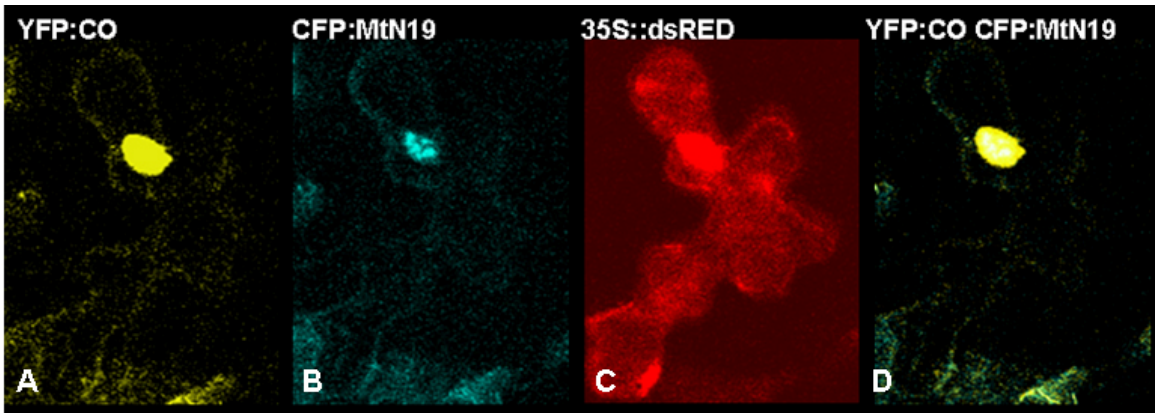

Figure 26. Co-localization studies in Arabidopsis epidermal cells bombarded with plasmids containing *35S::YFP:CO*, *35S::CFP:MtN19* and *35S::dsRED*. Both CO and MtN19 are present in the nucleus. A:YFP-channel, B: CFP-channel, C: dsRED-channel, D: YFP/CFP –channel overlay

YFP:CO and CFP:MtN19 both co-localize in the nucleus of Arabidopsis epidermal cells and MtN19 seems to localize to sub-nuclear aggregates, a feature often observed for CO. It was tested by *in vivo* FRET whether they also interact with each other in plant cells. FRET analysis showed this interaction and confirmed the results observed in yeast and *in vitro* (Figure 27).

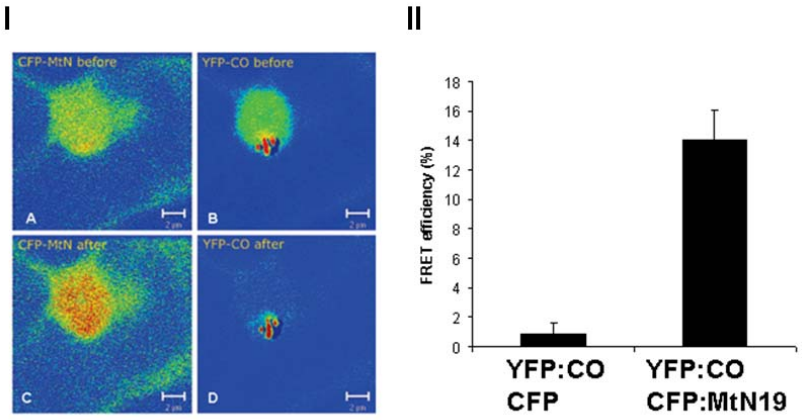

Figure 27. *In vivo* FRET analysis by acceptor photobleaching. A) After photobleaching an increase in CFP-expression can be observed indicating an interaction. B) FRET quantification comparing results obtained from measuring YFP:CO and CFP as FRET pairs with the positive pair YFP:CO and CFP:MtN19.

5.6. MtN19 interacts with the *FT*-promoter in yeast

In order to identify proteins able to interact with the promoter of *FT*, yeast-one-hybrid screening with a 300bp fragment of the *FT* promoter was carried out. MtN19 was isolated as an interactor of the promoter of *FT*. The interaction of MtN19 was reconfirmed by transforming the yeast strain Y187 containing the 300bp fragment of the *FT* promoter integrated into the genome upstream of the *HIS* gene. Since OsMtN19 was able interact with CO it was also tested whether it can bind to the *FT* promoter in yeast.

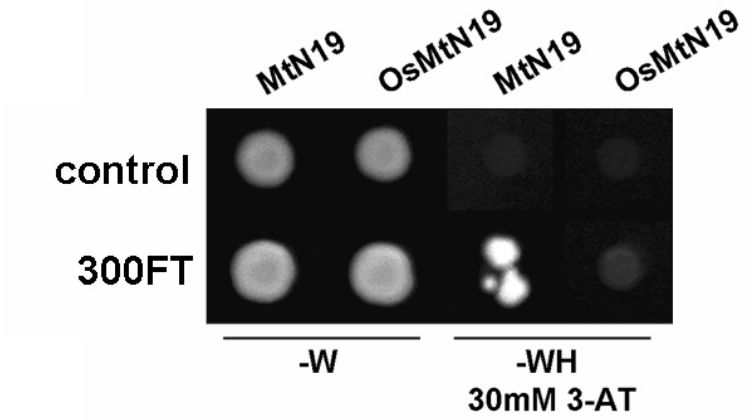

Figure 28. Yeast-one-hybrid analysis of MtN19 and OsMtN19 interacting with the *FT* promoter. The upper row shows the negative control (pHIS-1), the lower panel shows the interaction with *300FT*. – W: SD-medium lacking tryptophane; -WH 30mM 3-AT: SD-medium lacking tryptophane and histidine supplemented with 30mM 3-AT.

After transformation of the constructs, weak growth of yeast expressing MtN19 in the strain containing the *FT* promoter fusion was observed after an incubation of eight days at 30°C; no interaction was observed for OsMtN19 (Figure 28).

5.7. *MtN19* mRNA abundance at the end of the light period in long days

Since MtN19 was identified to interact with CONSTANS, a prerequisite for an interaction in planta is that both proteins are expressed at the same time and are localized to the same cells and compartments (e.g. nucleus). In order to examine the temporal expression pattern of *MtN19* mRNA, a time course experiment was done. Arabidopsis seedlings were grown in SD

(8 hours light, 16 hours dark) and LD (16 hours light, 8 hours dark) and samples of 12 day old seedlings were harvested every four hours over a 24 hour period. After RNA extraction and reverse transcription real-time quantitative PCR was performed to quantify *MtN19* mRNA (Figure 29).

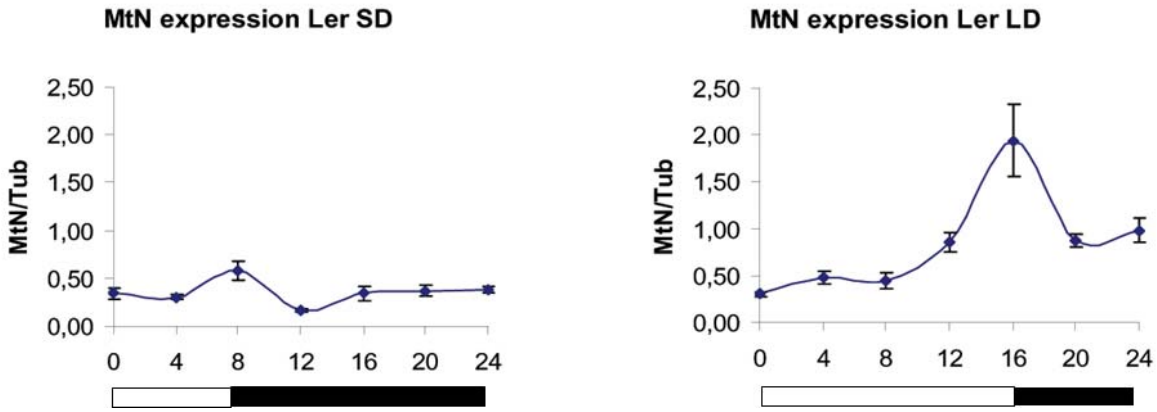

Figure 29. Temporal expression pattern of *MtN19* mRNA. Left picture shows MtN19 mRNA expression in SD, the right picture displays the expression in LD. Standard deviations calculated from four technical repeats of *MtN19/β-tubulin*. Bars represent day/night cycles.

Analyzing the temporal expression pattern of *MtN19* in short days revealed no obvious diurnal expression pattern. However, in LD the expression pattern of *MtN19* mRNA resembles that of *CO* mRNA with a peak at the end of the light phase, 16 hours after dawn.

5.8. *MtN19* mRNA can be induced by application of methyljasmonate

MtN19 expression is increased in different plant species in response to various stresses and chemicals indicating a possible role in stress signaling. Global expression analysis on microarrays also indicated that *MtN19* responds to a variety of conditions (Figure 22). Thus, the expression of *MtN19* mRNA in Arabidopsis was analyzed in response to various plant hormones and at different temperatures (Figure 30).

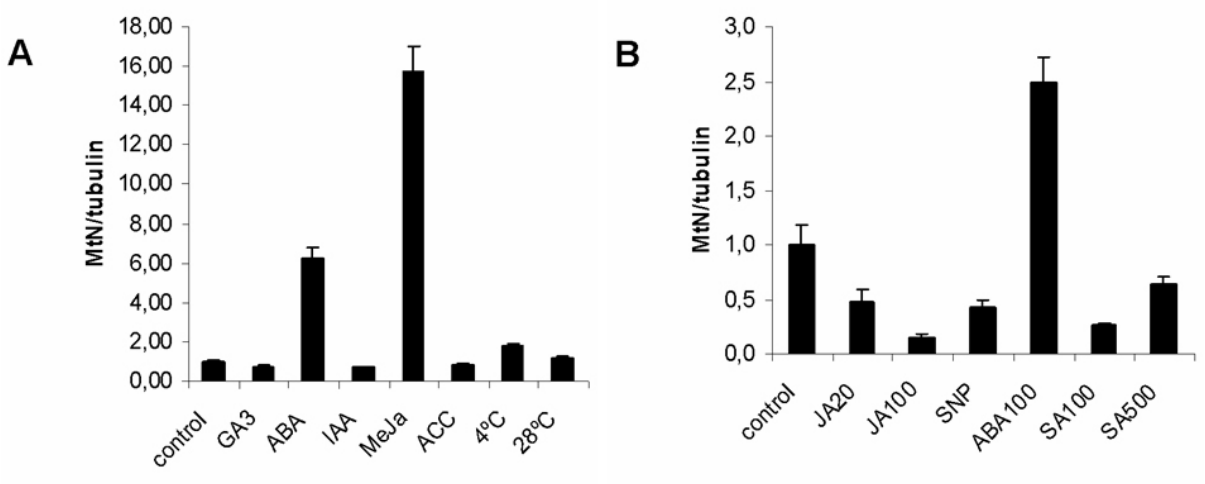

Figure 30. Hormone treatment experiments analyzing *MtN19* expression. A) Ten day old seedlings were treated on GM-plates with different hormone solutions containing each 50mM gibberellic acid (GA3), abscisic acid (ABA), auxin (IAA), methyljasmonate (MeJa) and the ethylene precursor 1-aminocyclopropan-1-carboxylic acid (ACC) for four hours. Additionally seedlings were incubated for four hours at 4°C and 28°C. Control plants were incubated with the same amount of water at room temperature. B) Hormone treatment experiment II. Ten day old Arabidopsis seedlings were incubated with 20mM and 100mM jasmonic acid (JA), 50mM sodium-nitroprusside (SNP), 100mM abscisic acid (ABA), 100mM and 500mM salicylic acid (SA) for 24 hours.

MtN19 is induced upon abscisic acid treatment which seems to correspond with its role in senescence in Alstroemeria (Breeze et al., 2004). Interestingly, the response is transient, since expression levels decrease from 6-fold after the four hour treatment (Figure 30a) to 2.5-fold after 24 hours (Figure 30b). Highest induction is elicited by treatment with methyljasmonate, where the *MtN19* transcript was induced 16-fold (Figure 30a). Treatment of Arabidopsis seedlings for 24 hours with 100mM jasmonic acid causes a 5-fold reduction in *MtN19*-expression (Figure 30b).

5.9. Analysis of transgenic plants in which *MtN19* expression is altered

To investigate the function of *MtN19* in Arabidopsis, transgenic plant lines were generated which overexpress the full length cDNA or which silence the gene using dsRNAi. Overexpressing *MtN19* with the *35S*-promoter resulted in plants showing WT phenotype. Analysis of these transgenic lines revealed that all the lines tested showed no increase in *MtN19* mRNA transcript (data not shown). Also expression of *MtN19* from the *SUC2*-promoter did not alter the WT phenotype (data not shown).

To identify plants with reduced *MtN19* expression, T-DNA insertion libraries were screened for insertions in *MtN19*. A phenotypic screen for *MtN19* knock-out lines from GABI-KAT and SALK did not result in plants showing alterations in flowering-time. An RNAi-approach was therefore used to knock-out *MtN19* in Arabidopsis. The *MtN19* cDNA was recombined into pJawohl8 vector. Since there is no other homologous gene in the Arabidopsis genome, the full length cDNA was used for this experiment. The first observation was that around 50% of T1 plants on BASTA selection trays showed an extreme early-flowering phenotype (Figure 31a).

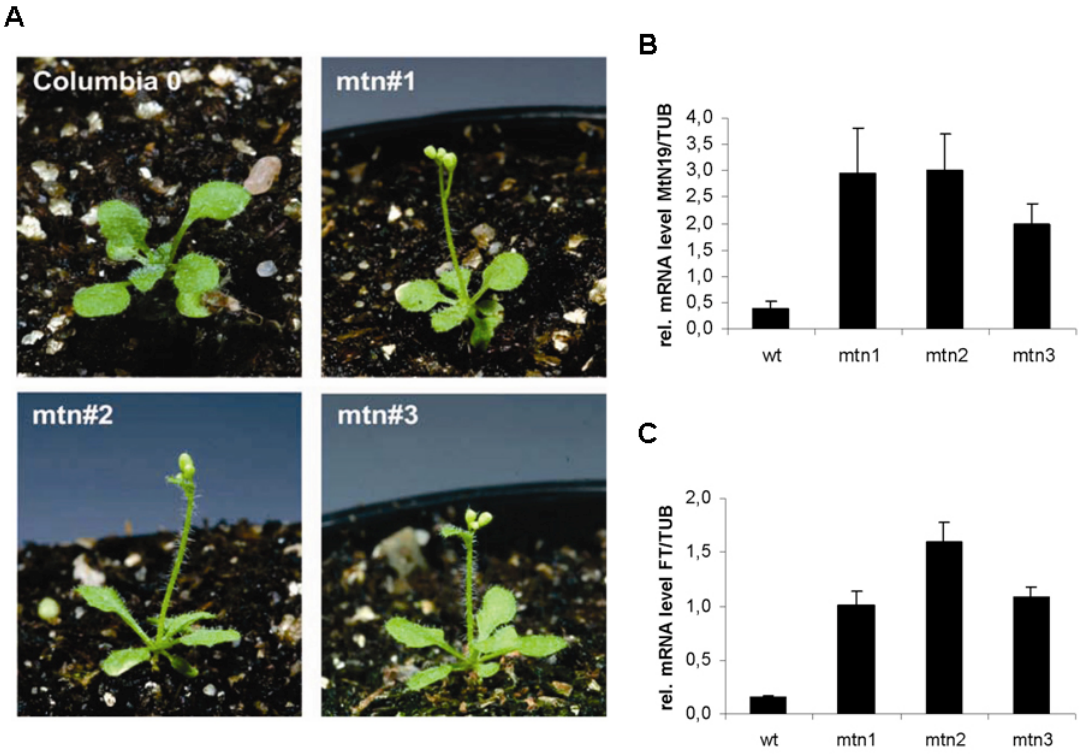

Figure 31. *MtN19 dsRNAi*-lines. A) Three independent T1-transformants in comparison to Columbia wild-type 14 days after germination in LD. B) *MtN19* expression analysis using a primer annealing in the 5' UTR to amplify specifically endogenous transcript and not the transgene. C) Expression analysis of *FT*.

The early-flowering lines isolated unexpectedly showed an increase in *MtN19* transcript (Figure 31b). Since the lines flowered extremely early, the expression level of *FT* was also analyzed, and this was increased as well. The other half of T1-transformants showed an extreme late-flowering phenotype. Several late and early lines were selected and analyzed for their segregation ratio of the transgene in the T2-generation. A 3:1 segregation indicated an insertion of a single transgene. After germination of T2-seeds on soil young seedlings were treated with BASTA to eliminate wild-type plants. Almost all lines showed a clear 3:1 segregation ratio, but interestingly several lines showed a conversion in their flowering-time

phenotype. In some early lines a few plants showed an extreme late-flowering phenotype, whereas in some late lines a few plants flowered extremely early. Early and late-flowering lines showing consistently late and early-flowering were selected for identification of homozygous lines. To investigate the function of MtN19, a late line (*MtL*) and an early line (*MtE*) were crossed with 35S::CO and a CO knock-out (*co-sail*). Figure 32 shows the phenotypes observed compared to *35S::CO* and *co-sail* and the F1 of a cross between *MtL* and *35S::CO*. Here, only *MtL 35S::CO* is shown, since both transgenes produce dominant phenotypes.

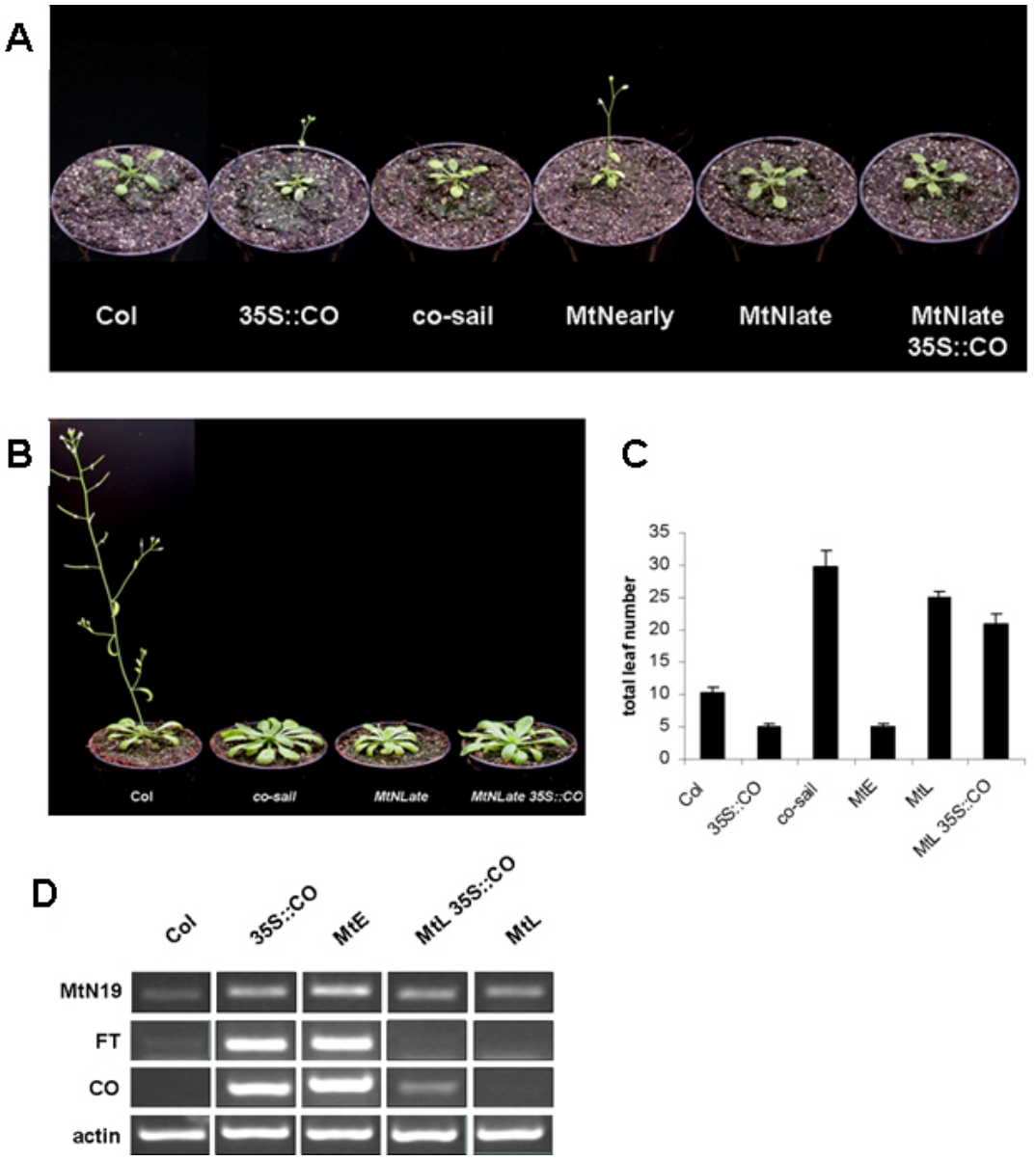

Figure 32. Analysis of *MtN* transgenic plants. A) Comparison of Columbia, *35S::CO*, *MtE*, *MtL 35S::CO* and *MtL* at 15 days after germination. B) Columbia, *co-sail*, *MtL* and *MtL 35S::CO* at 35 days after germination. C) Expression analysis of *MtN19*, *FT*, *CO* and *actin* by RT-PCR (28 cycles), 15 days after germination from leaf material.

Analysis of transgenic plants demonstrated that *MtE* and *35S::CO* both flower very early under long day conditions in comparison to *MtL* and *co-sail* which are late-flowering (Figure 32). An F1-cross between *MtL* and *35S::CO* revealed that *CO* is not able to complement the late-flowering phenotype of *MtL* (Figure 32). Gene expression analysis by RT-PCR demonstrated that both *35S::CO* and *MtE* express *MtN19*, *CO* and *FT* at high levels whereas the expression of *FT* and *CO* is under the level of detection in wild-type and *MtL* (Figure 32d). *MtN19* expression is weak in wild-type, intermediate in *MtL* and high in *MtE*. The *MtL35S::CO* double mutant expresses medium levels of *CO* but the level of *FT* is under the level of detection (Figure 32d). It can not be excluded that the reduced *CO* mRNA levels in *35S::CO MtL* lines could also be due to silencing of the 35S promoter in these plants.
A recent result obtained by overexpression of *MtN19* with a FLAG-epitope using the *pJAN* vector harboring a double *35S*-promoter resulted in early-flowering transgenic lines (Figure 33).

Figure 33. Analysis of *35S::FLAG:MtN19* transgenic plants. T1-line after BASTA selection, other transgenic plants showed similar phenotypes. The arrow indicates the floral bud.

