



Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

Control of floral meristem activity in the model plant *Arabidopsis thaliana*

Kamila Kwasniewska



**Trinity
College
Dublin**

The University of Dublin

Thesis submitted to the University of Dublin for the degree of
Doctor of Philosophy

Thesis supervisor: Prof. Frank Wellmer

Smurfit Institute of Genetics

Trinity College Dublin

Dublin, Ireland

2017



Thesis 11480

Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other University and it entirely my own work.

I agree to deposit this thesis in the University's open access institutional repository or allow the Library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgment.

Summary

Over the past 30 years, the flower development has been one of the main model to study the genetic control of organogenesis in higher plants. The work on *Arabidopsis thaliana* has led to the proposal of the ABC model of flower development. This model states the interaction of three classes of the genes that encode transcription factors specifying the four types of organs in the flower, *i.e.*, sepals, petals, stamens and carpels. While the activities of these master regulators have been extensively characterized in recent years, comparatively little is known about the functions of the target genes of these regulators.

One of the genes that is acting downstream of AGAMOUS (AG) – the transcription factor that specifies the formation of a carpel, is *KNUCKLES* (*KNU*). It has been reported that *KNU* is directly activated by AG and is believed to be a repressor of *WUSCHEL* (*WUS*) activity in the meristematic cells. To fully understand functions of *KNU* in the stem cells, several transgenic lines were generated. I used approaches allowing for knockdown of *KNU* functions in different regions of stem cells. I showed, that *KNU* is expressed earlier in the flower development and interacts with the gene regulating the size of meristem.

Although, the functions that *KNU* plays in flower development were described over a decade ago, its additional roles have not been proposed. The genome-wide binding data of *KNU* and the gene expression changes upon perturbation of *KNU* functions are still missing. To address this knowledge gap, the main focus of this thesis was on the characterization of the role of *KNUCKLES* (*KNU*) on a genome-wide scale. Using genomic technologies, I showed that *KNU* directly regulates sets of genes that act in the meristem development and maintenance. I also presented evidence that *KNU* regulates key factors that suppress or activate the trichome formation. I further showed that *KNU* is involved in regulation of the hormone metabolism and balancing their levels.

In the second part of this thesis, the dynamic distribution of epigenetic marks during early stages of flower development was investigated. The epigenetics is often defined as changes that are not caused by the modification in the DNA but instead by the DNA methylation or post-translational modification of the histones. These

changes have a substantial role in the processes that are involved in the formation and development of a flower. To better understand the mechanisms underlying the successful flower development, I used genome-wide approaches allowing for identification of interactions between the flower master regulator APETALA1 (AP1) and the epigenome. For that, the specific gene regions that are repressed or activated by the selected methylation marks were analyzed at different stages of flower development that is triggered by AP1. This work yielded in preliminary characterization of the interplay between the transcriptional regulation and the epigenetic mechanisms during the flower formation.

Acknowledgments

I would like to thank Prof. Frank Wellmer for all his guidance, advice and patience over the course of my PhD. I would like to thank to Dr. Emmanuelle Graciet, who helped me through the toughest times in the laboratory, while introducing me to the laboratory environment and discussing technical problems.

I would also like to thank current and former fellow lab members that worked with me over the past 6 years. To Darragh and Bennett for the laughter we had in the lab and the encouragement, especially over the last year. To Beibei and Pat for the help with the data analysis. To Andrea, Kevin, Liina and Sam for much appreciated scientific advice and great time in the lab. I would like to thank to Diarmuid, who was a real team leader and always took the time to answer the toughest questions. I would especially like to thank to Kasia, for all the meaningful conversations we had over the lunch times and for keeping me grounded and sane over the years.

I would like to thank the Amazing Climbing Group for keeping me busy outside of the lab, for all the support and great time during climbing. To Kasia, Jelle, Andy, Elisa and Tobi, Joe and Nico for teaching me how to reach the highest peaks.

I would also like to thank to all of my friends and especially Gosia, Madzia and Piotrek that supported me and listened even when I talked too much about science.

My special thanks and words of love go to my dearest Mom, who gave up her dreams to raise and educate her daughters. I am grateful for her support from overseas and believing in me, whatever I do.

I would like to thank to the most important person in my life, my beloved husband, Minde. I am grateful for his great patience and kindness and for putting up with me over the times of distress and when I doubted myself. Without him I would not be able to achieve my dreams.

Publications

Goslin K , Zheng B, Serrano-Mislata A, Rae L, Ryan PT, **Kwaśniewska K**, Thomson B, Ó'Maoiléidigh DM, Madueño F, Wellmer F and Graciet E. (2017) Regulatory interplay between LEAFY and APETALA1/CAULIFLOWER during floral initiation. Plant Physiology 174(2), 1097-1109

de Marchi R, Sorel M, Mooney B, Fudal I, Goslin K, **Kwaśniewska K**, Ryan PT, Pfalz M, Kroymann J, Pollmann S, Feechan A, Wellmer F, Rivas S, Graciet E. (2016) The N-end rule pathway regulates pathogen responses in plants. Scientific Reports 13;6:26020

Ryan PT, Ó'Maoiléidigh DS, Drost HG, **Kwaśniewska K**, Gabel A, Grosse I, Graciet E, Quint M, Wellmer F. (2015) Patterns of gene expression during Arabidopsis flower development from the time of initiation to maturation. BMC Genomics 1;16:488

Ó'Maoiléidigh DS, Thomson B, Raganelli A, Wuest SE, Ryan PT, **Kwaśniewska K**, Carles CC, Graciet E, Wellmer F. (2015) Gene network analysis of Arabidopsis thaliana flower development through dynamic gene perturbations. Plant Journal 83(2):344-58

Ó'Maoiléidigh DS, Wuest SE, Rae L, Raganelli A, Ryan PT, **Kwasniewska K**, Das P, Lohan AJ, Loftus B, Graciet E, Wellmer F. (2013) Control of reproductive floral organ identity specification in Arabidopsis by the C function regulator AGAMOUS. Plant Cell 25(7):2482-503

Wuest SE, O'Maoileidigh DS, Rae L, **Kwasniewska K**, Raganelli A, Hanczaryk K, Lohan AJ, Loftus B, Graciet E, Wellmer F. (2012) Molecular basis for the specification of floral organs by APETALA3 and PISTILLATA. Proceedings of the National Academy of Sciences of U S A 14; 109(33):13452-7

Abbreviations

35Spro	Cauliflower Mosaic Virus 35S promoter
AG	AGAMOUS
amiRNA	artificial microRNA
AP1	APETALA 1
AP2	APETALA 2
AP3	APETALA3
ASH1	absent, small or homeotic 1 protein
bHLH	basic Helix-Loop-Helix
BLAST	Basic Local Alignment Search Tool
bp	base pair
C2H2	Cys2His2
CAL	CAULIFLOWER
cDNA	complementary deoxyribonucleic acid
CDS	coding DNA sequence
ChIP	chromatin immunoprecipitation
CLF	CURLY LEAF
Cp software	Crossing-point, a value calculated by the Lightcycler480
CpG	5'-cytosine-phosphate-guanine-3'
DEG	differentially expresses genes
DNA	deoxyribonucleic acid
EAR	ethylene responsive element binding factor associated amphiphilic repression
EtOH	ethanol
FD	FLOWERING LOCUS D
FIS	floral induction system
FLC	FLOWERING LOCUS C
GFP	green fluorescence protein
GO	Gene Ontology
GR	Glucocorticoid Receptor
GUS	β-glucoronidase

h	hours
H2A	histone type 2-A
H2B	histone type 2-B
H3	histone type 3
H3K4me3	histone 3 lysine 4 trimethylation
H3K9me3	histone 3 lysine 9 trimethylation
H3K27me3	histone 3 lysine 27 trimethylation
H3K36me3	histone 3 lysine 36 trimethylation
H4	histone type 4
HAT	histone acetyltransferases
HAD	histone deacetylase
HDMs	histone demethylases
HKMTs	histone lysine methyltransferases
JmjC	Jumonji C domain
kb	kilobase
kDa	kilodalton
KNU	KNUCKLES
KYP	KRYPTONITE
<i>L-er</i>	Landsberg <i>erecta</i>
LFY	LEAFY
LSD1	lysine-specific demethylase
mRNA	messenger ribonucleic acid
ORF	open reading frame
PHD	plant homeodomain finger
PI	PISTILLATA
primer	oligonucleotide primer
RAM	root apical meristem
REF6	RELATIVE EARLY FLOWERING 6
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RT-PCR	reverse transcription polymerase chain reaction
SAM	shoot apical meristem
SEP	SEPALLATA
SUP	SUPERMAN