5.10. Discussion

MtN19 interacts with CONSTANS and the *FT* promoter in yeast

MtN19 was identified as an interactor with the CCT-domain of CONSTANS by yeast-two-hybrid screening and as a protein that is able to bind to the *FT* promoter in a yeast-one hybrid assay. Both screens were done independently and would be consistent with a role for MtN19 in the CO-mediated activation of *FT* expression. After isolation of the full-length cDNA the interactions were confirmed in yeast. MtN19 interacts with the CCT-domain of CO (Figure 24). Analysis of the homologous protein from rice shows that this protein also can interact with CO (Figure 24) indicating that these proteins have retained functional similarities during evolution (Figure 18). It could also be that both proteins share a structural similarity in a non-

conserved region. Analysis of the ability to interact with the *FT* promoter shows that MtN19 can bind to the 300bp fragment although the observed interaction appears to be rather weak (Figure 28). However, *Os*MtN19 does not bind to the *FT* promoter (Figure 28) suggesting that the DNA-binding domain recognized by of *Os*MtN19 might have changed during evolution. This might be related to the observation that CO activates *FT* expression in Arabidopsis, but the rice orthologue of CO (Hd1) represses expression of the rice *FT* orthologue (*Hd3*).

The interaction between MtN19 and CONSTANS was confirmed *in vitro* and in *vivo*

To confirm that the interaction of MtN19 with CO observed in yeast is valid, different independent assays were performed. *In vitro* co-immunoprecipitation using GAD-labeled CO and a GAD-specific antibody showed a weak precipitated band corresponding to the size of MtN19 (Figure 25). In the negative control lane testing the interaction between GAD and MtN19, the band was absent. Although the band appears to be very weak suggesting a weak interaction between the proteins, the result was obtained repeatedly, confirming that the interaction does occur under these conditions.

Co-transformation of fluorescent protein fusions showed that both MtN19 and CO co-localize in the nucleus of Arabidopsis epidermal cells (Figure 26). Additionally, we observed that MtN19 is not evenly distributed within the nucleus; it shows a localisation in sub-nuclear speckles, one of the features of CO. Testing the ability to interact *in vivo*, the acceptor photobleaching approach was applied and FRET was measured between YFP::CO and CFP::MtN19 (Figure 27). Quantification of the FRET efficiencies between YFP::CO and CFP::MtN19 compared to YFP and CFP::MtN19 showed a significant difference (Figure 27b).

***MtN19* shows a diurnal expression pattern**

In addition to localisation to the same compartment in the cell, another important prerequisite for a functional interaction *in planta* is expression at the same time. Because CO shows a diurnal rhythm, the temporal *MtN19* expression pattern was analyzed in 24-hour time courses both in SD and LD. Analysis of the temporal expression pattern revealed that in LD *CO* and *MtN19* are both expressed at the end of the light phase (Figure 29). In SD a slight peak of MtN19 expression appear also at the end of the light phase at eight hours after dawn (Figure 29). Since CO acts in LD conditions, both *CO* and *MtN19* are expressed at the same time of the day and could act together to control *FT* expression. The activation of FT by CO occurs in the phloem (Takada and Goto, 2003; An et al., 2004; Ayre and Turgeon, 2004), but so far the

spatial expression pattern of MtN19 is unknown although it seems to be expressed in leaves (Figure 20 and 21).

Induction of *MtN19* expression by application of plant hormones

MtN19 expression responded to the application of ABA with a 5-fold increase in mRNA levels, and to methyljasmonate with a 16-fold increase (Figure 30). It was shown, that methyljasmonate/jasmonate and abscisic acid signaling converge to activate the expression of several wound- and stress-inducible genes (Hildmann et al., 1992). Also the process of stomatal closure is regulated both by ABA and methyljasmonate indicating the convergence of two hormonal pathways on one physiological process. Plants carrying a recessive mutation in the *OST1* gene are impaired in stomatal closure in response to ABA (Mustilli et al., 2002). The methyljasmonate-insensitive mutant *jar1-1* is impaired in stomatal closure in response to methyljasmonate (MeJa) (Staswick et al., 2002; Suhita et al., 2004). Application of MeJa causes stomatal closure in *ost1-2* mutants and application of ABA causes stomatal closure in *jar1-1* indicating interplay between both signaling cascades (Suhita et al., 2004). Finally, it was shown that the interplay is at the level of reactive oxygen species (ROS) production and both *JAR1* and *OST1* act upstream of ROS production (Suhita et al., 2004). Production of ROS is a common feature of plant responses to biotic and abiotic stresses. ROS production triggers signaling cascades leading to various outputs such as stress tolerance, acclimation and cell death (Dat et al., 2000; Mittler, 2002; Vranova et al., 2002). In these various processes, ROS production is coupled to the action of several hormonal pathways, mainly ABA and methyljasmonate signaling. It is possible that also jasmonic acid induces *MtN19* after a short incubation and long time incubation results in a shut off of this signaling event. This would explain why *MtN19* is 5-fold reduced in response to jasmonic acid after 24 hour incubation (Figure 30b). However, several microarray studies revealed that the application of compounds eliciting ROS production cause upregulation of *MtN19* (Kimura et al., 2003; Navabpour et al., 2003; Breeze et al., 2004; Doss, 2005). We present evidence that application of MeJa and ABA, two plant hormones involved in ROS production also cause upregulation of *MtN19*. Since *MtN19* shows a diurnal expression pattern peaking at 16 hours after dawn in LD induces *FT* expression with CO and under stress conditions *MtN19* may be expressed to perform other functions. Nonetheless, the analysis of ROS in the process of flowering-time control might reveal a function since its production is controlled by various hormones and signalling cascades.

Transgenic plants overexpressing MtN19 flower early and silenced lines show early and late-flowering phenotypes

Plants expressing a double-stranded hairpin against the full length cDNA of *MtN19* show either extreme late or early-flowering. The distribution of late and early-flowering lines observed in the T1-generation was approximately 1:1. Analysis of the early-flowering lines showed that in these lines the *MtN19* transcript is overexpressed and not silenced (Figure 31a and b). In these lines, *FT* is highly induced likely explaining the early-flowering phenotype (Figure 31c). Analysis of both early (*MtE*) and late (*MtL*) flowering lines revealed that *MtE* has high levels of *MtN19*, *CO* and *FT* whereas in wild-type and *MtL* the expression levels of *FT* and *CO* were under the level of detection (Figure 32). The *MtN19* expression level is slightly upregulated in *MtL* and highly induced in *35S::CO* lines. *MtE* has high levels of *MtN19* compared to *MtL* where the transcript is not absent which would explain the late-flowering phenotype. In *MtL 35S::CO* plants the *CO* transcript is detected but not *FT*. MtN19 interacts with CO and overexpression of *MtN19* causes early-flowering by up-regulation of *FT*. The observation that *MtE* lines have high levels of *CO* suggests that MtN19 can also induced the expression of *CO* or the effect on *CO* is due to the existence of a positive feedback loop from *FT*. Since CO and MtN19 seem to interact to regulate *FT* expression they might also mutually induce their own transcription. The result that CO is unable to activate *FT* in *MtL 35S::CO* plants suggests that something CO requires is missing in these transgenic plants, which is most likely functional MtN19. However, since CO mRNA levels were reduced in these *35S::CO MtL* plants the late-flowering may also be partly due to a silencing effect between the two *35S* promoters.

In *35S::MtN19* lines, no elevated transcript levels were detected and the plants did not show early or late-flowering phenotypes (data not shown) suggesting a post-transcriptional mechanism controlling *MtN19* mRNA abundance. Overexpression of *35S::FLAG:MtN19* causes only early-flowering (Figure 33). This result suggests that either this vector produces higher levels of transcript that overcomes post-transcriptional regulation or the nucleotides encoding the FLAG epitope stabilize the mRNA of the transgene.

Is MtN19 post-transcriptionally regulated by naturally occurring antisense expression?

The variability observed in *MtN19-dsRNAi* lines and the failure to overexpress the *MtN19* mRNA could be explained by the existence of naturally occurring antisense transcription at the *MtN19* locus. Natural antisense transcripts were first described in prokaryotes where they regulate a variety of processes such as plasmid replication, conjugation and transposition (for

review see Wagner and Simons, 1994). First observations of the occurrence of natural antisense transcripts in eukaryotes were made in yeast and humans by analyzing the expression of mitochondrial DNA (Anderson et al., 1981; Bibb et al., 1981). After discovering overlapping sense / antisense transcription units of the Drosophila dopa decarboxylase gene and the role of the antisense transcript regulating the abundance of the sense transcript the function of antisense transcription became clear (Spencer et al., 1986). An example of transcriptional regulation is the human translation initiation factor eIF-2α whose abundance is regulated by the cyclic transcription of antisense RNA controlling the cell cycle in T lymphocytes (Noguchi et al., 1994). In the case of the human c-erbAα, belonging to the steroid/thyroid hormone receptor family, antisense regulation occurs at the post-transcriptional level by inhibition of transcript splicing (Miyajima et al., 1989; Munroe and Lazar, 1991). In *C. elegans* the heterochronic *lin-14* gene controls early developmental stages of the nematode. During development, the mRNA levels of *lin-14* remain constant but the protein decreases after the L2 stage. The decrease of the Lin-14 protein level is due to expression of *lin-4* yielding two small transcripts of 22 and 61bp that do not encode a functional ORF. *Lin-14* mRNA contains four *lin-4* complementary elements in its 3' UTR and sense/antisense basepairing results in translational inhibition (Lee et al., 1993). These results show that antisense RNA can interfere with gene expression at various levels of transcription and translation. Naturally occurring antisense transcripts were also identified in plants. In *Brassica oleracea* the *S LOCUS RECEPTOR KINASE* (*SRK*) gene which encodes for a protein involved in the regulation of self-incompatibility, is transcribed in both directions. Sense transcript accumulation is tissue-specific and depends on the absence of antisense RNA (Cock et al., 1997). Other members of this gene family are also controlled by antisense transcription as shown in maize (Ansaldi et al., 2000). A computational analysis aligning 32,127 rice cDNA sequences to rice genome sequences revealed the existence of 687 bidirectional transcript pairs (Osato et al., 2004). Global transcriptome analysis from mice provides evidence for the existence of 4,520 transcription units forming sense-antisense pairs, representing around 10% of all transcription units of the genome (Katayama et al., 2005). Analysis of the Arabidopsis transcriptome using whole-genome arrays revealed that out of around 26,000 genes tested, 3,000 show expression of sense-antisense pairs and 7,600 show tissue-specific sense-antisense expression (Prof. Dr. Joseph Ecker, personal communication). Moreover, antisense expression seems to show high dynamics in terms of spatial and temporal regulation implying that these numbers may only represent a small proportion of the antisense RNAs encoded in the genome.

The fact that transgenic plants overexpressing *MtN19* by the *35S*-promoter do not have elevated transcript levels nor flower early suggest an active mechanism downregulating *MtN19* mRNA levels. Furthermore, the observation that transgenic plants expressing a dsRNAi construct against *MtN19* show two extreme flowering phenotypes underline the hypothesis of natural antisense RNA. In *MtE*-lines the endogenous transcript is up-regulated probably due to silencing of the antisense-transcript. In *MtL*-lines the endogenous transcript is not downregulated but plants are late-flowering. Analysis of the *MtN19* locus on the Arabidopsis whole genome chip (signal.salk.edu) identifies antisense-sequences corresponding to the *MtN19* gene (Figure 34).

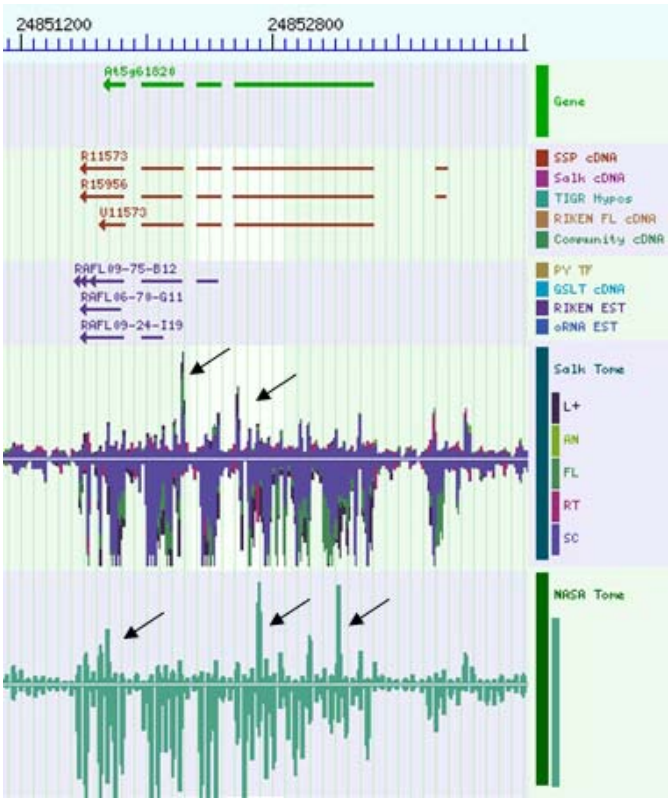

Figure 34. Detected antisense transcripts at the *MtN19* locus (At5g61820) using Arabidopsis whole genome arrays.

Legend:
L+ Light grown seedlings
AN Anthers
FL flowers
RT roots
SC suspension culture
Arrows indicate the detected antisense transcripts.
Data extracted from http://signal.salk.edu

Antisense transcripts are only detected for very specific regions and do not cover the whole locus. Due to the low spatial and temporal resolution of these few experiments it is likely that many more antisense transcripts remained undetected.

If the antisense transcript is polyadenylated, it is impossible to distinguish by RT-PCR between sense and antisense which might explain why the reduction of the sense transcript was not detected in *MtL* plants. However, the observation that some transgenic *MtN19*-

dsRNAi plants showing a flowering phenotype as T1-plants, showed the opposite flowering phenotype in the next generation suggests that during development a decision is made which transcript is silenced by the dsRNAi transgene. Wild-type and transgenic dsRNAi-lines should now be analyzed using northern blots with strand-specific probes to distinguish between sense and antisense transcripts. If in the late-flowering *MtL* plants the sense transcript is silenced and the antisense transcript stabilized this would support the hypothesis that antisense transcription is important in the regulation of *MtN19*.

5.11. Conclusion

We present evidence that CO interacts with the Arabidopsis MtN19 protein in yeast, *in vitro* and *in vivo*. Both proteins co-localize in the nucleus and we demonstrated that MtN19 is able to interact with the promoter of *FT* in yeast. Furthermore, the analysis of the temporal expression pattern of *MtN19* revealed that both *CO* and *MtN19* are expressed at the same time during the day in LD conditions. This is the timepoint where the CO protein is functional (Valverde et al., 2004). *MtN19* mRNA expression can be induced by the application of ABA and MeJa whereas application of jasmonic acid seems to reduce *MtN19* expression. Figure 31 summarizes our current knowledge on *MtN19* and places it in a model that describes how it could regulate *FT* together with CO.

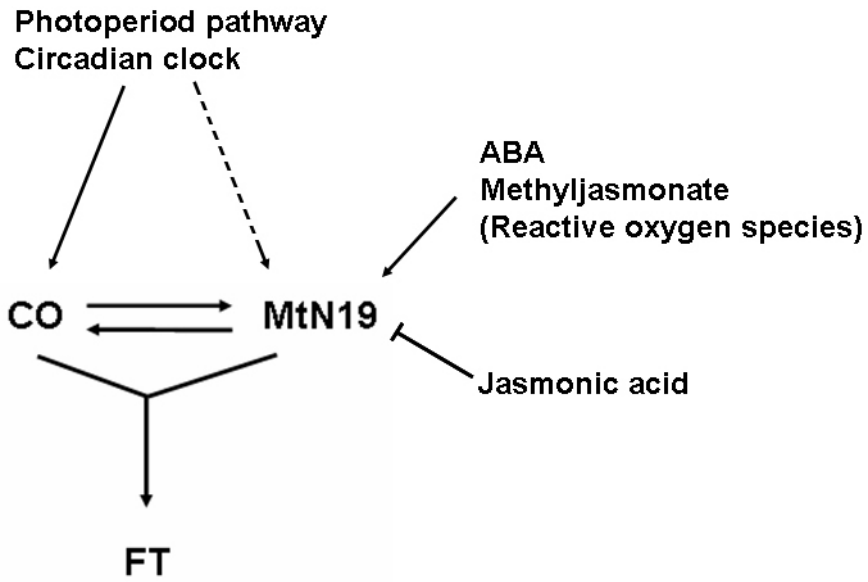

Figure 35. Putative model of the regulation of *MtN19* and its role in controlling *FT* expression together with CO.

Transgenic plants expressing a dsRNAi construct against *MtN19* exhibit two strong phenotypes, early and late-flowering. We have shown that the early-flowering dsRNAi-lines have high levels of *MtN19*, *FT* and *CO* explaining the early-flowering phenotype. All this information indicates that MtN19 is involved in controlling flowering-time together with CO. Further research on the biochemical function and post-transcriptional regulation of *MtN19* is needed to understand its precise role in the regulation of flowering in Arabidopsis.

6. FIDGET, an APETALA2-like protein involved in UV-light induced induction of *FLOWERING LOCUS T*

6.1 Introduction

Light is a key factor for plant development. In nature, sunlight provides positional information, regulates plant development and serves as the major energy source for production of sugar during photosynthesis. Sunlight consists of different wave lengths of light and depending on the location at which a plant grows, the spectrum of wavelengths it is exposed to differ. For example, under a canopy of other plants, light is enriched with far red light compared to the open field (Sessa et al., 2005). Plants have different photoreceptors that sense light inputs and convert these into physiological responses. One of the major physiological pathways that is regulated by light mediates the photoperiodic control of flowering-time. CONSTANS (CO) a key protein regulating the floral transition in response to photoperiod, activates the expression of *FLOWERING LOCUS T* (*FT*) within this pathway (Putterill et al., 1995; Samach et al., 2000; Suarez-Lopez et al., 2001). FT is a floral integrator on which several floral promoting pathways converge (Kardailsky et al., 1999; Kobayashi et al., 1999). FT mediates the floral transition by interacting with FD, a bZIP transcription factor and the FT/FD complex induces the transcription of the floral meristem identity gene *APETALA1* (Abe et al., 2005; Wigge et al., 2005).

In Arabidopsis, several photoreceptors are known to sense different light qualities and wavelengths. Phytochromes are involved in sensing the intensity and ratio of red/far-red light. The *Arabidopsis thaliana* genome encodes five phytochromes (PHYA-E) that mediate different developmental responses (Sharrock and Quail, 1989). Blue light is sensed by phototropins, protein kinases harboring two LOV domains that can perceive light and in response o these signals control phototropic responses (Christie et al., 1998; Briggs and Christie, 2002). Cryptochromes also perceive blue light and additionally respond to UV-A (320-400nm) light. Three cryptochromes are encoded in the Arabidopsis genome, named CRY1, 2 and 3. CRY1 is involved in mediating high fluence responses whereas CRY2 mediates low fluence responses (Ahmad and Cashmore, 1993; Guo et al., 1998; Lin and Shalitin, 2003). CRY3 is a more distantly related protein for which no function is known so far. Cryptochromes play an important role in the de-etiolation process, inhibiting hypocotyl

growth and promoting cotyledon expansion (Lin, 2002; Liscum et al., 2003). CRY2 also regulates photoperiod-dependent flowering, perhaps by interacting physically with PHYB in the nucleus (Cashmore et al., 1999; Mockler et al., 1999; Mas et al., 2000; Yanovsky and Kay, 2003). CRY1 and CRY2 both physically interact with the E3-ubiquitin ligase COP1, independent of the presence or absence of light (Wang et al., 2001; Yang et al., 2001). COP1 ubiquitinates the bZIP transcription factor HY5 in the dark but not in the light, and therefore HY5 promotes photomorphogenesis specifically in the light. In light, it is hypothesized, that CRY1 inhibits COP1 activity leading to the release of HY5 (Cashmore, 2003; Lin and Shalitin, 2003; Liscum et al., 2003). Beside its involvement in early light signaling (Osterlund et al., 2000), HY5 is required to regulate a subset of UV-B (280-320nm) responsive genes (Osterlund et al., 2000; Ulm et al., 2004).

Due to the anthropogenic destruction of the ozone layer, organisms living on the earth's surface must cope with increasing levels of UV-B light (Kerr and McElroy, 1993, 1994; Madronich et al., 1998; McKenzie et al., 1999). Plants growing in open field conditions are exposed to high levels of UV-light on sunny days and these cause damage and trigger stress responses such as the production of plant stress hormones and reactive oxygen species (Brosche and Strid, 2003; Ulm et al., 2004). Low fluence rates of UV-B initiate a photomorphogenic response, but the receptor that perceives UV-B has not been identified (Brosche and Strid, 2003). Continuous irradiation of Arabidopsis seedlings with low fluence UV-B light causes the inhibition of hypocotyl elongation and promotes cotyledon expansion (Kim et al., 1998). This photomorphogenic response requires the activities of both PHYA and PHYB (Kim et al., 1998). UV-B treatment followed by red light pulses was shown to enhance PHYB-mediated morphogenic responses (Boccalandro et al., 2001). However, phytochromes do not act as primary UV-light photoreceptors although they modulate UV-B responses (Wade et al., 2001). Apart from photomorphogenic responses, UV-B leads to the biosynthesis of UV-absorptive secondary metabolites such as flavonoids (Chappell and Hahlbrock, 1984; Landry et al., 1995; Kim et al., 1998; Boccalandro et al., 2001) and to gene expression (Christie and Jenkins, 1996; Lo et al., 2004). Elevated UV-B levels also increase the frequency of homologous DNA-rearrangements, a repair mechanism for UV-induced DNA-lesions such as cyclobutane pyrimidine dimers (Ries et al., 2000).
Although a UV-B receptor has not yet been identified, isolation of UV-B light insensitive mutants led to the identification of *UV-B LIGHT INSENSITIVE3* (*ULI3*). *ULI3* encodes an 80kD protein of unknown function harboring a predicted heme-diacylglycerol binding

domain. *ULI3* mRNA is highly induced upon UV-B treatment and ULI3:GFP localizes to the plasma membrane (Suesslin and Frohnmeyer, 2003). Molecular events following UV-B perception involve secondary messengers such as calcium, kinases and reactive oxygen species (Brosche and Strid, 2003). It is still unclear, whether these reactive oxygen compounds are side-products of a general stress response or whether they act as UV-B response specific signaling molecules. UV-C (wavelengths less than 280nm) is extremely harmful to plants and animals causing DNA-damage (Klaude et al., 1995). It was also shown, that UV-C treatment of Arabidopsis seedlings can accelerate the floral transition and that this stress signal is dependent on salicylic acid (Martinez et al., 2004).