T1	the first progeny that is derived from a transformed plant
T-DNA	transfer DNA
TAIR	The Arabidopsis Information Resource
TBA	tetr-butyl alcohol
TF	transcription factor
TRX	trithorax protein
UFO	UNUSUAL FLORAL ORGANS
ULT1	ULTRAPETALA 1
UTR	untranslated region
V	volts
w/v	weight/volume
ZFP	ZINC FINGER PROTEIN

Table of Contents

CHAPTER 1 – GENE REGULATORY NETWORKS UNDERLYING FLOWER DEVELOPMENT IN *ARABIDOPSIS THALIANA* 5

1.1 Introduction 5

1.1.1 The evolutionary origin of flowers6

1.1.2 Initiation of flower formation8

1.2 The ABC model of floral organ identity specification 10

1.2.1 Floral organ identity factors and the quartet model14

1.2.2 Identification of genes acting downstream of floral organ identity factors15

1.3 Role of C2H2-type zinc finger transcription factors in flower development 18

1.3.1 Function of KNUCKLES in flower development20

1.4 Strategies for analyzing the gene network controlling flower development 24

1.4.1 Perturbation of floral regulators24

1.4.2 A system for the synchronization of flower development27

1.4.3. Gene expression profiling to identify downstream targets of floral regulators28

1.4.4 Genome-wide localization studies of floral regulators28

1.5 Aims of the project..... 29

CHAPTER 2 – MATERIALS AND METHODS 32

2.1 Strains and growth conditions 32

2.1.1 Plant lines32

2.1.2 Bacterial strains32

2.1.3 Plant growth media.....33

2.1.4 Bacterial growth media33

2.2 Cloning and genotyping 33

2.2.1 Plasmid DNA preparation33

2.2.2 cDNA synthesis.....33

2.2.3 Preparation and transformation of competent *Escherichia coli*33

2.2.4 Preparation and transformation of competent *Agrobacterium tumefaciens*34

2.2.5 Quick *Arabidopsis* genomic DNA preparation34

2.3 Plant transformation and selection of transformants 34

2.3.1 Plant transformation and seed sterilization34

2.3.2 Selection of transgenic plants35

2.4 Crosses and isolation of higher order mutants 35

2.4.1 Genotyping assays for mutant alleles.....35

2.5 Microscopy techniques 36

2.5.1 Light microscopy36

2.5.2 Epifluorescence microscopy.....36

2.6 GUS staining	36
2.7 Tissue fixation and chloralhydrate clearings.....	37
2.8 Treatments of lines encoding inducible promoters	37
2.8.1 Ethanol treatment of plants.....	37
2.8.2 Treatment of plants with a dexamethasone-containing solution	37
2.8.3 Treatment of plants with a dihydrotestosterone-containing solution	38
2.8.4 Collection of floral tissue	38
2.9 Generation and cloning of artificial miRNAs	38
2.10 Quantitative real-time PCR.....	39
2.11 Western blotting	40
2.12 Chromatin immunoprecipitation.....	40
2.12.1 Crosslinking of plant tissue	40
2.12.2 Chromatin extraction from plant tissue and chromatin shearing.....	41
2.12.3 Immunoprecipitation	42
2.12.4 Elution, de-crosslinking and DNA isolation.....	42
2.12.5 Quantification of genomic DNA.....	43
2.12.6 Diagenode ChIP-seq library preparation	43
2.13 Microarray experiments	43
2.13.1 Sample preparation.....	43
2.13.2 Hybridization.....	44
2.13.3 Washes and slide scanning	44
2.14 Data analysis and bioinformatics.....	44
2.14.1 Microarray data processing and Gene Ontology analysis.....	44
2.14.2 Read alignment, quality control and general data handling for ChIP-seq.....	45
2.15 Generation of transgenic lines	46
2.15.1 Generation and expression of an amiRNA that targets the <i>KNU</i> mRNA.....	46
2.15.2 Crossing the 35S _{pro} : <i>KNU</i> -amiRNA line into the floral induction system and isolation of homozygous line.....	47