Several classes of Arabidopsis transcription factors are implicated in converting biotic and abiotic stress responses into a transcriptional output. One class contains members of the APETALA2-like (AP2-like) family of proteins, which are transcription factors involved in various stress responses (Chen et al., 2002). Furthermore, it has been shown that AP2-like proteins act as integrators of hormonal signals such as those mediated by jasmonic acid and ethylene during pathogen threat (Fujimoto et al., 2000; Brown et al., 2003; Lorenzo et al., 2003). These proteins are named after the *apetala2* homeotic mutant, which shows defects in flower development (Jofuku et al., 1994). Important processes are controlled by the APETALA2 (AP2) protein including the establishment of flower meristem identity (Irish and Sussex, 1990; Bowman et al., 1993), floral specification and organogenesis (Komaki et al., 1988; Bowman et al., 1989, 1991; Jofuku et al., 1994) and regulation of ovule and seed development (Leonkloosterziel et al., 1994); (Modrusan et al., 1994). Recently, APETALA2 was also shown to control seed mass and yield in Arabidopsis (Jofuku et al., 2005; Ohto et al., 2005). The Arabidopsis genome comprises 145 AP2-like proteins that can be subdivided into four major classes; one with two AP2-domains such as APETALA2, two with one AP2-domain that comprise the drought response element binding proteins (DREB) and ethylene response factors (ERF), and finally RAV1-like proteins, which have an AP2-domain and a B3-domain (Riechmann and Meyerowitz, 1998; Kagaya et al., 1999; Sakuma et al., 2002; Gutterson and Reuber, 2004). DREB-proteins bind to dehydration responsive elements (DRE), an element found in a variety of low-temperature stress and dehydration inducible genes (Sakuma et al., 2002). ERF-proteins bind to GCC-boxes that are often found in ethylene inducible pathogenesis-related genes (Sakuma et al., 2002).

The AP2-domain itself can be subdivided into two functional domains, the YRG-element consisting of a 20-amino acid stretch containing basic and hydrophilic residues and the RYAD-element, forming a carboxy terminal amphipathic alpha-helix (Riechmann and

Meyerowitz, 1998; Kizis et al., 2001). AP2-domain proteins were also identified in cyanobacteria, ciliates and viruses, suggesting that plants may have acquired these genes by horizontal gene-transfer via a transposition and homing process (Magnani et al., 2004; Wessler, 2005). Furthermore it was reported, that the ciliate AP2-protein can bind to poly(dG)/poly(dC) stretches indicating a functional conservation with the plant orthologues (Magnani et al., 2004).

Several AP2-like proteins are involved in regulating the transition to flowering in Arabidopsis. Among those, two *AP2*-like genes, named *SCHLAFMUETZE* (*SMZ*) and *SCHNARCHZAPFEN* (*SNZ*) were isolated by activation-tagging. These two genes exhibit a late-flowering phenotype when overexpressed (Schmid et al., 2003). Another activation tagging approach led to the identification of microRNA172 (miR172), which causes early-flowering upon overexpression (Aukerman and Sakai, 2003). A set of AP2-domain proteins named TOE1, TOE2, TOE3 as well as SMZ and SNZ were identified as floral repressors downregulated by miR172 (Aukerman and Sakai, 2003; Schmid et al., 2003). AP2 is also repressed by miR172 and this seems to be due to translational rather than transcriptional inhibition (Aukerman and Sakai, 2003; Chen, 2004b).

In this chapter, an AP2-like protein that regulates *FT* is described. Using a 300-bp fragment of the *FT* promoter for yeast-one hybrid analysis FIDGET (FIT) an APETALA2-like protein that belongs to the subfamily of ethylene response element binding proteins was identified. FIT shows highest similarities to Pti6, an AP2-like transcription factor isolated from tomato (Gu et al., 2002). Evidence is presented that FIT interacts with the *FT* promoter in yeast, *in vitro* and *in vivo*. Overexpression of *FIT* by the *SUC2* promoter specifically in the phloem, the tissue in which *FT* is expressed, causes early-flowering due to upregulation of *FT*. *FIT* mRNA is strongly induced by UV-B light and analysis of the expression pattern by promoter-GUS fusions reveals that expression is detected in the vasculature upon UV-B treatment. Furthermore, we demonstrate that UV-B accelerates the floral transition and that this transition relies on the activity of *FT*.

6.2. FIT interacts with the promoter of *FT* in yeast

In order to isolate novel DNA-binding proteins interacting with the *FT* promoter we conducted yeast-one-hybrid screening. A 300-basepair fragment (*300FT*), which was identified in a systematic *FT* promoter analysis to be most responsive for induction by CO, was used for this screen. The *300FT* fragment extends from -539bp upstream of FT ATG to -207bp upstream. *300FT* was cloned upstream of the yeast *HIS*-gene and recombined into the genome of the Y187 yeast strain.

An APETALA2-like protein was the only DNA-binding protein that was found to interact with the *FT* promoter fragment. This protein was named FIDGET (FIT) because in further studies it behaved in an opposite way to two floral repressors named SCHLAFMUETZE (night-cap, sleepyhead) and SCHNARCHZAPFEN (snorer) (Schmid et al., 2003). After isolating the full-length cDNA of *FIT*, the ability of FIT to bind to *300FT* was retested and the interaction observed in the primary screen was confirmed. FIT can bind to the *FT* promoter fragment and induce the transcription of the *HIS* gene in yeast, which allows in growth on selective medium (Figure 36).

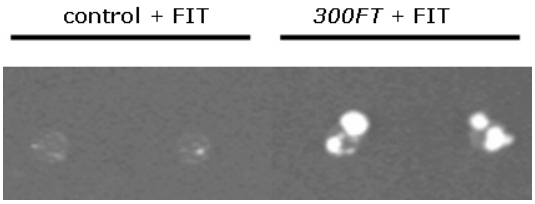

Figure 36. FIT binds to a 300bp fragment of the *FT* promoter in yeast. The control shows Y187 yeast expressing FIT which can not grow (left) compared to the yeast expressing *FIT* and containing 300bp of the *FT* promoter upstream of the *HIS* gene (right). Cells were grown for seven days on SD-medium lacking histidine and leucine supplemented with 15mM 3-AT.

6.3. Purification of recombinant HIS-tagged FIT protein

To further characterize the binding of FIT to the *FT* promoter we purified recombinant HIS-tagged FIT protein from *E. coli*. Unfortunately HIS:FIT was insoluble and targeted to inclusion bodies in *E. coli*. Therefore denaturing conditions for protein purification were applied.

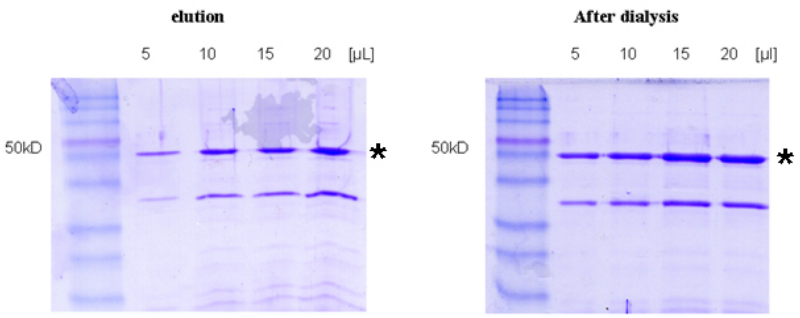

Figure 37. Purification of HIS:FIT using affinity chromatography with Ni-NTA resin. The left picture shows HIS:FIT protein after elution on a polyacrylamide gel, asterisk indicates the FIT-band, the lower band on the gels seems to be a degradation product. The size of HIS:FIT is around 40kD but it seems to migrate at around 50kD. The right picture shows HIS:FIT protein after dialysis.

After eluting the protein from the nickel column a four step dialysis was carried out (1h against 6M ura pH 5, 1hour 4M urea pH 5.5, 1h 2M urea pH 6.5, overnight against 100 mM NaP, 10% glycerol pH 7.0) to allow the protein to refold. The protein was subsequently concentrated on an Amicon centrifugation column. The isolated protein contents of both the elution and after dialysis followed by concentration were analyzed by SDS-PAGE (Figure 33). The isolated protein migrates at around 50kD which is in the range of the actual size of HIS:FIT (39kD).

6.4. *in vitro* DNA-binding assays

The interaction of FIT with *300FT* observed in yeast was confirmed by *in vitro* DNA-binding assays. In a first approach the 300 base pair fragment labeled with ^{32}P and used for *in vitro* binding. FIT was found to bind to the *300FT* fragment but not to the *nos* terminator which was used as a negative control (Figure 38a).

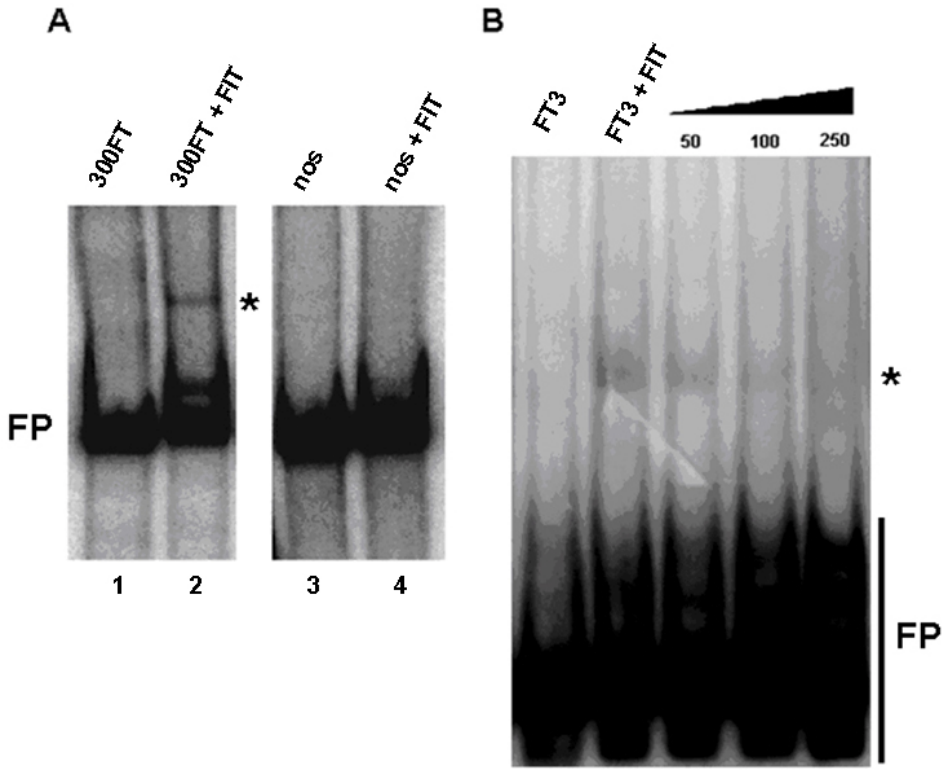

Figure 38. *In vitro* DNA binding assays using HIS:FIT protein. A) Gelshifts using the 300bp fragment from the *FT* promoter (left) in comparison to the nos terminator (right) to exclude unspecific DNA binding. Lane 1: *300FT* free probe Lane 2: 300FT plus HIS:FIT, lane 3: nos free probe, lane 4: nos plus HIS:FIT. Asterisk shows the shifted band indicating an interaction only between FIT and *300FT*. B) Smaller fragments of *300FT* were tested for FIT-binding. *FT3* is a 50bp fragment located within 300FT and extending from -267bp to -191bp upstream of *FT* ATG. A competition experiment was performed with unlabeled fragments (50, 100 and 250 times more unlabeled *FT3*-DNA) which caused a gradual decrease in the DNA-binding of FIT to labeled FT3. Asterisk indicates the shifted band; FP: free probe.

The putative FIT-binding site was located in the 5' region of *300FT* using the 50bp oligonucleotide *FT3* labeled with ^{32}P. It was possible to compete the positive interaction by the addition of unlabeled *FT3* excluding non-specific binding of FIT to *FT3* (Figure 38b).

6.5. FIT induces *FT* expression in a transient *Luciferase* based system

In order to gain more insight as to the effect of FIT on *FT*-expression, a transient Luciferase expression assay was applied using *FT* promoter-*Luciferase* (*LUC*) fusions. A fragment containing the promoter region 2.7kb upstream of the transcriptional start of *FT* was isolated and fused to the *LUC* gene (constructed by Dr. Aidyn Mouradov). This construct was coated on gold microcarriers along with *35S::GFP* and *35S::FIT*. To reveal whether the action of FIT is specific, the next closest homolog (*FIDGET-LIKE1*, *FTL1*) was isolated and a second

experiment was carried out using *35S::FTL1*, *FT::LUC* and *35S::GFP*. A negative control was performed using the empty expression vector, *FT::LUC* and *35S::GFP*. After particle bombardment on Arabidopsis leaves, the Luciferase expression was measured using a photon counter.

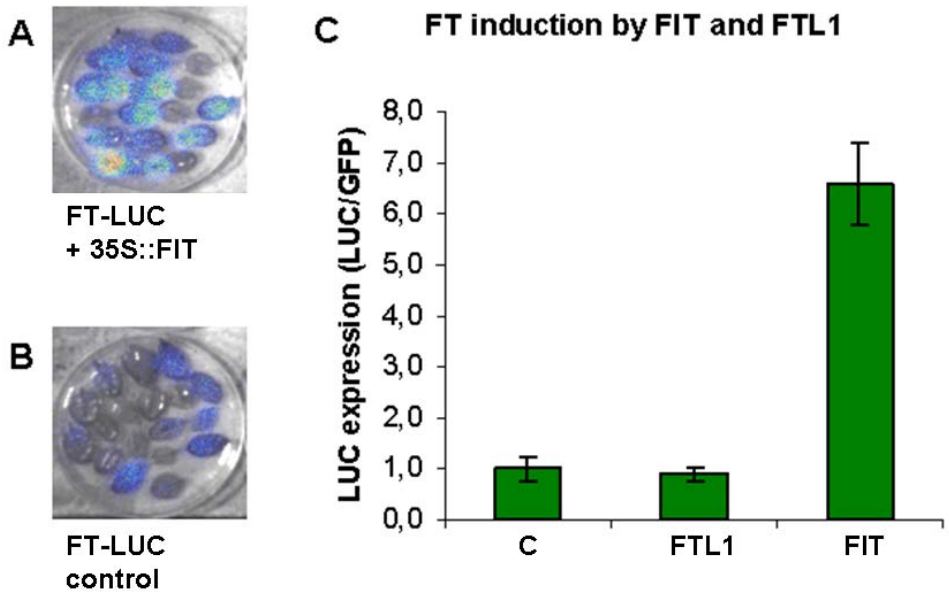

Figure 39. Transient *pFT::LUC* assay after particle bombardment. A) and B) show pictures of transformed leaves expressing *LUC*. C) Quantification of LUC signals compared to GFP-signals. C = *FT::LUC* control; *FTL1=FIDGET-like 1*; *FIT = FIDGET*

In transient promoter-*Luciferase* assays FIT was shown to induce *FT*-expression seven-fold as seen by an increase in LUC/GFP ratio (Figure 39). FTL1, the next closest homolog to FIT is unable to induce *FT* (Figure 39C).

6.6. Fine-mapping of the FIT-responsive element in the *FT*-promoter

To define the site through which FIT activates *FT* expression, different *FT* promoter fragments fused to *LUC* (constructed by Dr. Aidyn Mouradov) were bombarded with and without the *FIT*-effector plasmid.

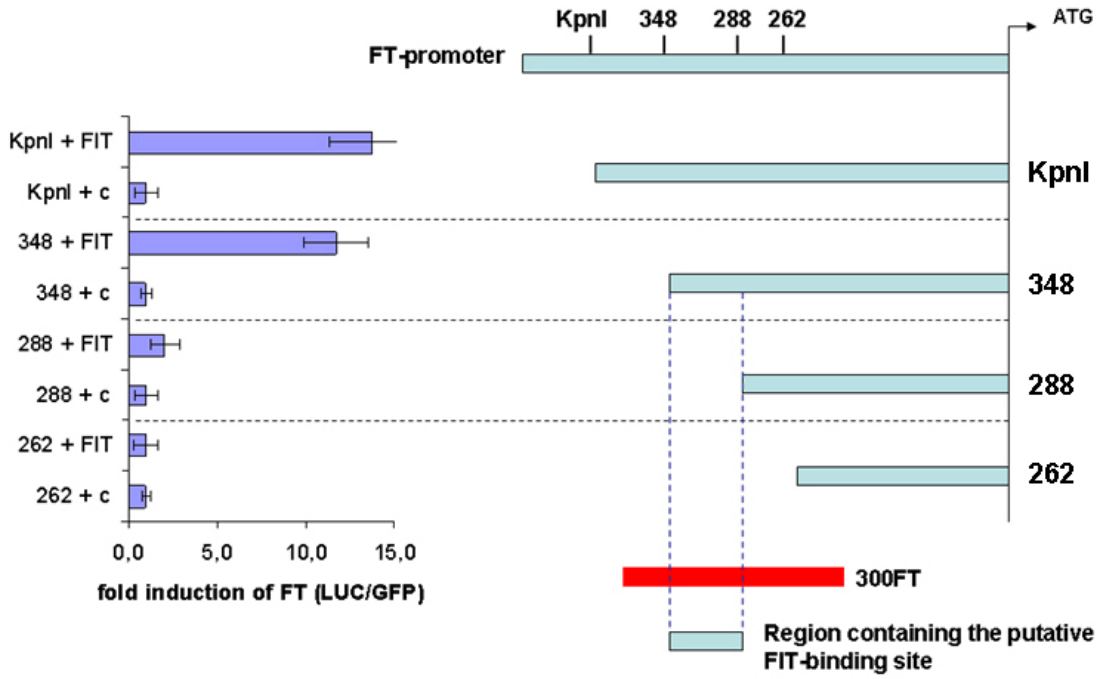

Figure 40. Transient Luciferase-system using small promoter fragments. Top right is a schematic drawing of the *FT* promoter with positions of the small fragments used (KpnI, 348, 288, 262). On the left is plotted the fold activation with and without effector (*35S::FIT*). *300FT* indicates the fragment used for yeast-one-hybrid screening.

A significant decrease in inducibility is detected using fragment 288 compared to 348 (Figure 40). After calculating the fold-activation the putative FIT-binding site was narrowed down to around 60bp, which was also present in the *300FT* fragment that was used for yeast-one-hybrid screening.

6.7. FIT binds to the *FT*-promoter *in vivo*

To test whether FIT can bind to the *FT* promoter *in vivo* transgenic plants overexpressing HA:FIT from the *35S*-promoter were generated. A homozygous line was chosen for chromatin immunoprecipitation and the expression of HA:FIT was analyzed by western blot (Figure 41a).

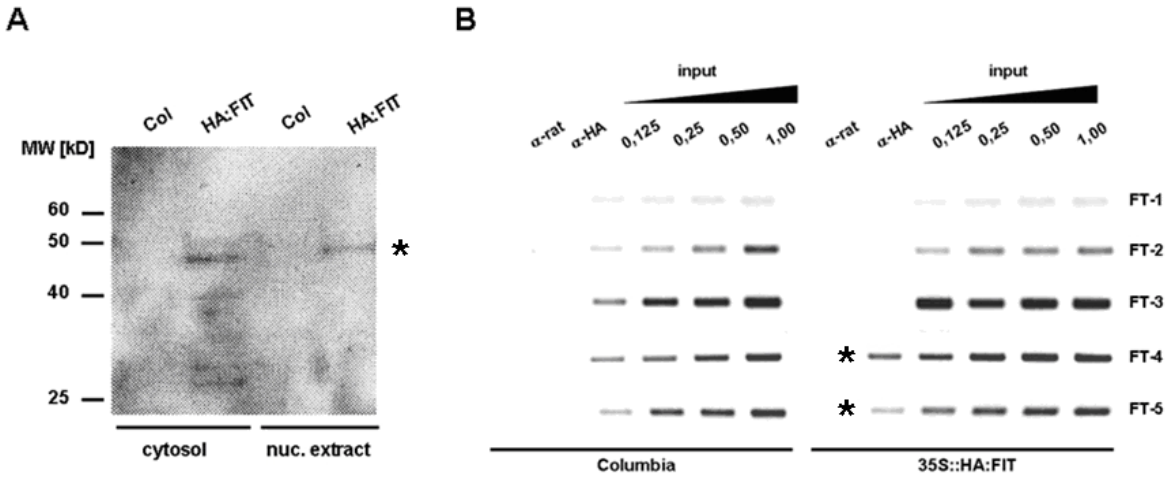

Figure 41. Plant Chromatin immunoprecipitation. A) Plants expressing HA:FIT were grown 12 days under LD condition on SD-plates. After nuclear extraction, the cytosolic and nuclear fractions were analyzed by western blotting comparing HA:FIT plants and Columbia (Col) wild type plants. FIT protein is detected both in cytosolic and nuclear fractions (asterisk). B) *In vivo* ChIP-analysis using different primers *FT-1* (-0,3kb), *FT-2* (- 2,0kb), *FT-3* (-4.0kb), *FT-4* (+1.0kb), *FT-5* (+0,1kb). Numbers in brackets are the amplified regions of the *FT* promoter related to the position of the transcriptional start site. Asterisks indicate immunoprecipitated fragments using α-HA-antibody. Input is a 1/20 dilution of the chromatin used for immunoprecipitation, numbers represent the amount (μl) used for PCR-amplification.

After chromatin immunoprecipitation, PCR-reactions were carried out to amplify different regions in the *FT*-promoter. The region that FIT is thought to bind (*FT-1*, Figure 41b) could not be efficiently amplified. PCR-amplifications using primer *FT-4* and *FT-5* which anneal in closer proximity to the putative FIT-binding site were successful compared to *FT-2* and *FT-3*, which amplify regions of the *FT*-promoter located further upstream (Figure 41b). Using Columbia extracts and the α-HA antibody or the α-rat antibody, no immune precipitation was detected. Also the α-rat control antibody did not precipitate any chromatin from *HA:FIT* lines.

6.8. FIT can homodimerize *in vivo*

CO activates *FT* expression by an unknown mechanism. Since FIT is a transcription factor which can bind to the *FT* promoter, it was tested whether CO is able to interact with FIT to activate *FT*. CO was fused to YFP and FIT to CFP and transient FRET experiments in Arabidopsis were carried out. However, an interaction was not detected nor was co-

localization in sub-nuclear speckles observed (data not shown). In contrast, testing of YFP:FIT with CFP:FIT showed FRET signals in living cells (Figure 42).