2.15.3 Generation of plants expressing the <i>KNU</i> -amiRNA in specific meristematic domains	47
2.15.4 Over-expression of the <i>KNU</i> coding sequence	48
2.15.5 Generation of and epitope-tagged over-expression line of <i>KNU</i>	48
2.15.6 Generation of a line allowing for inducible over-expression of <i>KNU</i>	49
2.15.7 Generation of an epitope-tagged amiRNA-resistant version of <i>KNU</i>	49
2.15.8 Crossing <i>KNU</i> _{pro} :r <i>KNU</i> -GFP, 35S _{pro} : <i>KNU</i> amiRNA into the FIS.....	50
2.15.9 Isolation of AlcA _{pro} : <i>KNU</i> -amiRNA1 AP1 _{pro} :AP1-GR <i>ap1-1 cal-1</i> plants	50
2.15.10 Cloning of a construct for over-expression of <i>KNU</i> -VP16	51
CHAPTER 3 – IDENTIFICATION OF FUNCTIONS PLAYED BY KNUCKLES IN THE MERISTEMATIC CELLS OF THE FLOWER.....	52
3.1 Introduction	52
3.1.1 Organization of the shoot apical meristem	52
3.1.2 Regulation of meristem maintenance	53

3.1.3 CLV signal transduction	55
3.1.4 CLV receptor localization and interactions	56
3.1.5 Regulation of floral stem cell termination	58
3.1.6 Epigenetic mechanisms that control meristem regulation	62
3.2 Results	64
3.2.1 Perturbation of <i>KNU</i> function	64
3.2.2 Identification of an amiRNA that perturbs <i>KNU</i> activity	64
3.2.3 Effects of <i>KNU</i> perturbation in different domains of the floral meristem	70
3.2.3.1 Expression of the KNU-amiRNA in the <i>CLV1</i> domain	71
3.2.3.2 Expression of the KNU-amiRNA1 in the <i>WUS</i> domain	72
3.2.3.3 Expression of the KNU-amiRNA in <i>CLV3</i> domain	74
3.2.4 Validation of the meristem-specific LhG4 driver lines	76
3.2.5 Analysis of <i>KNU</i> expression in lines expressing KNU-amiRNA1 in specific domains of the floral meristem	79
3.2.6 Genetic interactions between <i>KNU</i> and <i>CLV3</i>	84
3.3 Discussion	88
3.3.1 Identification of a functional amiRNA against <i>KNU</i>	88
3.3.2 Effects of KNU-amiRNA expression in the <i>CLV1</i> , <i>CLV3</i> and <i>WUS</i> domains	89
3.3.3 Genetic interactions between <i>KNU</i> and <i>CLV3</i>	91
CHAPTER 4 - CHARACTERIZATION OF THE TRANSCRIPTIONAL NETWORK CONTROLLED BY KNUCKLES	94
4.1 Introduction.....	94
4.2 Identification of genes acting downstream of C2H2 zinc finger transcription factors.....	94
4.3 Results	96
4.3.1 amiRNA-mediated perturbation of <i>KNU</i>	96
4.3.1.1 Characterization of an ethanol-inducible KNU-amiRNA line	96
4.3.1.2 Characterization of a dexamethasone-inducible KNU-amiRNA1 line	98
4.3.2 Generation of an inducible <i>KNU</i> over-expressing line	103
4.3.2.1 Characterization of a dexamethasone-inducible <i>KNU</i> over-expression line	103
4.3.3 Characterization of a line ectopically expressing KNU-GFP	106
4.3.4 Testing the activity of the EAR-like domain in KNU	111
4.3.4.1 Characterization of plants expressing a KNU-VP16 fusion protein	113
4.3.4.2 Characterization of plants expressing a KNUmEAR-VP16 fusion protein	114
4.3.5 Translational fusion of an mGFP5 reporter to KNU	118
4.3.6 Characterization of plants containing 35S _{pro} :KNU-amiRNA and KNU _{pro} :mKNU-mGFP in an AP1 _{pro} :AP1-GR <i>ap1 cal</i> background.....	120
4.3.7 Global identification of genomic regions bound by KNU	122
4.3.7.1 Generation of ChIP-seq libraries	122
4.3.7.2 Quality assessment of ChIP-seq data	124
4.3.7.3 Detection of KNU binding sites in the <i>Arabidopsis</i> genome	127
4.3.8 Genes acting downstream of KNU	133
4.3.8.1 Mutations in <i>CRABS CLAW</i> enhance the over-proliferation of ectopic tissue in <i>knu-1</i>	133

4.3.8.2 <i>knu-1</i> partially rescues the supernumerary stamen phenotype of <i>sup-5</i>	135
4.3.8.3 Gynoecia of <i>knu-1</i> flowers are sensitive to exogenous cytokinin treatment	136
4.3.9 Identification of genes acting downstream of KNU through inducible gene perturbation.....	138
4.3.10 Comparison of ChIP-seq and microarray data.....	145
4.4 Discussion	147
4.4.1 Identification of an amiRNA that efficiently targets <i>KNU</i>	148
4.4.2 Inducible and constitutive over-expression of <i>KNU</i> resulted in novel phenotypes	149
4.4.3 An activated form of KNU influences floral architecture	150
4.4.4 Functionality of the mKNU-GFP fusion protein.....	151
4.4.5 Genomic regions bound by KNU	152
4.4.6 Genes acting downstream of KNU.....	153
4.4.7 Future work	154
CHAPTER 5 - DYNAMIC DISTRIBUTION OF EPIGENETIC MARKS DURING EARLY FLOWER DEVELOPMENT	155
5.1 Introduction	155
5.1.1 Histone methylation in plants	155
5.1.1.1 H3K9 methylation is linked to DNA methylation	157
5.1.1.2 Role of H3K27 methylation in plant development.....	158
<i>The FIS-containing PRC2 complex</i>	160
<i>The EMF2-containing PRC2 complex</i>	160
<i>The VRN-containing PRC2 complex</i>	161
5.1.1.3 H3K4 methylation and Trithorax Group Proteins	162
5.1.1.4 H3K36 methylation and ASH-related Group proteins	164
5.1.2 Histone acetylation in plants	165
5.2 Aim of the project.....	167
5.3 Results	167
5.3.1 Genome-wide H3K27me3 distribution at early stages of flower development	167
5.3.1.1 Generation of ChIP-seq libraries from chromatin associated with repressive H3K27me3 marks	169
5.3.1.2 Quality assessment of data from the H3K27me3 ChIP-seq experiments.....	171
5.3.1.3 H3K27me3 distribution in the <i>Arabidopsis</i> genome	173
5.3.2 Genome-wide distribution of activating H3K4me3 marks at early stages of flower development.....	178
5.3.2.1 Generation of ChIP-seq libraries from chromatin associated with H3K4me3	178
5.3.2.2 Quality assessment of data from the H3K4me3 ChIP-seq experiment	180
5.3.2.3 H3K4me3 distribution in the <i>Arabidopsis</i> genome	182
5.4 Discussion and future work.....	184
REFERENCES	184
Appendix 1	211
Appendix 2	214

CHAPTER 1 – GENE REGULATORY NETWORKS UNDERLYING FLOWER DEVELOPMENT IN *ARABIDOPSIS THALIANA*

1.1 Introduction

Over the past 30 years, our understanding of the genetic mechanisms that control plant development has made rapid advances. This progress was made possible largely through the invention of new experimental methods, ranging from advanced imaging techniques to genomic approaches. One area that has greatly benefited from this development was reproductive plant biology. In particular, the formation of flowers has become one of the most widely studied aspects of plant development. This focus on flower formation not only comes from the importance of flowers as the reproductive units of angiosperms, but also because flowers and the fruits and seeds they produce are pivotal for human nutrition. Furthermore, the intriguing variations in floral shape and architecture found among the ~350,000 extant angiosperm species make flowers an excellent study object for understanding the genetic basis of the evolution of developmental processes.