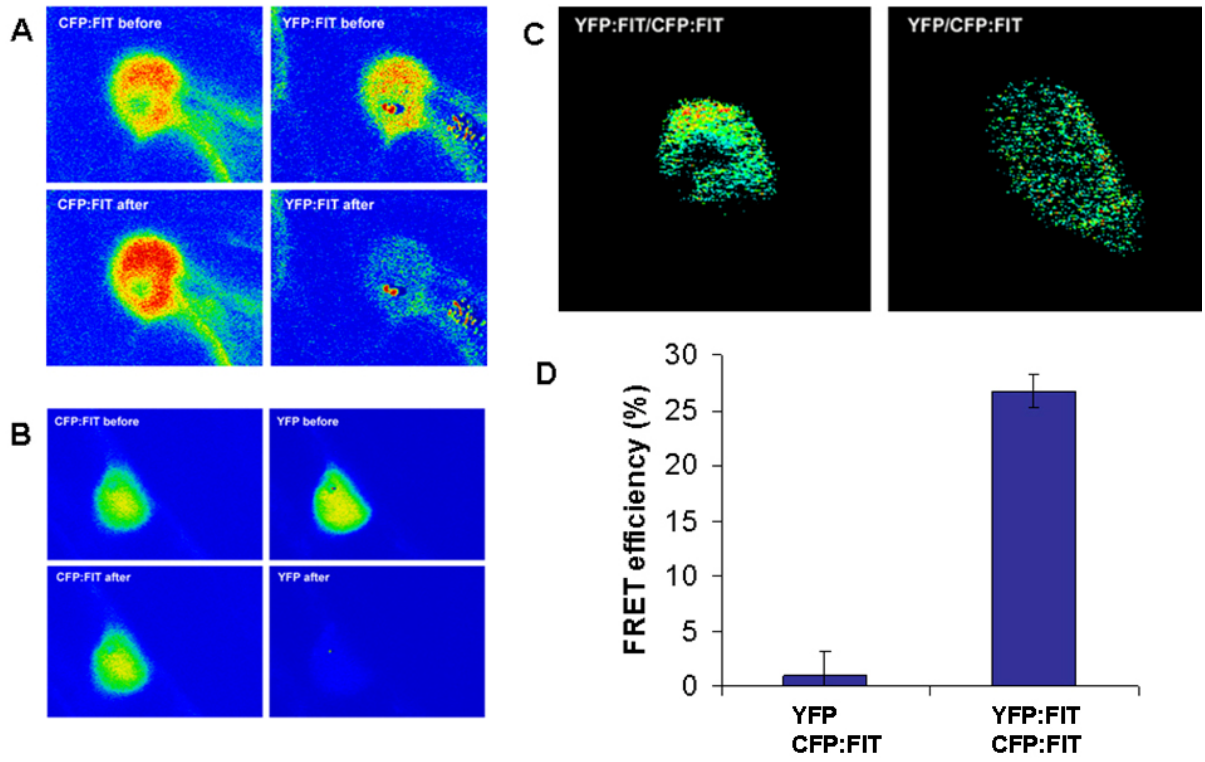

Figure 42. FRET analysis of FIT homodimerization. A) YFP:FIT and CFP:FIT interact as seen in the increase in intensity of the CFP spectrum after photobleaching YFP:FIT. B) negative control analyzing YFP and CFP:FIT, no interaction was observed. C) False color images showing the distributions of FRET efficiencies in the nucleus. D) Quantification and comparison between both FRET pairs shown in A) and B).

This suggests that FIT is able to dimerize, a phenomenon which has so far not been assigned to APETALA2-like proteins. The physiological impact of this interaction is unknown.

6.9. *FIT* mRNA shows a circadian expression pattern that is altered in *lhy/cca1* double mutants

In order to analyze whether the expression pattern of *FIT* shows circadian regulation, timecourse experiments were performed, harvesting 12 day old seedlings every four hours over a time period of 24 hours in short day and long day conditions.

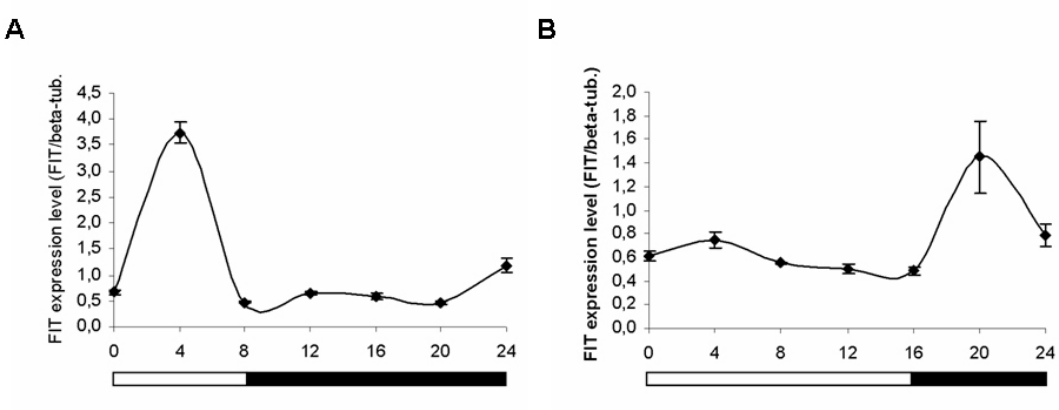

Figure 43. *FIT* timecourse experiment. A) short days and B) long days. The figures show experiments from four technical repetitions. *FIT* mRNA levels were quantified against beta-tubulin. Bars below the graphs represent the photoperiod (in hours).

Analysis of the temporal expression pattern shows that *FIT* is expressed at higher levels in SD peaking 4 hours after dawn compared to LD where the peak appears in the dark phase (Figure 43). Depending on the age of the seedlings the peak in LD is slightly shifted and appears in 9 day old seedlings at the end of the light phase (data not shown).

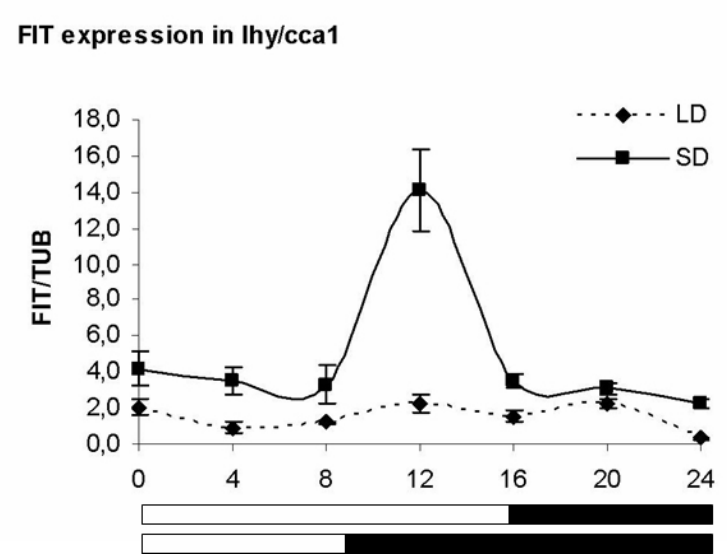

Figure 44. Analysis of the *FIT* expression pattern in the *lhy/cca1* double mutant in short days and long days. *FIT* mRNA levels were quantified against beta-tubulin. Bars below the graph represent the photoperiod (in hours).

FIT shows a diurnal expression pattern and therefore whether this expression pattern is altered in mutants in which the circadian system is distorted was tested. Analysis of the *FIT* mRNA expression in *lhy/cca1* double mutants revealed that the LD peak is abolished (Figure 44). In SD a very strong peak can be observed which is shifted to the dark phase at 12 hours after dawn (Figure 44).

6.10. *FIT* expression in response to plant hormones

In order to gain more insight into the physiological function of FIT and to be able to place it within a physiological pathway, the responses to *FIT* mRNA levels to various plant hormones and changing temperatures were tested. Therefore, 10 day old Arabidopsis seedlings were exposed to different plant hormones or different temperatures.

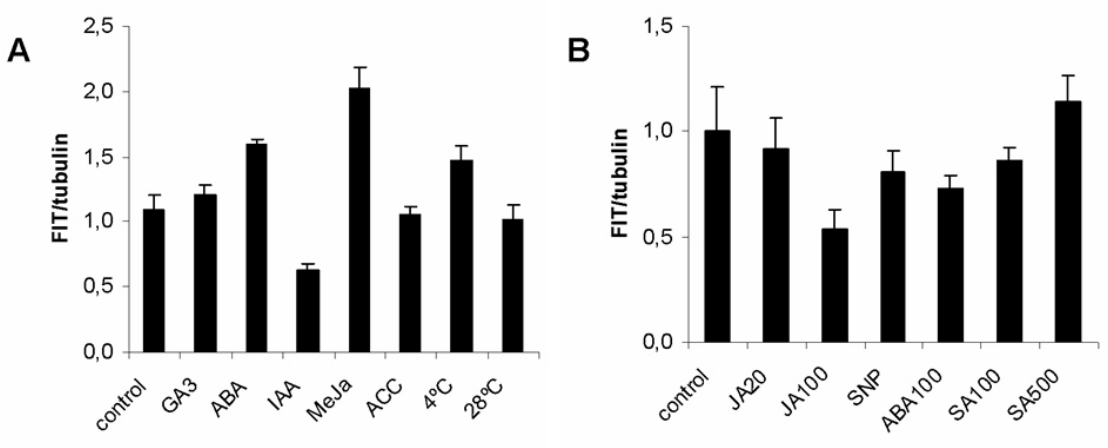

Figure 45. Analysis of *FIT* expression in response to hormone treatments. A) 10 day old seedlings were treated with 50mM gibberellic acid (GA3), abscisic acid (ABA), auxin (IAA), methyljasmonate (MeJa) and the ethylene precursor 1-aminocyclopropan-1-carboxylic acid (ACC) for four hours. In parallel experiments seedlings were incubated for 4 hours at 4°C or 28°C. Control plants were incubated with water at room temperature. B) Hormone treatment experiment II. 10 day old Arabidopsis seedlings were incubated with 20mM and 100mM jasmonic acid (JA), 50mM sodium nitroprusside (SNP), 100mM abscisic acid (ABA),100mM and 500mM salicylic acid (SA) for 24 hours. *FIT* mRNA levels were quantified against beta-tubulin.

The expression level of *FIT* did not change dramatically upon hormone treatment. The strongest effects were a 50% reduction, observed after a four hour treatment with auxin (Figure 45a) and a two-fold upregulation, observed by methyljasmonate treatments for four hours. In the 24 hour treatments only 100mM jasmonic acid caused a two-fold reduction.

6.11. Isolation of *fit-1*, a loss of function mutation in *FIT*

Loss-of-function mutants often provide insight into the physiological pathways the corresponding genes are involved in. A T-DNA knock out in the *FIT* gene (GABI-KAT line 541G11) was found in the GABI-KAT collection and the disruption of the gene was confirmed by PCR on genomic DNA and by RT-PCR (Figure 46).

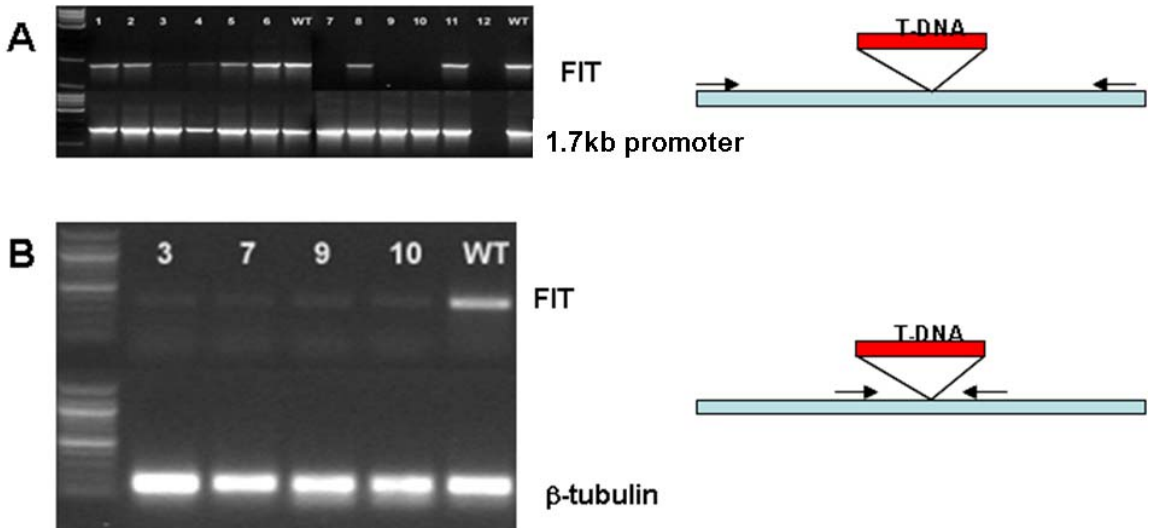

Figure 46. Confirmation of the presence of a T-DNA insertion in the *FIT*-gene. A) PCR on genomic DNA amplifying the *FIT*-gene with primers that flank the proposed insertion site of the T-DNA. The transgenic lines 3, 7, 9, 10 show no amplification indicating the insertion of the T-DNA is homozygous in these lines. Line 12 shows also no PCR product but the control PCR amplifying a 1.7kb *FIT*-promoter fragment also failed, which might be due to poor DNA quality. B) Lines 3, 7, 9 and 10 were analyzed by RT-PCR and only a very faint PCR product was detected which might be background or contamination of wild-type DNA or RNA.

The *fit-1* mutant contains a single-insertion of the T-DNA and this is inserted in the middle of the *FIT* gene, which contains no introns. Furthermore, RT-PCR analysis shows that the *FIT* transcript is dramatically reduced (Figure 46) indicating that *fit-1* may retain some *FIT* RNA. The analysis of this line will be extended by performing PCR with primers annealing to the T-DNA and the FIT gene, and by analyzing the structure of the *FIT* mRNA present in the *fit-1* line.

6.12. Misexpression of *FIT* in the phloem companion cells results in early-flowering under long days

Overexpression of *FIT* by the *35S*-promoter causes a slight acceleration of the floral transition, whereas expression of *FIT* from the *SUC2*-promoter, which results in high ectopic expression in the phloem, shows a clear early-flowering phenotype. *FT* is expressed in the phloem and misexpression of *FIT* where *FT* is expressed enhances the flowering phenotype compared to plants overexpressing *FIT* from the *35S*-promoter. The *fit-1* mutant plants seem to flower later, but this is dependent on the light conditions used (Figure 47).

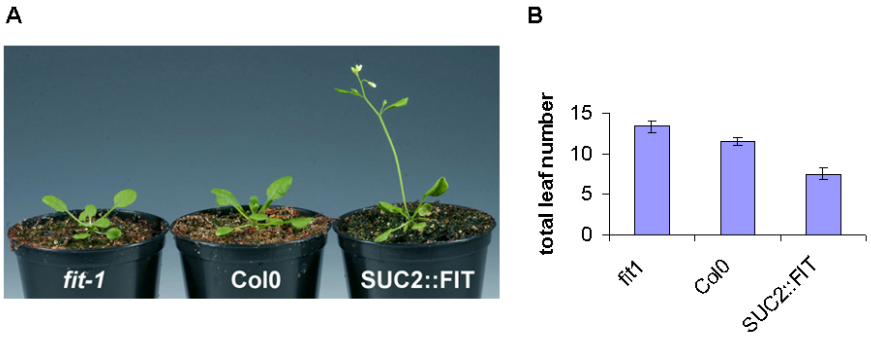

Figure 47. Flowering-time analysis of *fit-1*, Col-0 and *SUC2::FIT* in LD conditions. B) Quantification of leaf numbers at bolting.

The observed late-flowering phenotype is not consistent in all cabinets, which indicates that light fluence or quality is important and that under some conditions the loss of *FIT*-function is compensated for by other regulatory pathways. Growing the same plants in Percival grow banks under LD condition resulted in later flowering Col-0 plants and therefore the flowering phenotypes of *fit-1* and *SUC2::FIT* were less strong (Figure 48).

6.13. The early-flowering phenotype of *SUC2::FIT* is *FT*-dependent

FIT has the strongest effect on flowering when expressed in the phloem companion cells where *FT* and *CO* are expressed. This suggested that FIT activity might depend on the activity of these characterized flowering-time genes. Therefore, the *SUC2::FIT* transgene was introduced into mutant plants impaired in the photoperiod pathway (*gi-3*, *co-2* and *ft-7*) by transformation and the flowering-time behavior of homozygous double mutants was analyzed (Figure 48).

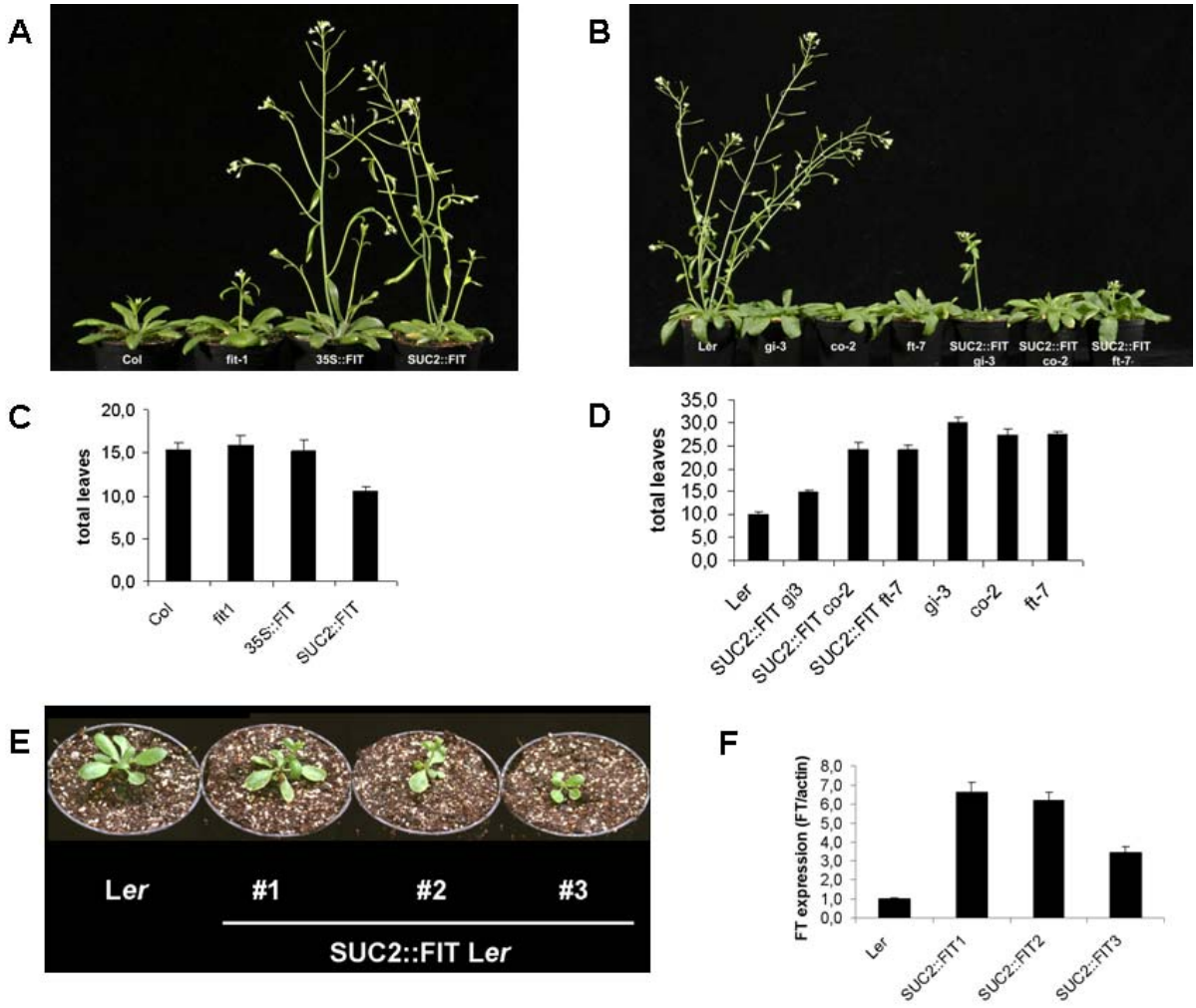

Figure 48. Analysis of SUC2::FIT in different genetic backgrounds under LD conditions. A) Flowering phenotypes of transgenic plants (*fit-1*, *SUC2::FIT*, *35S::FIT*) in Columbia background compared to Col-0. B) Flowering phenotypes of *SUC2::FIT* in *gi-3*, *co-2* and *ft-7* (all in L*er*) compared to *gi-3*, *co-2* and *ft-7*. C) and D) show leaf numbers at the transition to flowering. E) Flowering-time phenotypes of *SUC2::FIT* in L*er*, three independent T1-plants grown in LD compared to L*er*. F) *FT* expression in SUC2::FIT transgenic lines.

Under LD conditions 35S::FIT and SUC2::FIT accelerate the floral transition compared to wild-type and *fit-1* (Figure 48a and c). However, under these growth conditions the effect of *35S::FIT* on flowering-time is weaker than in previous experiments. The late-flowering phenotype of *gi-3* can be suppressed by misexpression of *FIT*. Also the late-flowering phenotypes of *co-2* and *ft-7* are rescued by *SUC2::FIT* but only to a much lesser extent than *gi-3* (Figure 48b and d).

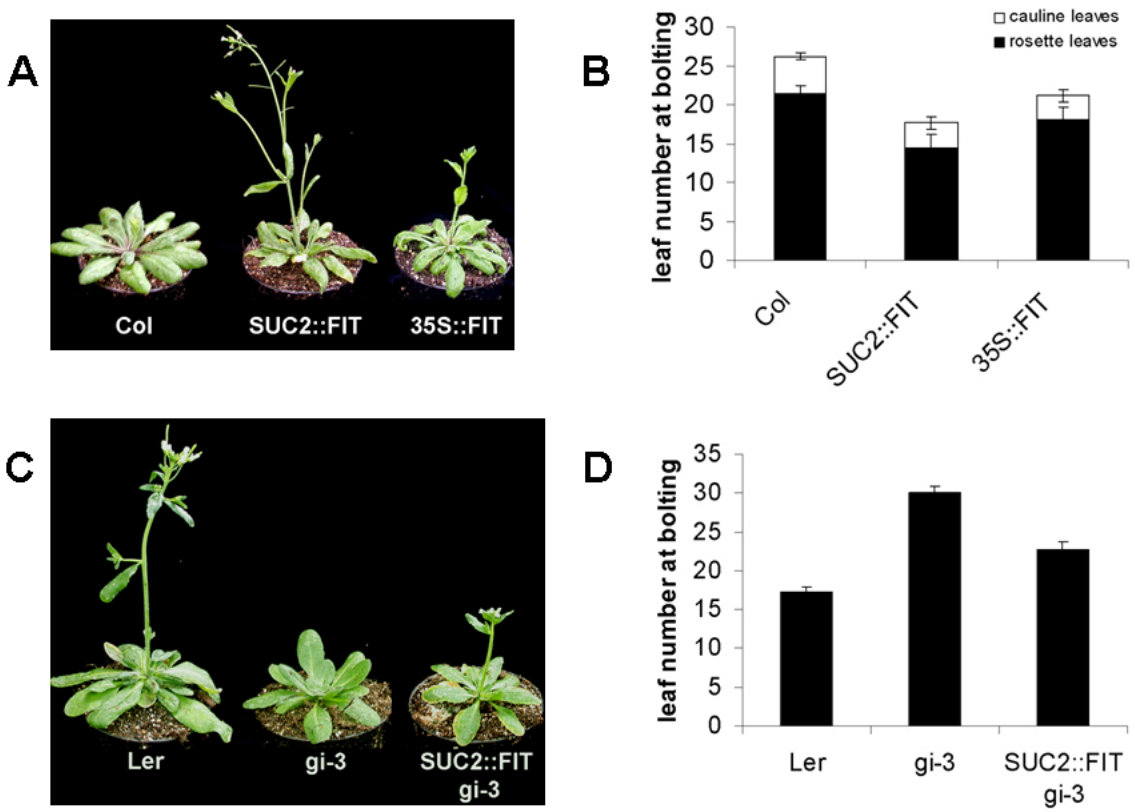

Figure 49. Analysis of SUC2::FIT in different genetic backgrounds under SD conditions. A) Flowering phenotypes of transgenic plants (*SUC2::FIT, 35S::FIT*) in Columbia background compared to Col-0. C) Flowering phenotypes of *SUC2::FIT* in *gi-3* (in L*er*) compared to *gi-3* and L*er* B) and D) Quantification of flowering-time by counting the number of leaves at the transition to flowering.

35S::FIT is also early-flowering in SD (Figure 49a). *SUC2::FIT* in Landsberg *erecta* background is early-flowering both in LD and SD compared to Landsberg *erecta* and the early-flowering phenotype correlates with higher levels of *FT* mRNA (Figure 48 e and f; Figure 49a). Also in SD the late-flowering phenotype of *gi-3* is suppressed by *SUC2::FIT* (Figure 49c). However, in SD the late-flowering phenotypes of *co-2* and *ft-7* mutants are not affected by *SUC2::FIT* (data not shown).

6.14. *FIT* mRNA is induced by UV-light and expressed in vascular tissue

Analysis of publicly available microarray data (www.genevestigator.ethz.ch) suggests that *FIT* is induced by UV-B and UV-C light. The response of *FIT* to UV-B and shorter wavelengths of light (UV-C) was confirmed by another microarray study conducted by Dr.

Roman Ulm and colleagues from the University of Freiburg, Germany (Dr. R. Ulm, personal communication). In order to confirm these results 12 day old Arabidopsis seedlings were treated for 30 minutes with UV-B light and RNA extracted prior and after UV-B treatment. The expression levels of *FIT* and *FT* were then compared in treated and non-treated plants (Figure 49).

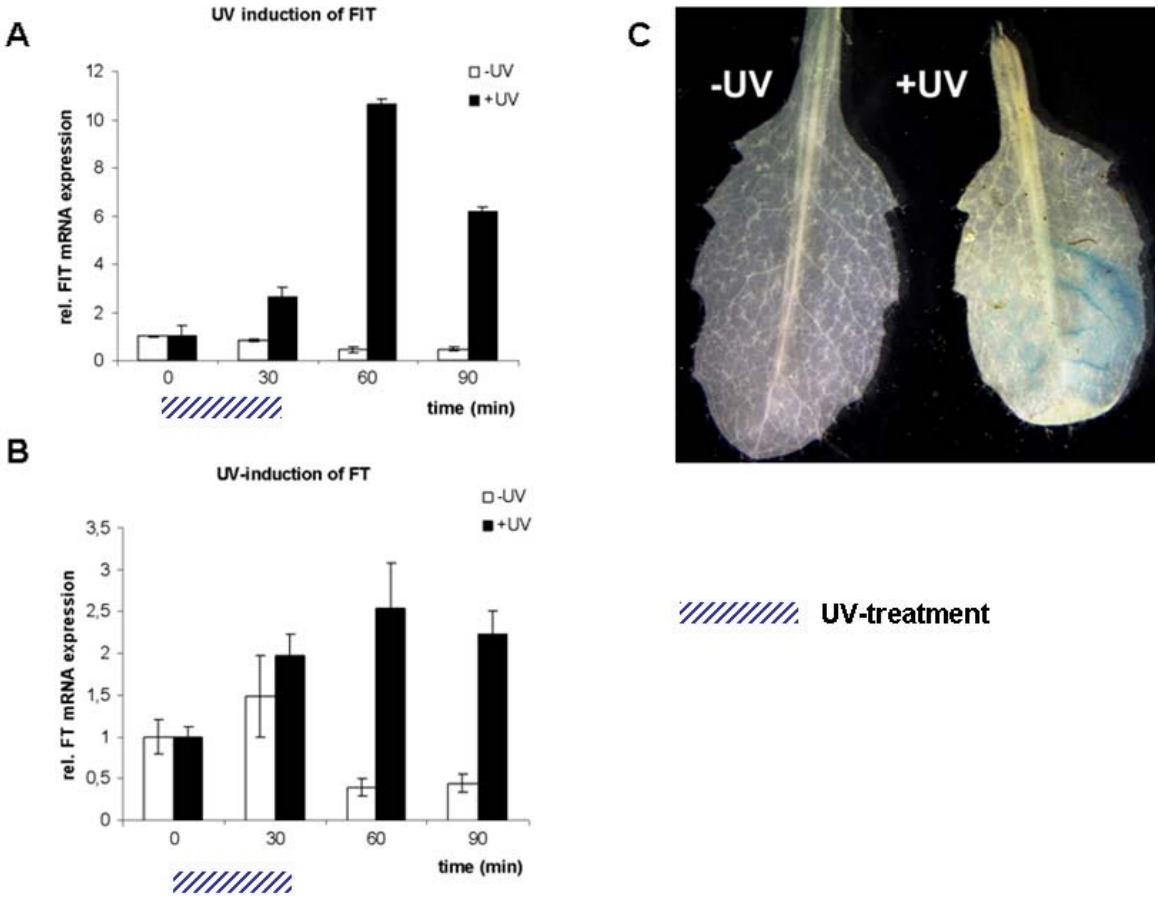

Figure 50. *FIT* responds to UV-B light. A) Expression analysis of *FIT* in response to UV-B. Plants were grown for 12 days in LD and a UV-B pulse was applied at ZT6 for 30 minutes (between 0 and 30 minutes in the graph). The 0 minutes value was before the pulse and the 30 minutes immediately after the pulse. 60 and 90 minute values are half an hour and one hour after the pulse. Bar represents the time of the UV-pulse. B) Expression analysis of *FT*. Same conditions as described for A). C.) UV-B treatment of *pFIT::GUS* plants. GUS expression is only detectable after UV-B treatment in the vasculature.

UV-treatments confirmed the microarray data, demonstrating that *FIT* is upregulated in response to UV-light. In response to a 30-minute pulse of UV-B a 10-fold increase is detected (Figure 50a). For *FT* the expression analysis was less conclusive because it was difficult to detect *FT* by quantitative RT-PCR (qRT-PCR) due to primer dimer formation. Nevertheless, there was a tendency for elevated *FT* transcript levels after UV treatment (Figure 50b).

To examine the spatial expression pattern of *FIT*, transgenic plants expressing the *GUS* gene under the control of the *FIT* promoter were constructed. Under normal growth conditions GUS expression was almost undetectable. However, treatment of the *pFIT::GUS* plants with UV-B light caused GUS expression in the vascular tissue, confirming our genetic and molecular data (Figure 50c).

6.15. UV light can accelerate the floral transition in Arabidopsis

Because *FIT* is expressed in the vascular tissue in response to UV-light and can induce *FT* expression, whether UV-light can induce early-flowering in Arabidopsis was examined. The experiment was carried out under short days (10 hours light, 14 hours darkness) in order to avoid a strong influence of the photoperiod pathway.

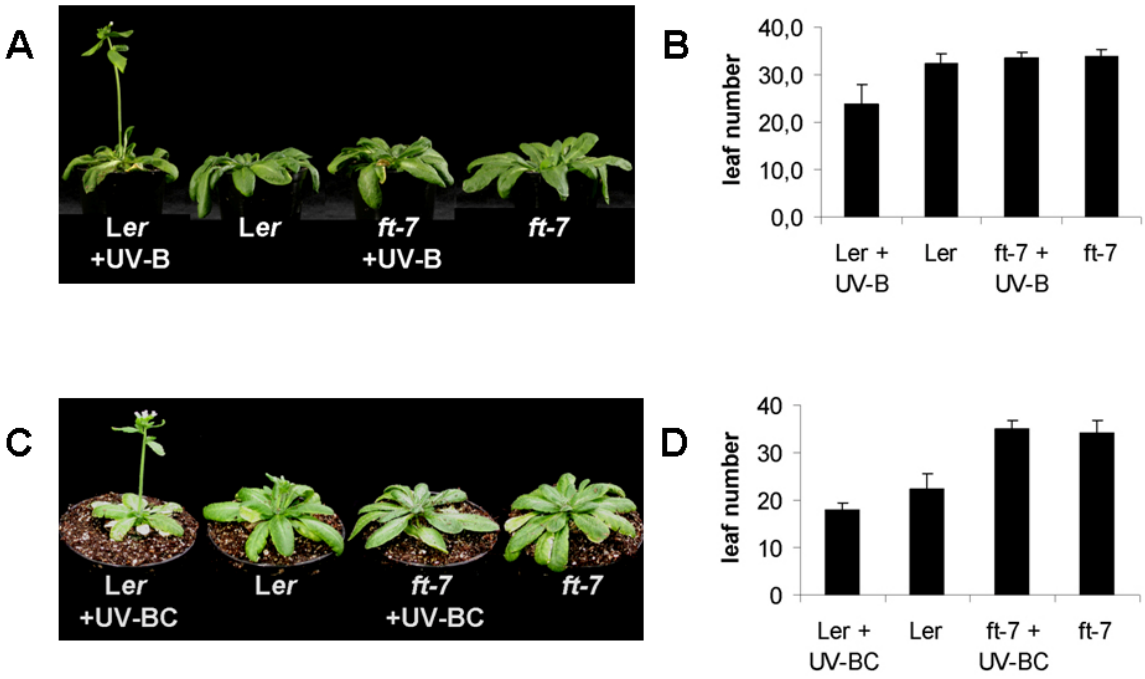

Figure 51. Flowering-time experiment in response to UV-light. A) Phenotypes observed after UV-B (0,5W/m^2) treatment of L*er* and *ft-7*. C) Flowering-time analysis in response to UV-B/C. B) and D) Quantification of flowering-time by counting the number of leaves at the transition to flowering. (In both experiments the comparison of L*er* treated to non-treated plants is significant, student's t-test P<<0.05).

The experiment was carried out by treating wild-type and *ft-7* plants every second day with UV-B for 30 minutes. When stress symptoms (yellowish leaves) were observed, the treatment

was stopped for a few days. The results from this experiment indicate that flowering can be induced by treating plants with UV-B and that this signaling pathway requires the activity of *FT* (Figure 51a and b). Additionally, we analyzed whether flowering is also accelerated by a mixture of UV-B and UV-C light. Since UV-C light causes sever damage, plants were irradiated daily for 5-10 minutes with a mixture of UV-B and UV-C light. Also in response to this stress, wild-type plants responded by acceleration of flowering. Plants carrying a mutation in the *FT* gene (*ft-7*) did not respond to UV-B/C irradiation (Figure 51c).

6.16. Targeted misexpression of Arabidopsis transcription factors demonstrates that AP2-like proteins influence the floral transition when expressed in vascular tissue

CO and *FT*, two of the major regulators of the floral transition, are both expressed in the phloem companion cells and mutations in these genes can be rescued by misexpressing them in the phloem from the *SUC2* promoter. In order to find other regulators of flowering-time around 1.000 transcription factor genes from the REGIA transcription factor library were misexpressed from the *SUC2* promoter (work from Dr. Lionel Gissot). After transformation several lines were identified showing abnormal flowering-time phenotypes. Interestingly, several *AP2-like* genes exert flowering-time abnormalities when expressed in the phloem. Three *AP2-like* genes are early-flowering when mis-expressed in the phloem, one of them being *FIT*, which was an independent confirmation of the results described in this chapter. Four *AP2-like* genes exhibit a late-flowering phenotype upon misexpression.
To find out whether other AP2-like transcription factors can bind to the *FT* promoter all known AP2-like genes having an effect on flowering such as *TOE1*, *2*, and *3*, *SMZ*, *SNZ* and all genes identified as causing late or early-flowering in the above mentioned screen, were tested in yeast by one-hybrid screening.

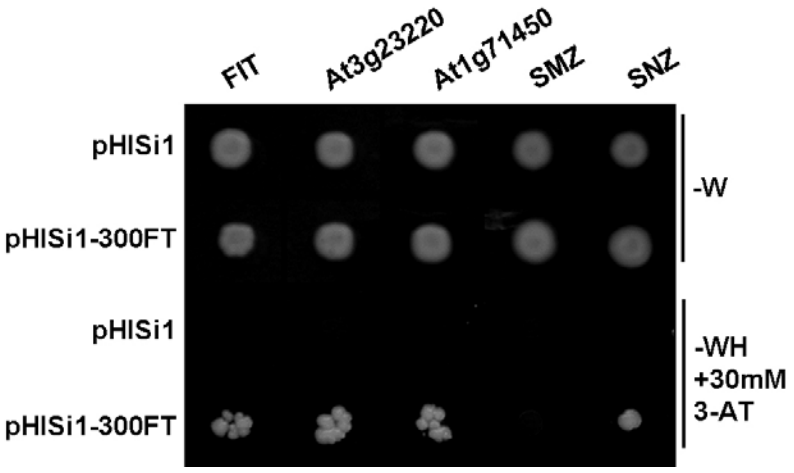

Figure 52. Systematic yeast-one hybrid analysis of the interaction of AP2-like proteins having an effect on the floral transition using the 300-bp *FT* promoter fragment. The upper panel shows yeast growth on SD-medium lacking tryptophan. The lower panel shows yeast growing on SD-medium lacking histidine and tryptophan supplemented with 30mM 3-AT.

Two AP2-like proteins (At3g23220, At1g71450 and FIT) showed strong growth induction by yeast-one-hybrid screening (Figure 52). The induction by SNZ was weak and yeast growth was only visible after ten days incubation at 30°C. Phylogenetic analysis of all AP2 proteins showed that one of the two AP2-like proteins causing late-flowering, At3g23220, is closely related to FIT (not shown) indicating that these two proteins could compete for the same cis element in the *FT* promoter. These results point towards the existence of a regulatory node in the *FT* promoter where positively or negatively acting AP2-like proteins bind to modulate the expression of *FT* in response to environmental stimuli. This would allow a fine-tuning of the floral transition at the level of floral integration.

Various studies have shown that microRNA172 targets mRNAs of *AP2* and *AP2*-like genes and interferes with their expression by translational inhibition (Aukerman and Sakai, 2003; Schmid et al., 2003; Chen, 2004a). The model Aukerman and Sakai propose is that early in development repressors of flowering such as *TOE1*, *TOE2* and *TOE3* are expressed. Later in development miR172 is expressed and inhibits *TOE1*, *TOE2* and *TOE3* allowing the transition to flowering to occur (Aukerman and Sakai, 2003). *FIT* acts as a floral activator and is probably not targeted by miR172 since it can induce flowering at the time where miR172 is expressed. Moreover, analysis of the microRNA-binding site reveals seven mismatches which probably impair microRNA-association and translational inhibition (Figure 53).

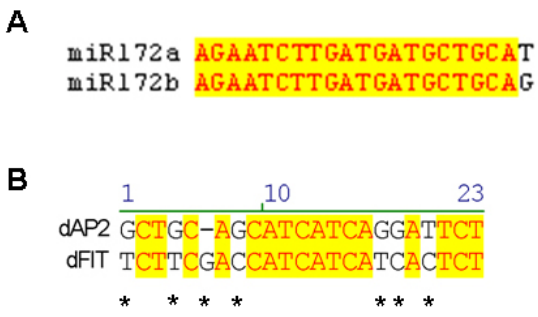

Figure 53. Alignment of miR172 and the microRNA-binding sites in AP2 and FIT. A) Alignment of miR172a and miR172b showing a difference in one nucleotide. B) Sequence alignment of the miR-binding site in AP2 and FIT showing seven mismatches.

Analysis of publicly available microarray data (www.genevestigator.ethz.ch) of *FIT*, *At3g23220* and *At1g71450* which can bind to the *FT* promoter and affect flowering-time, shows that their mRNAs are induced by salt stress in root tissue (not shown). This effect is probably unrelated to *FT*. The array data indicate that *FIT* is most highly expressed in senescent leaves (not shown), which is in good agreement with the observation that *FIT* is induced in leaves after UV-induction since UV-light is known to evoke senescence-associated symptoms (Brosche and Strid, 2003; Ulm et al., 2004). Apart from expression in roots upon salt stress, *At3g23220* is induced by treatments with cycloheximide and *Botrytis cinerea* (Genevestigator; data not shown). *FIT* is the only gene among these three that is induced by UV-light and acts by upregulation of *FT* expression.

6.17. Discussion

FIT interacts with the *FT* promoter in yeast, *in vitro* and *in vivo*

FIT was isolated in a yeast-one hybrid screen and found to interact with a 300 base pair fragment of the promoter of the flowering-time gene *FT*. The interaction initially identified in yeast with a clone present in the library was verified in yeast using a clone expressing the full length protein (Figure 32). Independent methods were used to confirm the interaction observed in yeast . *In vitro* gelshift assays were carried out using recombinant HIS:FIT protein (Figure 37; Figure 38a and b). The shifted band appeared very weak, which might be due to small amounts of active protein being present or to improper buffer conditions. Because of the formation of insoluble inclusion bodies HIS:FIT could not be purified under native conditions, which might have improved the *in vitro* gelshifts. Using smaller *FT* promoter fragments, the interaction was narrowed down to a 60bp region and competition experiments indicated a specific interaction (Figure 38b). More competition experiments

using mutant fragments are required to confirm these results. In order to verify the interaction observed in yeast and *in vitro*, *35S::HA:FIT* plants were used to test the interaction *in vivo* by chromatin immunoprecipitation (ChIP). Chromatin from wild-type and *35S::HA:FIT* was prepared and precipitated with an HA antibody. PCR reactions flanking the FIT binding site confirmed that the interaction occurs *in vivo* since we obtained amplification products only with *35S::HA:FIT* chromatin when the HA-antibody was used (Figure 41b). In all negative controls no amplification was observed. Due to low resolution of ChIP-PCR reactions because of the length of DNA fragments precipitated, this assay is less well suited to determine the exact binding element. The fact that chromatin is efficiently precipitated from regions upstream of the transcriptional start site suggests a second binding site for FIT in the first intron of *FT*. Recently it was shown, that FLC binds in the first intron of *FT* to suppress its expression (Dr. F. Turck, unpublished).

FIT induces *FT* in a *Luciferase*-based promoter assay

To identify the function of the interaction of FIT with the *FT* promoter, an *in vivo Luciferase* (*LUC*)-based promoter assay was carried out. Transient bombardments with *35S::FIT*, and *2.7 kb FT-Luc* revealed that FIT can up-regulate *FT* (Figure 39). The next closest homolog, named *FTL1*, was neither able to induce *FT* expression (Figure 39) nor did misexpression in the phloem by the *SUC2*-promoter result in early-flowering. This indicates that the effect of FIT on *FT* is specific. To narrow down the FIT responsive element in the *FT* promoter, deletions from the 5' towards the 3' end of the promoter were used (constructed by Dr. Aidyn Mouradov). Analysis of the *LUC* activation of these deletion fragments revealed that the FIT response element is located in the middle of the 300bp fragment used for one-hybrid screening (Figure 40). These results are consistent with the *in vitro* gelshifts (Figure 38). Figure 54 summarizes the findings from yeast, *in vitro* and the *LUC*-assay.

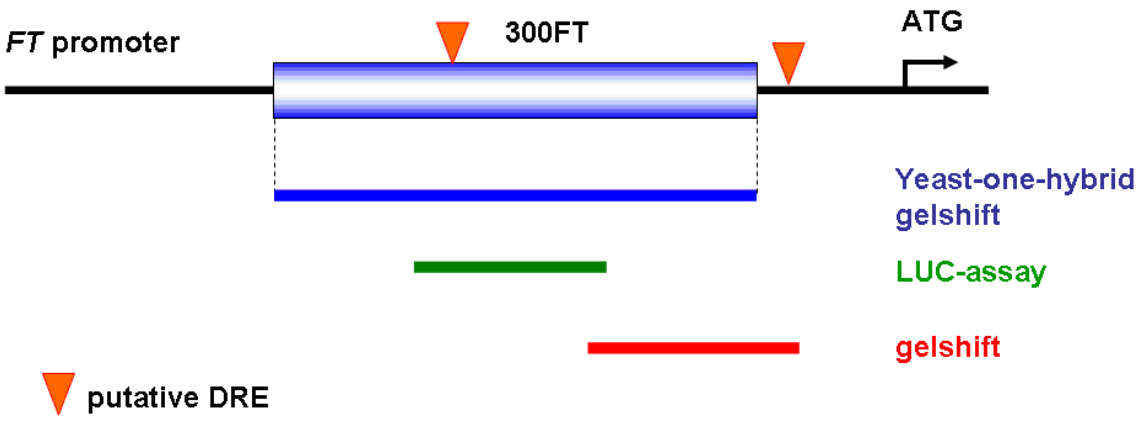

Figure 54. Schematic overview of the proximal *FT* promoter showing the distribution of putative AP2-binding sites. The blue bar shows the fragment with which FIT interacted in gelshifts and in yeast. The green bar represents the region where FIT was able to activate LUC-expression in the transient promoter assay. The red bar represents the FT-3 fragment used for *in vitro* gelshifts.

In summary, these results indicate that FIT binds to a region in the *FT* promoter with no known AP2-like binding element. The 300bp fragment used for yeast-one-hybrid screening contains a putative drought response element (DRE) which could be targeted by FIT. Also the *LUC*-experiment is consistent with the involvement of this element but not the gelshift. Since the intensity of the shifted band using the *FT-3* oligonucleotide was very weak and competition experiments with mutant oligonucleotides were not yet performed, the band shift experiments might still be misleading. Future experiments will be directed to answer the question whether FIT interacts with the putative DRE or binds to a novel element.

FIT homodimerizes, a novel feature of AP2-like proteins?

The flowering-time gene *CO* causes upregulation of *FT* expression by an unknown mechanism. It was tested by *in vivo* FRET analysis whether CO can interact with FIT mediating an interaction between CO and DNA. Such an interaction was not detected but testing the interaction of FIT with itself revealed that it is able to homodimerize (Figure 42). So far homodimerization of AP2-like protein such as FIT has not been described, and the functional significance of this homodimerization is still not clear. The observed interaction might not take place under natural conditions and occurs only in the artificial testing conditions. Nonetheless, homo- or heterodimerization might be important for the function of AP2-like proteins in general. This could also be a feature of EREBP-proteins like FIT, since these have only one AP2-domain. Homo- or heterodimerization would then yield a two AP2-domain complex which would increase the complexity of the interaction with DNA and

potentially generate enhanced specificity. This effect might help to explain their roles in various developmental and stress related pathways.

FIT mRNA shows a circadian expression pattern which is shifted in *lhy*/*cca1* mutant plants

Analysis of the temporal expression pattern of *FIT* revealed that its mRNA shows a circadian expression pattern. In SD *FIT* mRNA peaks around 4 hours after dawn, compared to LD where the peak occurs at the beginning of the night (Figure 39a and b). Whether *FIT* is regulated by the circadian clock of the plant was tested by analyzing the expression pattern in *lhy*/*cca1* mutants. These plants show severely altered clock function and clock gene expression peaks at an earlier phase in light/dark cycles (Alabadi et al., 2001; Mizoguchi et al., 2002). In *lhy*/*cca1* mutant plants a peak of *FIT* expression is almost absent in LD whereas in SD the peak is shifted towards the night at 12 hours after dawn (Figure 40). Moreover, the expression in SD is significantly higher, indicating that *LHY* and *CCA1* might regulate FIT expression by repression. These results suggest that *FIT* is a circadian clock regulated gene whose expression is affected by mutations in *lhy/cca1*.

The natural expression pattern of *FIT* does not resemble the expression pattern of a gene directly involved in *FT* regulation. The expression of *FIT* is low in LD and the major peak appears in SD at a time at which *FT* is not expressed. This expression pattern suggests that *FIT* performs functions other than *FT* regulation. However, in response to UV stress the expression of *FIT* is ectopically induced resulting in *FT* induction. This is detected in response to a 30-minute UV-pulse given to wild-type plants grown in LD. UV-treatment led to a ten-fold increase of *FIT* expression in LD at a time when the gene is naturally expressed at low levels (Figure 49).

Analysis of transgenic plants reveals a role for FIT in flowering-time control

Analysis of *fit-1* knock out plants indicated that they might be delayed in flowering under LD conditions (Figure 47). Overexpression of *FIT* by the *35S* promoter caused acceleration of flowering both under short day (data not shown) and long day conditions (Figure 48). The strongest effect is observed when *FIT* is ectopically expressed in the phloem companion cells by the *SUC2* promoter. *SUC2::FIT* plants also flower early in short days (not shown) and long days (Figures 47 and 48). The *SUC2::FIT* transgene was also introduced in mutants impaired in the photoperiod pathway. The *gi-3* mutant can be rescued by *SUC2::FIT* whereas the extent of complementation is less pronounced in *co-2* and *ft-7* mutant plants. This

indicates that FIT functions in addition to the photoperiod pathway and a basal activity of CO is necessary. Since the *co* mutation is only rescued to a small extent it is possible that the presence of CO changes the chromatin structure of the *FT* promoter allowing an access of alternative factors to modulate *FT* expression. The fact that also *ft* mutants can be partially rescued by *SUC2::FIT* indicates that *FIT* can modulate the expression of other flowering-time genes (Figure 48). Since high UV-levels are mostly perceived in summer days where the photoperiod is long, might explain that additional factors need an active photoperiod pathway to accelerate the floral transition.

Analysis of factors to which *FIT* responds in wild-type plants

Transient experiments treating Arabidopsis seedlings with different plant hormones and exposing them to different temperatures were carried out to identify conditions under which *FIT* mRNA expression is induced. Knowledge of these conditions could place *FIT* within physiological pathways. Treating Arabidopsis seedlings for four hours with different hormones revealed a 1.5-fold induction by ABA, a 2-fold induction with methyljasmonate and a 2-fold decrease by treating with auxin (Figure 45a). All these effects are very mild and do not allow *FIT* to be placed within one of these hormone pathways. However, the effects of ABA and methyljasmonate indicate a possible function in stress signaling.

Analysis of publicly available microarray experiments (Zimmermann et al., 2004; Zimmermann et al., 2005; www.genevestigator.ethz.ch) indicated that *FIT* might be induced by UV-B and UV-C light. Microarray experiments of *hy5* mutants and wild-type in response to UV-light (Ulm et al., 2004) have shown that *FIT* expression is *HY5* independent and induced by UV-B and UV-C light (Dr. Roman Ulm, University of Freiburg, personal communication). These results place *FIT* in the stress signaling pathway rather than being involved in a UV-B dependent photomorphogenic response. Treatment of Arabidopsis seedlings with a 30 minute UV-B pulse elicited a 10-fold increase in *FIT* expression 30 minutes after the pulse (Figure 50a). The upregulation of *FT* in these conditions is less clear, but a trend towards elevated expression levels after the UV-pulse was observed (Figure 50b). Analysis of *FIT* promoter-*GUS* fusion lines showed almost no expression at various developmental stages. Treatment of the *pFIT::GUS* lines with UV-B light showed that *FIT* is induced in vascular tissue (Figure 50c). These results confirm the misexpression results where *FIT* shows the strongest flowering-time phenotypes when ectopically overexpressed in vascular tissue (Figures 47-49).

Exposure of plants to UV-light accelerates the floral transition

To investigate the effect of UV-light on the floral transition, Arabidopsis plants were grown under SD conditions of ten hours day and 14 hours night. 14 days after germination plants were treated with 30 minute UV-B pulses every two to three days. The frequency of these treatments was dependent on obvious stress symptoms such as the development of chlorotic leaves. The aim was to avoid these symptoms to allow proper development. In our experiment, wild-type plants responded to these UV-B pulses by acceleration of the floral transition (Figure 51). However, not all plants responded similarly to the UV-pulses. There was some overlap between the latest flowering plants treated with UV-light and the earliest non-treated plants (student's T-test $P<<0.05$). Nevertheless, measuring the average leaf number produced at the transition to flowering clearly showed a significant difference between the average leaf numbers of treated and non-treated plants. Homozygous *ft* mutant plants do not respond to the UV-stimulus which indicates that the UV-signal evoking flowering requires the activity of *FT*. Preliminary results show that treatment of plants with a combination of UV-B/C is also able to accelerate the floral transition (Figure 51c). Current activities aim to identify optimal conditions for the acceleration of the floral transition in response to UV-B and UV-C or a combination of both wavelengths. Furthermore, mutants in *FT*, *FIT* and *CO* are being analyzed to place these genes within a genetic network of genes controlling the floral transition in response to UV-light and/or abiotic stress.

Other AP2-proteins causing late flowering can bind to the *FT* promoter in yeast

Analysis of AP2-like proteins that cause alteration of the flowering-time when expressed in the vascular tissue revealed that some of these proteins can bind to the *FT* promoter (Figure 52). Because most of these genes are expressed in response to different environmental stimuli, suggests the existence of a regulatory node in the *FT* promoter. In response to certain environmental cues positively or negatively acting AP2-proteins are expressed which can influence the floral transition at the level of floral integration.

6.18. Conclusion

The present data suggest the existence of a fifth flowering-time pathway that regulates flowering in reponse to stress signals. *FIT* is not epistatic to *CO* suggesting that this new flowering-time pathway acts together with or involves the photoperiod pathway. Under LD conditions where the photoperiodic pathway induces the transition to flowering, the stress pathway could be activated by UV-light, high-light or drought stress, leading to an acceleration of flowering induction (Figure 55).

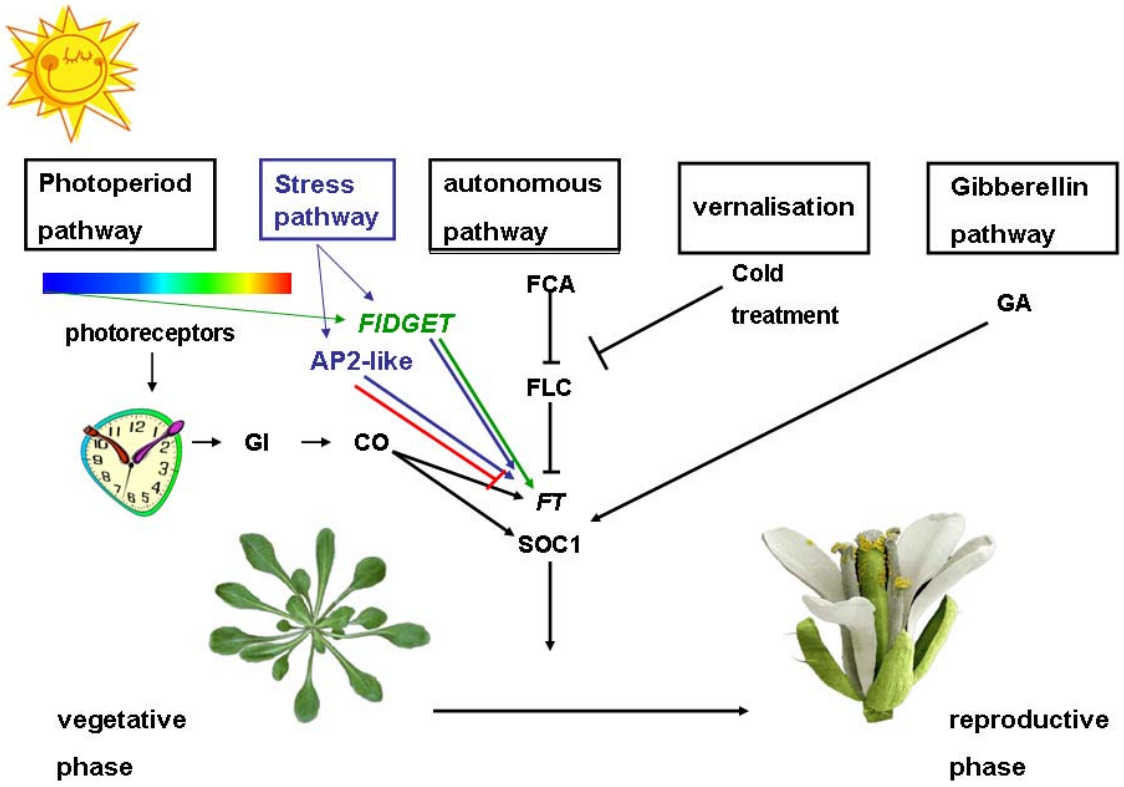

Figure 55. Five flowering-time pathways control the floral transition in Arabidopsis.

Several AP2-like transcription factors were identified which repress or activate the floral transition when overexpressed or ectopically expressed in the phloem. Among those we presented evidence that *FIT* can accelerate the floral transition by direct activation of *FT*, probably in response to UV-light or maybe also other stress factors. Current research focuses on the determination of the FIT-binding site in the *FT* promoter and analysis whether other AP2-like proteins interact with the same motif. Furthermore, the response of *FIT* to various UV-light treatments in wild-type and various mutant backgrounds will be analyzed.

7. General conclusion and perspectives

The transition to flowering is one of the most important changes during plant development. Several pathways are known to be involved in mediating this transition. Among them, the photoperiod pathway mainly involves the flowering-time genes *GI*, *CO* and *FT* (Boss et al., 2004; Searle and Coupland, 2004). Activation of *CO* results in transcription of *FT* (Samach et al., 2000). *CO* is transcriptionally regulated by the circadian clock of the plant (Suarez-Lopez et al., 2001) and post-translationally by the 26S-proteasome in response to different light qualities (Valverde et al., 2004).

Identification of proteins involved in recruiting CO to the *FT* promoter

The main aim of the thesis was the investigation of proteins interacting with CO and to identify potential candidates that are involved in mediating the transcriptional regulation of *FT*. Since CO acts as a transcription factor but does not contain any known DNA-binding motif we searched for proteins mediating such interaction with DNA.

We present evidence that CO interacts with all three subunits of the heterotrimeric HAP complex. The interaction with the HAP3 and HAP5 subunits are direct and were confirmed in yeast, *in vitro* and *in vivo*. The interaction with the HAP2 subunit seems to be indirect but mutations in *CO* that result in late-flowering abolished an interaction with HAP2. Sequence analysis comparing HAP2 proteins and CCT-domain proteins revealed that both proteins share a common domain named NF-YA2. Mutations affecting highly conserved residues in this NF-YA2 domain result in loss-of-function both in CCT-domain and in HAP2 proteins. Testing several isoforms of HAP2, HAP3 and HAP5 in a systematic yeast-two-hybrid screen with the CCT-domains of CO and COL15 unraveled several positive interactions. These results indicate that a general function of the CCT-domain is to interact with the HAP complex. In addition, we found that overexpression of *FLAG:HAP3a* by the *35S*-promoter results in a delay of flowering that correlates with low levels of *FT* mRNA. Suggesting the FLAG-epitope creates a dominant-negative effect we can not explain whether CO becomes sequestered into non-flowering promoting complexes because of a disruption of the formation of the HAP complex or because the HAP-complex is not involved in the transcriptional regulation of *FT*. However, the flowering-time data suggest that CO and FLAG:HAP3a interact *in vivo*. Mutations affecting highly conserved residues in the domain of homology

result in late-flowering and cause a disruption of the interaction with HAP2, which suggests that HAP proteins are involved in flowering-time control.

Arabidopsis MtN19, a protein of unknown function, can interact with CO and with the promoter of *FT* unraveling a potential mechanism as of how CO interacts with DNA. It has been shown that *MtN19-like* genes are expressed in response to various stresses mainly involving the production of reactive oxygen species. Transgenic plants expressing a dsRNAi construct against *MtN19* show extreme flowering-time phenotypes. In some of the transgenic plants, the endogenous *MtN19* mRNA level is higher compared to wild-type and those plants flower extremely early. The late-flowering *MtN19* dsRNAi plants (*MtL*) can not be rescued by overexpression of *CO* suggesting that functional MtN19 protein is lacking in these lines. This strongly indicates that MtN19 is involved in the control of the floral transition and functions by interacting with CO and the promoter of *FT*.

Isolation of FIDGET, an APETALA2-like protein that interacts with the promoter of *FT*.

Using yeast-one-hybrid screening we isolated FIT, a protein that binds to the promoter of *FT*. FIT does not interact with CO indicating that it acts as a CO-independent regulator of *FT*. Misexpression of *FIT* in the phloem by the *SUC2* promoter, the place where *FT* is expressed, resulted in early flowering. Analysis of the *SUC2::FIT* transgene in various flowering-time mutants indicates that *FIT* requires an active photoperiod pathway to fulfill its function. This suggests that *FIT* acts independently of *CO* but requires active CO to upregulate *FT*. Furthermore, we demonstrate that UV-light can accelerate the floral transition and that FIT is induced in response to UV in the phloem probably triggering the expression of *FT*. Analysis of AP2-like proteins that delayed the floral transition when expressed in the phloem demonstrated that these proteins can interact with the *FT* promoter in yeast. This suggests the existence of a regulatory node in the *FT* promoter where repressors and activators can bind to modulate the expression of *FT*. Future research will focus on the determination of the exact AP2-binding site in the *FT* promoter. Moreover, cues that elicit the expression of these repressive *AP2-like* genes will be analyzed whether they negatively influence the transition to flowering.

Identification of proteins involved in the post-translational regulation of CO

In addition to the HAP complex, FIT and MtN19, I identified several other proteins that appear to be involved in the regulation of CO function. Here we describe that the degradation of CO most likely involves the E3-ubiquitin ligase COP1. We present evidence that both CO and COP1 co-localize in sub-nuclear speckles and we detected protein-protein interaction by FRET *in vivo* within these speckles. This sheds light on the degradation process which was previously unknown (sections 9.1 and 9.2). Another candidate that might be involved in the post-translational regulation of CO is a small ZZ-type zinc finger transcription factor named PHD. A close homolog of PHD is the ZZ-type zinc finger protein HRB1 that is involved in red and blue light signaling (Kang et al., 2005). Here we presented evidence that PHD exists in two splice variants and that CO interacts with PHD in yeast and *in vivo*. Furthermore, we have shown that the interaction appears only with the longer splice variant indicating that the interaction is via the carboxy terminal part of PHD. Overexpression of *PHD* resulted in elongation of the hypocotyl under red-light. PHD was also found to interact with GI which is involved in PHYB-mediated red-light signaling and *gi* mutants exhibit long hypocotyls in red-light. PHD is perhaps a negative regulator of red-light signaling. The ZZ-type zinc finger domain is closely related to the PHD-domain and RING-finger domain. It is known that RING-finger proteins can act as E3-ligases mediating the degradation of target proteins (Imaizumi et al., 2003; Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Imaizumi et al., 2005; Kepinski and Leyser, 2005). Since a close homolog of PHD is involved in red and blue signaling and the stability of CO protein is dependent on the light quality suggests that one of the functions of PHD might be modulating stabilization/degradation of CO (sections 9.6-9.10.).

It is known that CO protein can be phosphorylated and preliminary data suggest that the phosphorylated form of CO is the active form (Dr. Wim Soppe, unpublished). We isolated a nuclear PP2C phosphatase and have shown that CO and PP2C interact in yeast and *in vivo* (sections 9.3. and 9.4.). Transgenic plants expressing *35S::PP2C* do not show a flowering-time phenotype in our test conditions (data not shown). Comparing plants expressing *35S::PP2C 35S::CO* with plants expressing *35S::CO*, indicated that the double mutants flower later compared to plants expressing *35S::CO* (Section 9.5.). This could be a dosage effect since the F1-cross was analyzed. Future experiments are directed to unravel whether plants expressing *35S::PP2C 35S::CO* are later flowering and whether this correlates with the amount of non-phosphorylated CO protein. Furthermore, we aim to analyze the stability of the non-phosphorylated form of CO.

Summary of functions of identified proteins interacting with CO

We present evidence that a variety of proteins including proteins of so far unknown function interact with CO. Figure 56 shows the identified candidates organized into their putative functions.

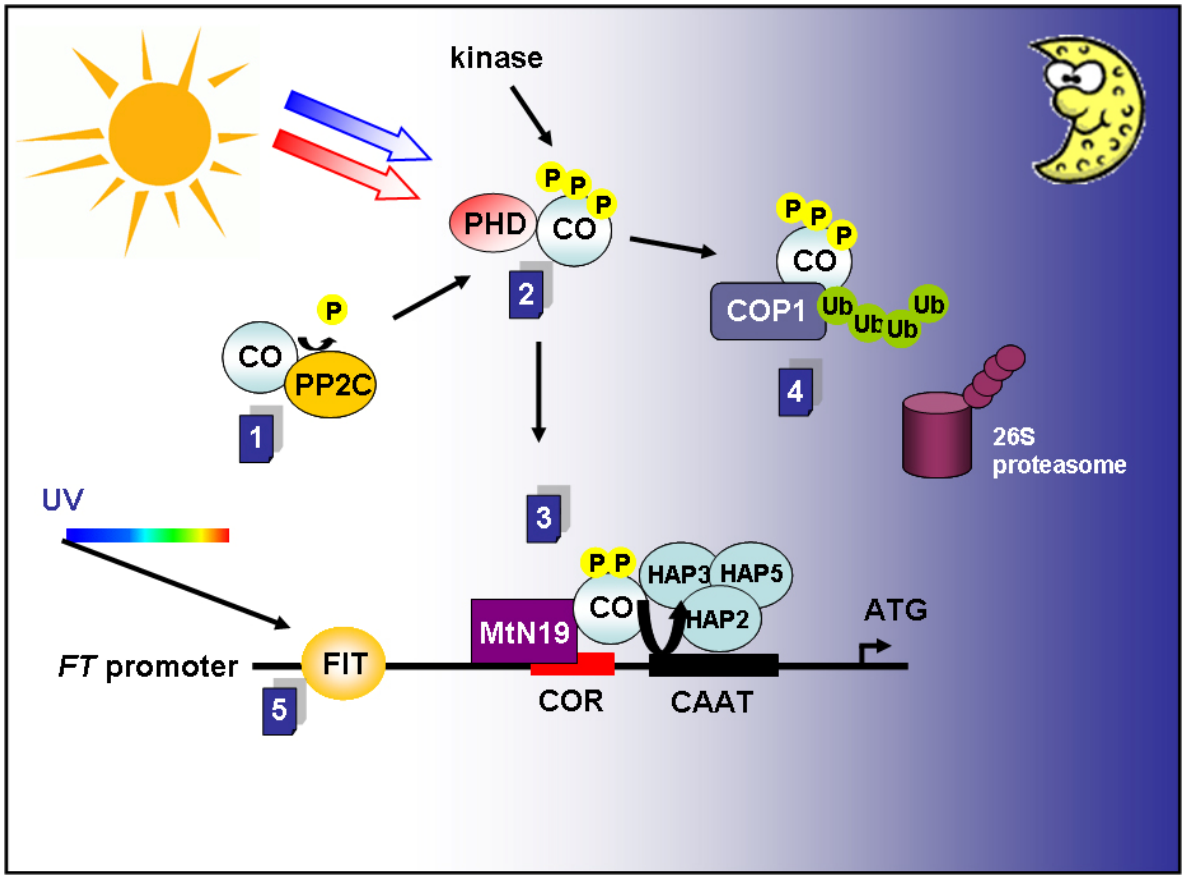

Figure 56. Schematic drawing of the identified proteins interacting with CO- and the *FT* promoter. COR: CO-responsive element; P: phosphate-group; Ub: ubiquitin.

The nuclear PP2C phosphatase interacts with CO probably to remove phosphate groups and maintains CO in an unphosphorylated state. This interaction is likely to take place in the early light phase keeping CO in an inactive state (Figure 56.1) since later in the day CO can be phosphorylated (Dr. Wim Soppe, unpublished). PHD might also interact with CO during the light phase, probably in response to red and/or blue light but the function of this interaction is unknown (Figure 56.2). CO can interact with MtN19 which is able to bind to the *FT* promoter. It is possible that CO binds together with MtN19 to a so far unidentified CO-response element in the *FT* promoter (Figure 56.3). In addition CO might interact via its CCT-domain with the heterotrimeric HAP complex and this interaction induces the expression of *FT* at the end of the light phase in LD. In the absence of light CO interacts with the E3-ubiquitin-ligase COP1 that initiates ubiquitination of CO followed by the degradation by the

26S-proteasome. Finally, FIT an AP2-like protein is able to induce the expression of *FT* by binding to its promoter in response to UV-light (Figure 56.5).

8. Literature

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9. Appendix

The results that are shown in this chapter were obtained by yeast-two-hybrid screening with domains of CO. In the case of COP1 the interaction was directly tested, since *cop1*-mutant plants show early flowering in LD and SD which might be due to a failure in the degradation of CO protein.

9.1. CONSTANS and COP1 co-localize in the nucleus in sub-nuclear speckles

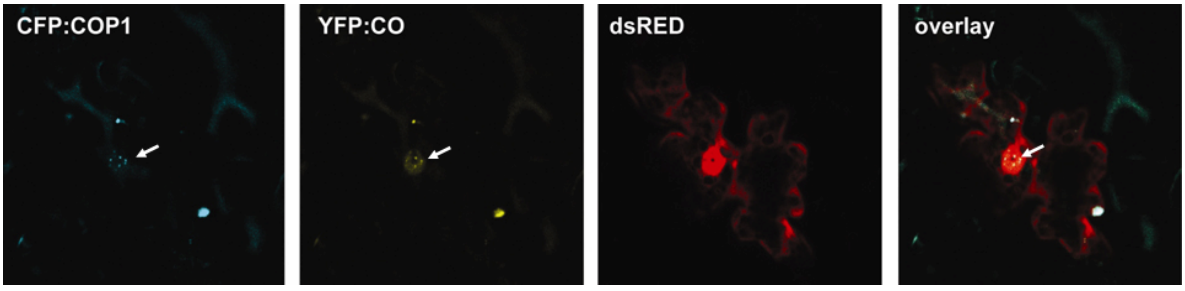

Figure 57. Co-localization of *35S::YFP:CO* and *35S::CFP:COP1 and 35S::dsRED.*

The bombardment revealed that both proteins co-localize in sub-nuclear speckles. *35S::dsRED* was co-transformed to highlight the transformed cell. CO and COP1 co-localize in big nuclear speckles, as indicated by the arrows. In addition, some accumulation of fluorescent CO and COP1 is found to be located at the plasma membrane. The function of these plasma-membrane located aggregations is unknown.

9.2. CONSTANS and COP1 interact *in vivo*

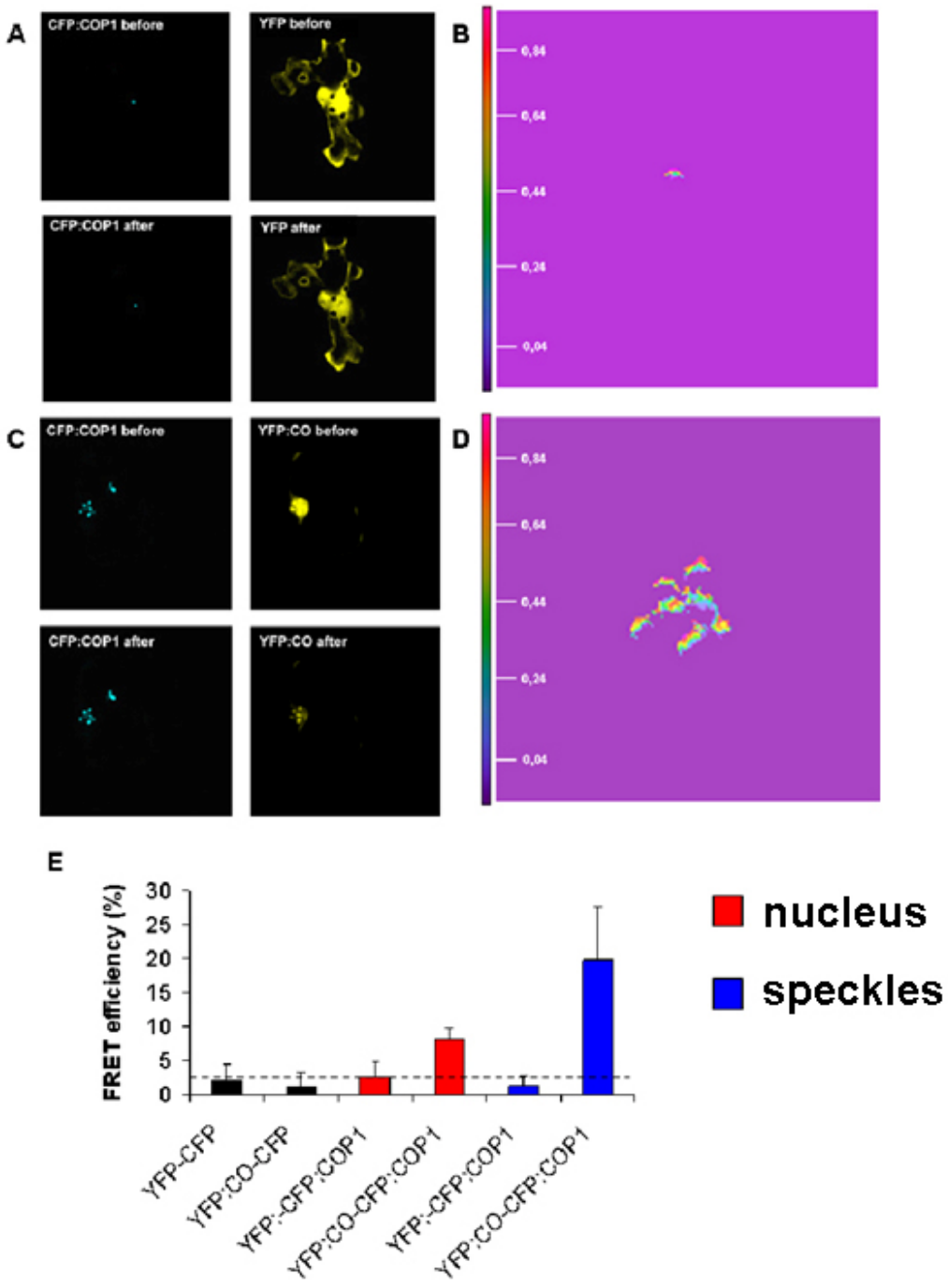

Figure 58. Acceptor-photobleaching experiment using *35S::YFP:CO* and *35S::CFP:COP1*. CFP:COP1 does not interact with YFP (A and B). YFP:CO interacts with CFP:COP1 as seen by an increase in CFP-fluorescence after YFP-photobleaching (C and D). Quantification of the FRET-efficiencies in the whole nucleus indicated a weak interaction but analysis of sub-nuclear speckles revealed a strong interaction between CO and COP1 (E).

9.3. CONSTANS and PP2C interact in yeast

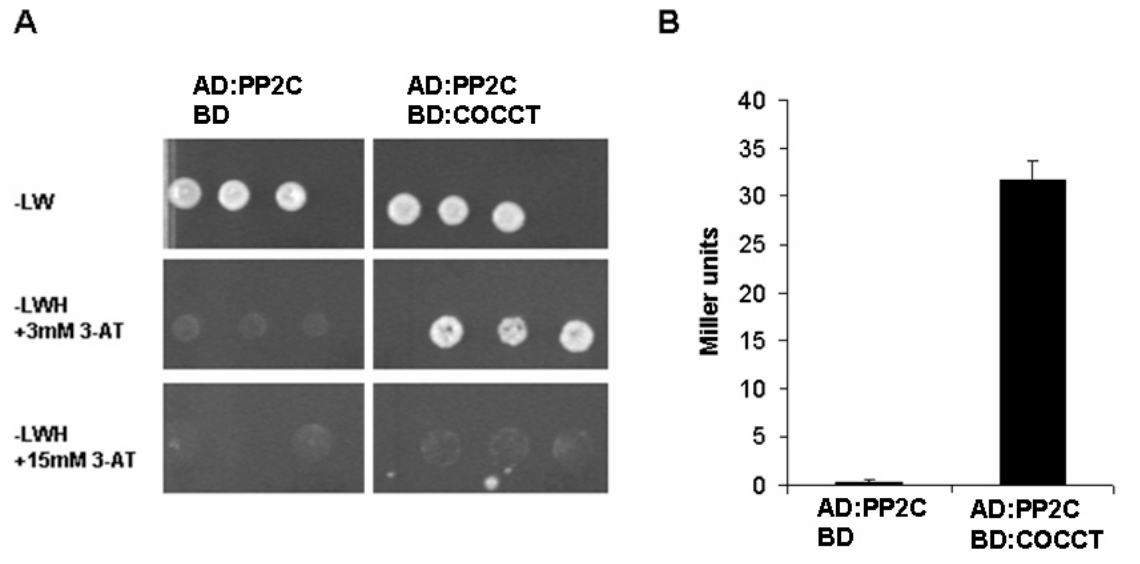

Figure 59. PP2C confirmation tests in yeast. A nuclear PP2C phosphatase was isolated by yeast-two-hybrid screening interacting with the CCT-domain of CO. A) selection of transformed yeast on HIS-lacking media in yeast strain AH109. The interaction between CO and PP2C is weak, because it is inhibited by 15mM 3-AT. B) Liquid β-Galactosidase assay (yeast strain MAV 203).

9.4. CONSTANS and PP2C interact *in vivo*

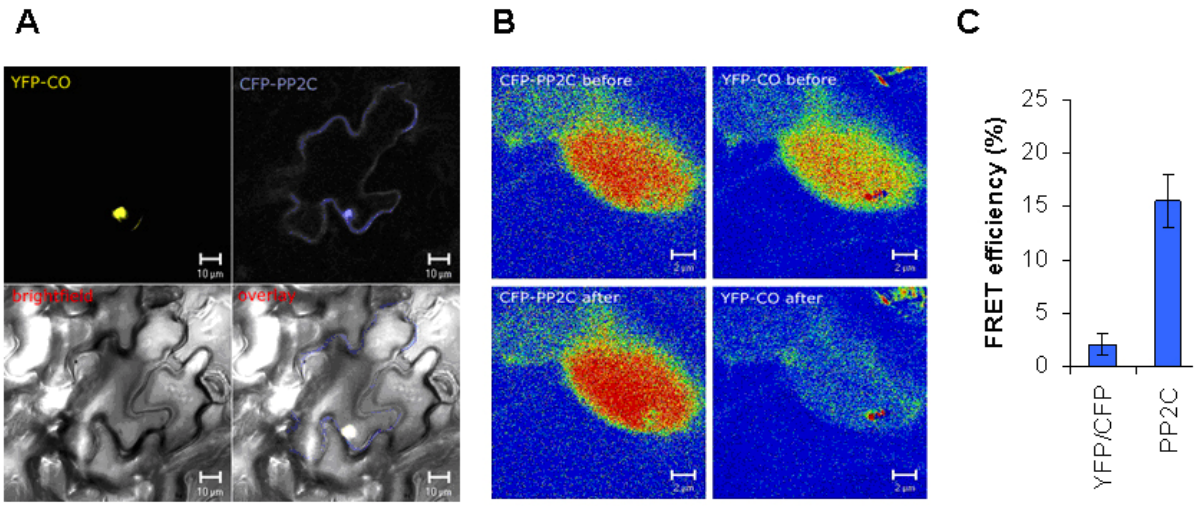

Figure 60. A) Co-bombardment of *35S::YFP-CO* and *35S::CFP-PP2C*. CO is clearly localized in the nucleus, whereas the PP2C protein is in the cytoplasm and in the nucleus. B) Acceptor photobleaching experiment to determine the FRET efficiency as a measure of the strength of the interaction. An increase of CFP-PP2C fluorescence after photobleaching was observed. C) FRET efficiencies of interactions of CO with the PP2C phosphatase.

9.5. Overexpression of *PP2C* by the *35S*-promoter slightly delays flowering of *35S::CO* plants

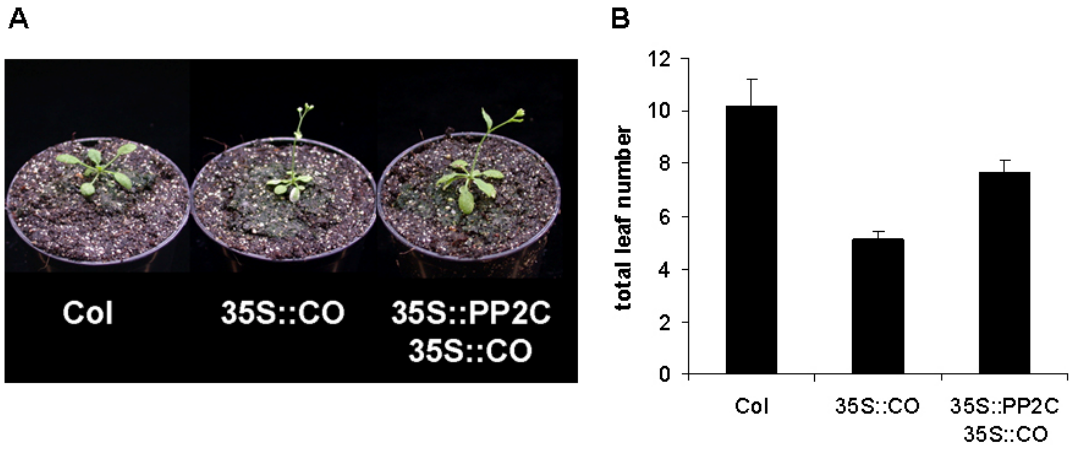

Figure 61. Crossing *35S::PP2C* to *35S::CO* double-heterozygote F1 plants show a small delay in the floral transition compared to homozygote 35S::CO plants (A and B).

Overexpression of *PP2C* by the *35S*-promoter does not significantly alter flowering time in long day conditions. This delay could be due to lower *CO* expression in F1-crosses because of heterozygosity of the transgene. Future experiments will reveal whether CO protein is less phosphorylated in these lines and whether this affects the floral transition or the degradation of CO.

9.6. CO and PHD interact in yeast

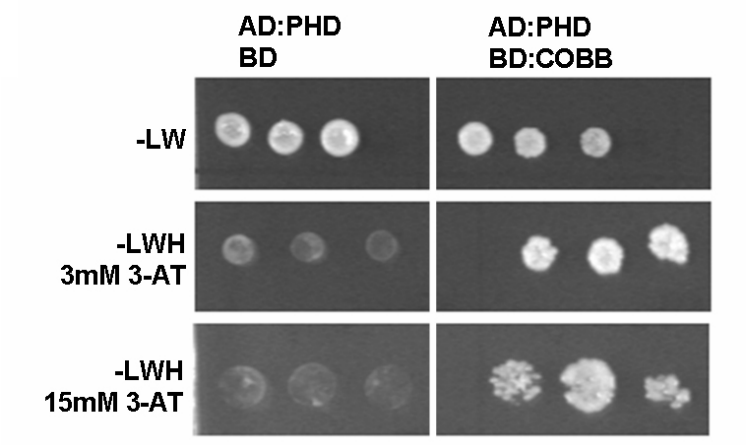

Figure 62. Reconfirmation of the CO-PHD-finger interaction tested in yeast. The left panel shows the control experiment testing the interaction of AD:PHD with the GAL4-binding domain (BD). The right panel shows the interaction of AD:PHD with BD:COBB. PHD interacts with the B-boxes of CONSTANS very strongly, up to 15mM 3-AT.

9.7. The PHD-finger exists in two isoforms, PHD and PHDS, which are produced by alternative splicing

Figure 63. Analysis of the amino acid sequences revealed that the isolated PHD-finger belongs to a small family of ZZ-domain proteins. BLAST search revealed that the PHD-finger exists in two splice variants.

```
          (1) 1          10         20         30         40         55
PHD       (1) ATGGACGCTGATTCCAAGAGATTTCTAGCTACGCTTCGATCCCGATCTGAAATGT
PHDS      (1) ATGGACGCTGATTCCAAGAGATTTCTAGCTACGCTTCGATCCCGATCTGAAATGT

         (56) 56         70         80         90         100        110
PHD      (56) TAATGGGCTTTGAAGAAATAGATGGAGATGATGATTTCCAGGAGGAGTTTGCTTG
PHDS     (56) TAATGGGCTTTGAAGAAATAGATGGAGATGATGATTTCCAGGAGGAGTTTGCTTG

        (111) 111        120        130        140        150        165
PHD     (111) CCCGTTCTGTGCAGAATCGTATGATATCATCGGGTTGTGCTGTCACATAGATGAT
PHDS    (111) CCCGTTCTGTGCAGAATCGTATGATATCATCGGGTTGTGCTGTCACATAGATGAT

        (166) 166        180        190        200        210        220
PHD     (166) GAACATACTTTAGAGTCGAAGAACGCGGTATGCCCTGTTTGTTCTCTTAAAGTGG
PHDS    (166) GAACATACTTTAGAGTCGAAGAACGCGGTATGCCCTGTTTGTTCTCTTAAAGTGG

        (221) 221        230        240        250        260        275
PHD     (221) GAGTTGATATCGTAGCACACAAACGCTTCACCATGGGA-----------------
PHDS    (221) GAGTTGATATCGTAGCACACAAACGCTTCACCATGGGAGTTTATTTAAGTTGCAG

        (276) 276        290        300        310        320        330
PHD     (259) CGAAAGAGAAAGTCACGAAAAAGCGGTACCAATTCGACACTTTCACTTCTTCGGA
PHDS    (276) CGAAAGAGAAAGTCACGAAAAAGCGGTACCAATTCGACACTTTCACTTCTTCGGA

        (331) 331        340        350        360        370        385
PHD     (314) AAGAACTAAGAGAAGGAGATCTACAAAGGTTGTTAGGATTTACTTCTCGTAATGG
PHDS    (331) AAGAACTAA-------------------------------------------------

        (386) 386        400        410        420        430        440
PHD     (369) TTCTGTTGCGTCAAGTGTAACTCCTGATCCTCTCCTGTCGTCATTTATCTCACCT
PHDS    (340) ------------------------------------------------------------

        (441) 441        450        460        470        480        495
PHD     (424) ACACGATCACAGAGTTCTCCTGCGCCACGCCAAACCAAAAACGTATCCGAGGATA
PHDS    (340) ------------------------------------------------------------

        (496) 496        510        520        530        540        550
PHD     (479) AACAGATAGAGCGCAAGAGACAAGTGTTCATCTCACCGGTCTCGTTAAAAGATCG
PHDS    (340) ------------------------------------------------------------

        (551) 551        560        570        580        590        605
PHD     (534) GGAAGAGAGGAGGCACAAATCAGAGTTTGTTCAGAGGCTCTTGTCATCAGCCATT
PHDS    (340) ------------------------------------------------------------

        (606) 606        620
PHD     (589) TTCGATGAAGTCTAA
PHDS    (340) ---------------
```

Figure 64. Nucleotide alignment of the small variant PHDS and PHD identifies mis-splicing at position 258 resulting in precocious termination.

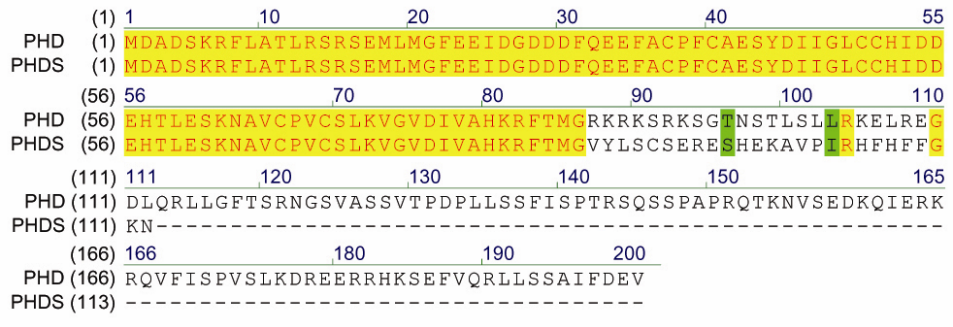

Figure 65. Amino acid alignment revealed that PHD and PHDS mainly differ in their carboxy terminus which is deleted in PHDS. Yellow: conserved residues; green: similar residues.

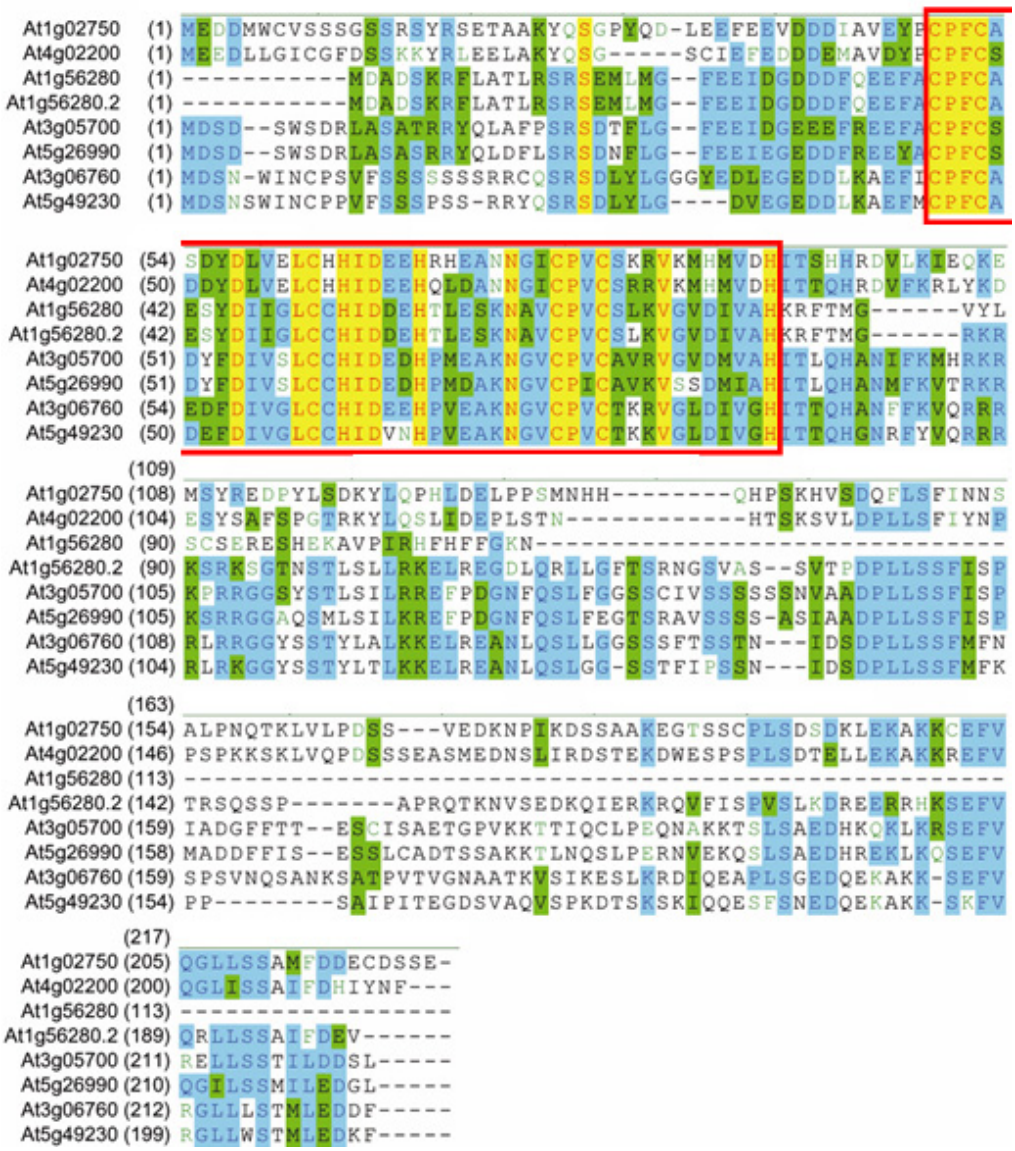

Figure 66. Analysis of the amino acid sequences of all ZZ-domain proteins encoded in the Arabidopsis genome revealed that both PHD and PHDS contain the ZZ-domain (red box). Furthermore the proteins differ in their carboxy terminus. Color code: Yellow: conserved residues; blue: identical residues; green: similar residues.

9.8. CO interacts with PHD in vivo but not with PHDS indicating that the interaction is via the carboxy terminus of PHD

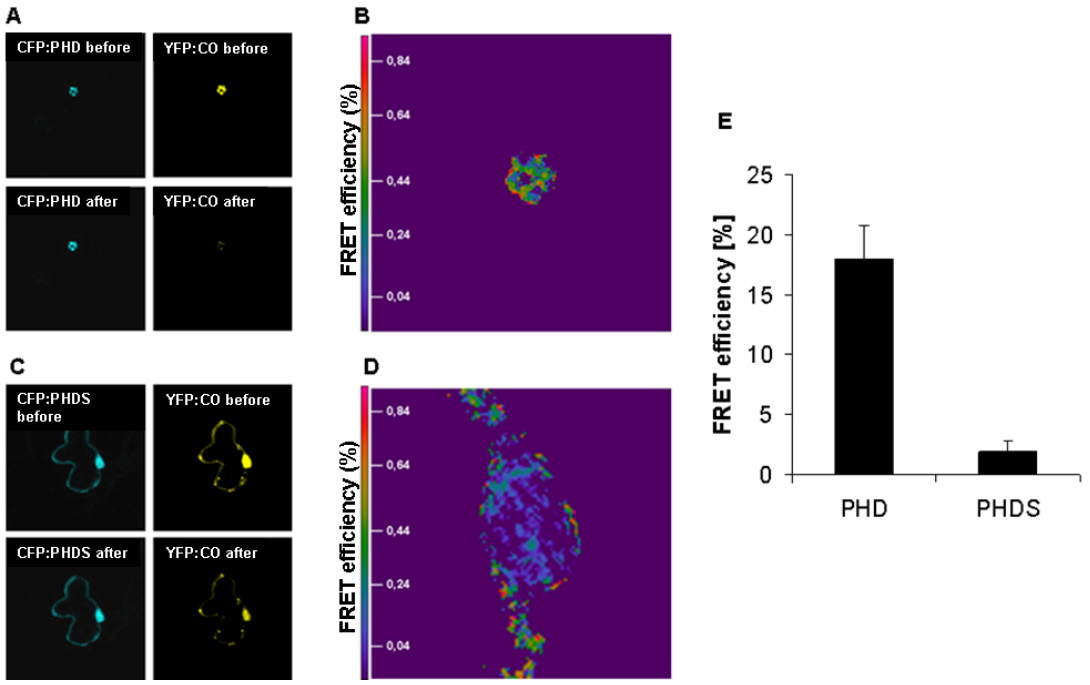

Figure 67. *In vivo* FRET analysis between CO and PHD and CO and PHDS. A+B) FRET analysis between PHD and CO. C+D) FRET analysis between PHDS and CO. B+D) FRET efficiency maps displayed in false color images. E) Quantification of FRET efficiencies acquired in different independent experiments.

Interestingly, CO only interacts with PHD and not with PHDS indicating that the interaction is with the carboxy terminus of PHD. The carboxy termini of the ZZ-protein family show more divergence than the amino termini, this suggests that the interaction of CO and PHD is specific.

9.9. PHD can mediate an interaction between CO and GI *in vivo*

PHD was also isolated as an interactor of GI (Hugo Konijn, unpublished). To answer the question whether CO and GI could be present in a higher order protein complex mediated by the small PHD-protein, in vivo FRET experiments were carried out.

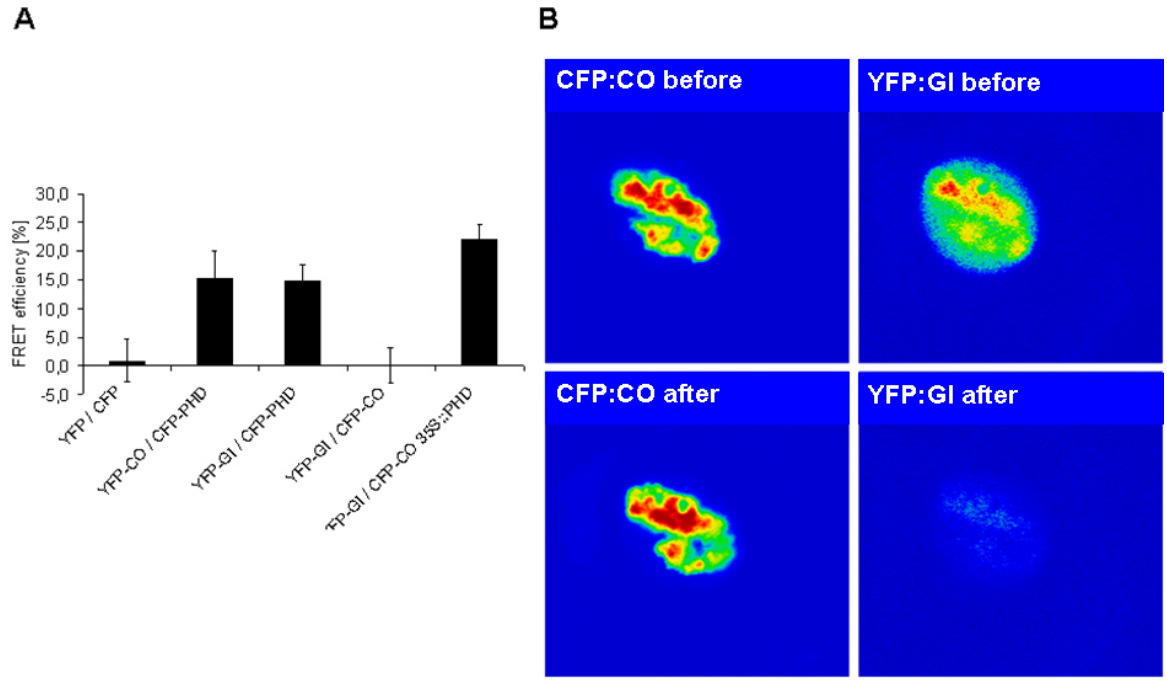

Figure 68. A) Quantification of several FRET experiments are shown. B) FRET experiment of a nucleus transformed with *35S::YFP:GI*, *35S::CFP:CO* and *35S::PHD*.

FRET analysis of the PHD-finger protein PHD with CO and GI revealed that PHD is able to interact both with CO and GI. No FRET was observed between CO and GI indicating that these two proteins can not directly interact with each other. The co-transformation of *35S::PHD* revealed indirect FRET signals between CO and GI. These experiments indicate that PHD can function as a molecular linker mediating an interaction between CO and GI. The physiological consequence of this interaction is unknown.

9.10. Overexpression of *PHD* causes hypocotyl-elongation in red light conditions

The effect of different wavelengths of light on the elongation of the hypocotyl was analyzed. Since GI acts in the PHYB pathway and also shows a hypocotyl phenotype in red light we analyzed whether transgenic PHD-lines show a phenotype in these conditions. Furthermore, from one member of the ZZ-domain family, HRB1, it is known that it functions in red- and blue-light signaling (Kang et al., 2005). Since GI has a function in red light and its known that CO protein is degraded in red light we analyzed what effect this putative interactor has. No effect was observed in blue, far-red and white light.

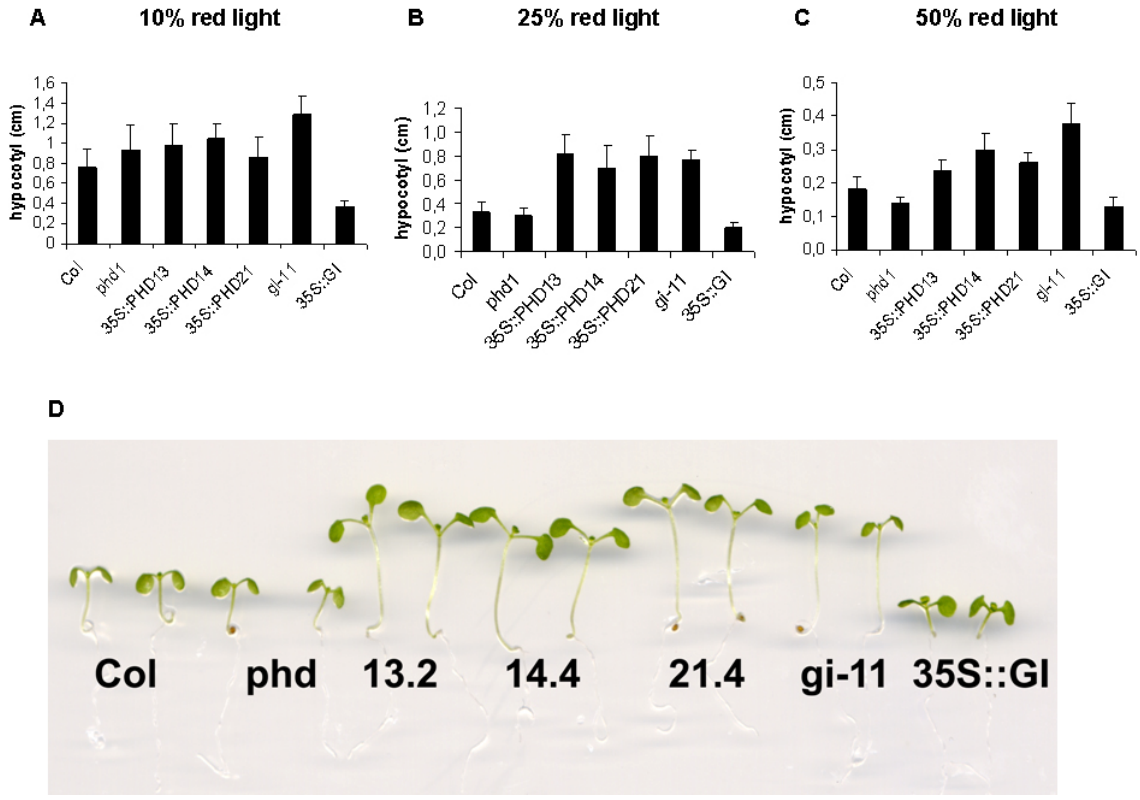

Figure 69. Hypocotyl length measurements of different mutants A)-C) Quantification of hypocotyl lengths (in collaboration with Hugo Konijn).

Analysis of plants overexpressing *PHD* revealed that these lines have an elongated hypocotyl when grown under red light. This phenotype is similar to the *gi*-mutant. However, a knock out of *PHD*, *phd*, does not show a phenotype (experiment in cooperation with Hugo Konijn).

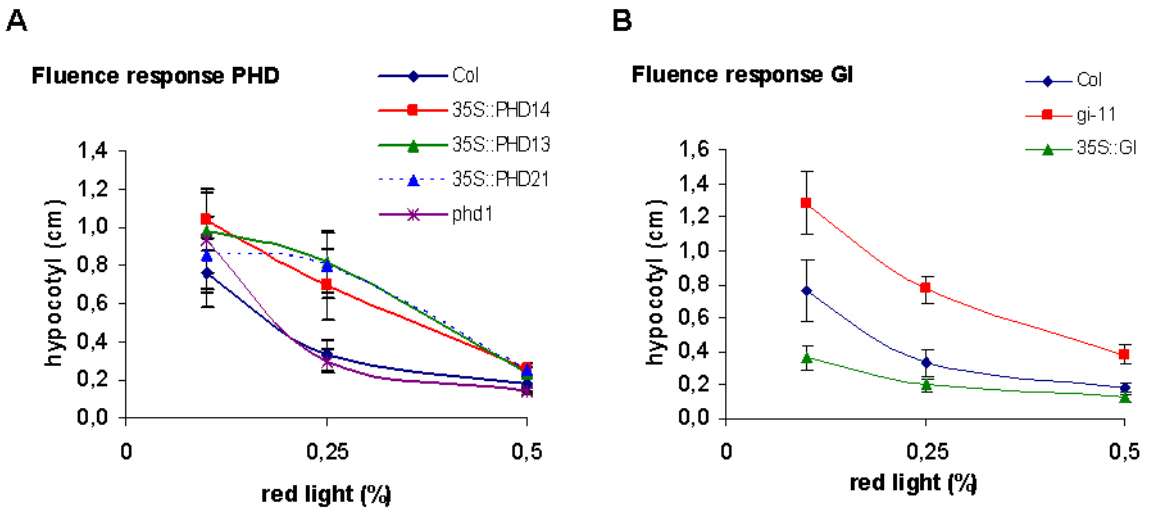

Figure 70. Analysis of red-light responses depicted as fluence response curves. PHD shows the strongest effect in 25% red light (A). The effects of GI in the same light conditions (B) (together with Hugo Konijn).

Apart from this phenotype *PHD* transgenic plants did not exhibit any flowering time phenotype in the conditions analyzed so far. Recent experiments focus on the analysis of double and triple mutants (crosses to *gi, co, phyB, phyA, 35S::CO, 35S::GI*) in light signaling and flowering time control. Furthermore the stability of the PHD protein in those conditions will be analyzed using a PHD-specific antibody.

9.11. Overexpression of *HAP3a* causes late-flowering and this correlates with a reduction in *FT* transcript levels

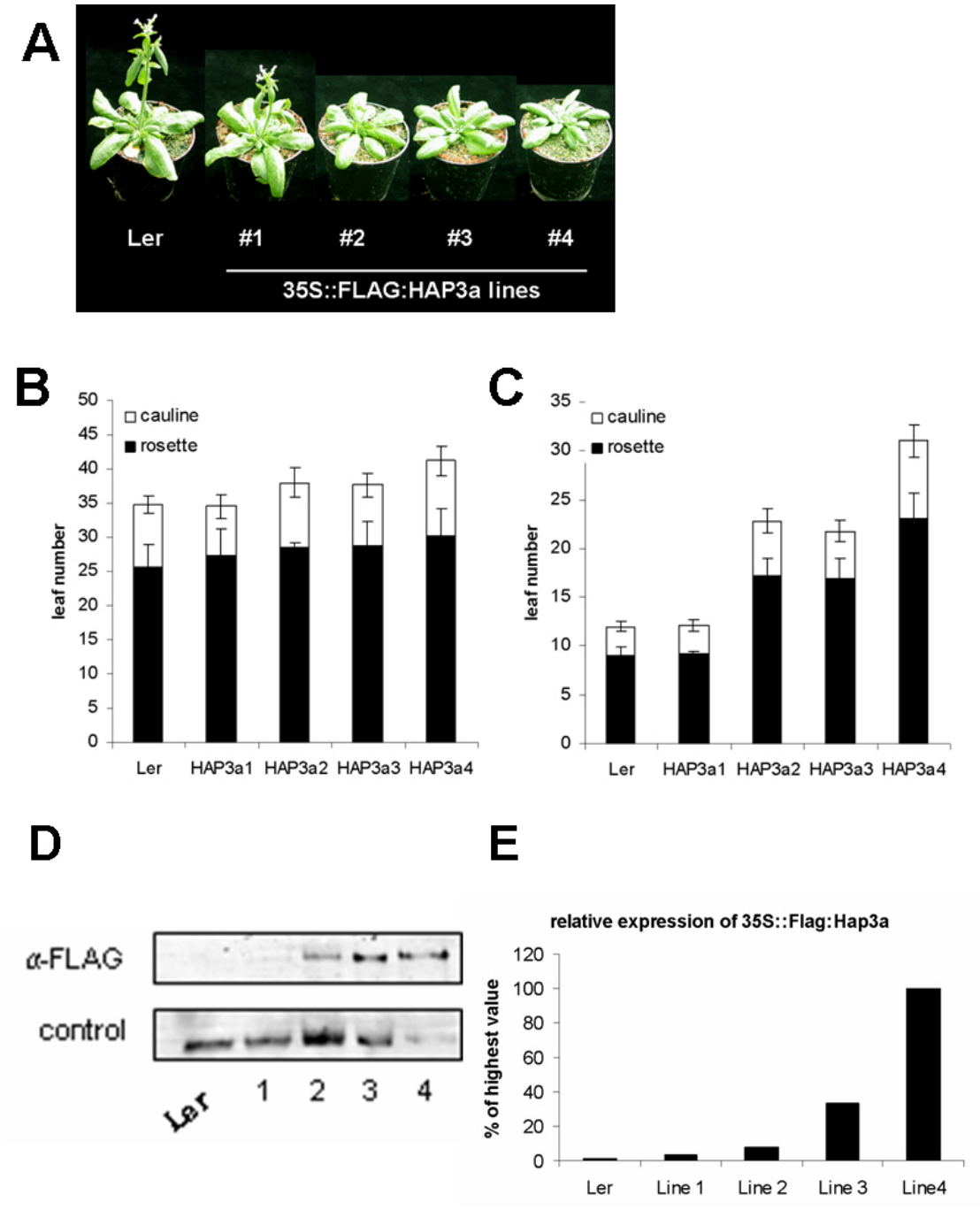

Figure 71. A) Flowering-time phenotypes of four independent homozygote *35S::FLAG:HAP3a* transgenic plants in comparison to wild-type grown in LD. B) Quantification of flowering-time by counting the numbers of leaves produced at bolting in LD. C) Flowering-time in SD. D) Western blot using α-FLAG antibody. Lane labeled with 1-4 show protein extracts from transgenic *35S::FLAG:HAP3a* lines. The late flowering phenotype correlates with FLAG:HAP3a protein levels. E) Quantification of protein levels compared to the control lane (background detected by the secondary antibody). (Figure A-E from Dr. Franziska Turck).

9.12. CO partially suppresses the late flowering phenotype of *35S::FLAG:HAP3a*

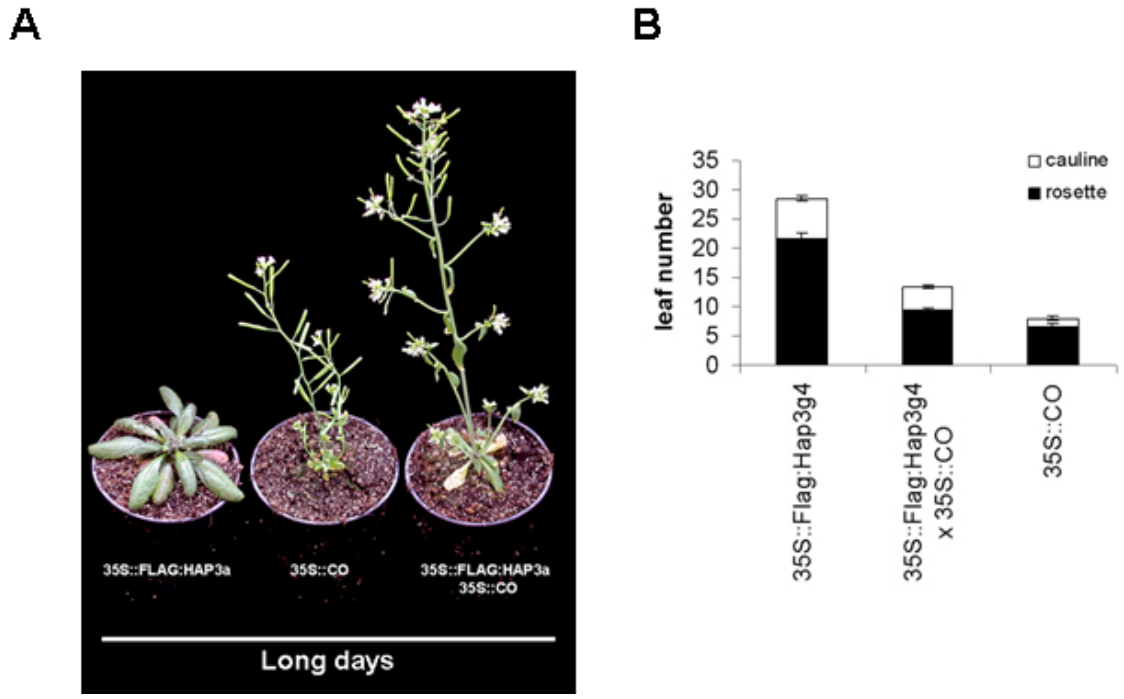

Figure 72. A) Flowering-time analysis in LD comparing *35S::FLAG:HAP3a*, *35S::CO* and *35S::FLAG:HAP3a 35S::CO*. B) Quantification of flowering-time by counting the numbers of leaves produced at bolting shows that overexpression in the late-flowering *35S::FLAG:HAP3a* lines can be partially complemented by *CO*. (Data from Dr. F. Turck)

Erklaerung

"Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. George Coupland betreut worden."

Köln, den Dezember 2005

Teilpublikationen

Stephan Wenkel, Franziska Turck, Jose Gentilhomme and George Coupland
Physical interaction and sequence similarities between CONSTANS and the CCAAT-binding complex (manuscript in preparation).

Lebenslauf

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