Genetic analyses, in particular those using the model plant *Arabidopsis thaliana*, have led to the identification of dozens if not hundreds of genes that are involved in the control of different processes during flower formation. Since the early 1990s, the activities of many of these genes have been characterized in molecular detail. One of the most significant concepts introduced into the field of flower development is the ABC model of floral organ identity specification (Bowman et al., 1991; Coen and Meyerowitz, 1991). This model has fundamentally changed the understanding of the complex genetic mechanisms that control flower development and has guided research in this area over the past 25 years. Additional insights into the control of flower development came from studies that employed genome-wide approaches such as transcript profiling by microarray analysis (e.g., (Gomez-Mena et al., 2005; Kaufmann et al., 2009; Kaufmann et al., 2010; Wellmer et al., 2006; Wellmer et al., 2004; Wuest et al., 2012)). This work also led to the identification of downstream targets of several key

floral regulators and highlighted that extraordinary complexity of the gene regulatory network that underlies the formation of flowers.

In the following sections, I will describe the current understanding of the origin of flowers and outline what is known about the molecular basis of flower development in *Arabidopsis*. In particular, I will write on the role of *Cis2-His2* zinc-finger transcription factors as floral regulators, which include *KNUCKLES* (*KNU*), the main focus of this thesis. In the other chapters of this thesis, I will present the results of a perturbation analysis of *KNU*, where *KNU* was inactivated in different regions of the floral meristem (Chapter 3), and outline data obtained from the genome-wide identification of genes that act downstream of *KNU* (Chapter 4). Lastly, I will discuss the results of an epigenomics analysis, in which I studied the distribution of selected epigenetic marks during early stages of flower development (Chapter 5).

1.1.1 The evolutionary origin of flowers

One of the most fascinating questions in evolutionary biology is that of the origin of flowers. Charles Darwin, puzzled by this problem, called it an “abominable mystery” in one of his letters to Hooker (see for review in: (Friedman, 2009)). This exchange between the 19th century scientists ignited exciting and important discussions on the evolutionary history of angiosperms (Frohlich, 2003). Over the course of the last century, different theories about the origin of flowers and flowering plants have been formulated, which were initially based exclusively on fossils known at the time (reviewed in: (Friedman, 2009)). However, molecular biology techniques, which became widely available in the mid to late 20th century, have provided a vast amount of molecular data that can now be used to complement results obtained from analyzing the fossil record (Frohlich and Chase, 2007).

Like most theories in the early days of botanical research, the Anthophyte theory was based on fossils alone and used only a cladistics approach (Doyle and Donoghue, 1986). This theory proposed that the first flowering plants were linked to similarly structured gymnosperms, like Gnetales (Doyle and Donoghue, 1986). A more recent theory, which is based not only on the fossil record but was in part derived using molecular evidence from living plants, is the Mostly Male theory (Frohlich and Parker, 2000). This theory suggests that the angiosperm flower developed from the male

reproductive organ with the female reproductive structure placed ectopically on the male structure of the gymnosperm ancestor. This event could be the beginning of the precursor of carpels, which is the most important and complex flower organ (Frohlich, 2003). This theory is based on molecular evidence of loss of one of the floral meristem identity genes, the *LEAFY* (*LFY*) ortholog *NEEDLY*. It has been reported that *NEEDLY* is lost in angiosperms and may be partially responsible for the production of both male and female reproductive organs, on a single growth axis (Baum and Hileman, 2006; Frohlich, 2006).

Further studies of *LFY* activity and its role in determining the difference between the male and female reproductive organs, led to the discovery of other genes likely responsible for the formation of bisexual flowers (Theissen and Becker, 2004; Theissen et al., 2002). It was proposed that B-class MADS-box genes (described in detail in Section 1.2.1) are involved in specifying male and female cones of gymnosperms. However, this theory did not discuss formation of the carpel itself but suggested the origin of the flower from the male or female cone, by the reduction of the expression of the B-class genes in the higher region of the cone. That is how the out-of-male/out-of-female theory was founded (Theissen and Becker, 2004; Theissen et al., 2002).

More recent studies expanded on the abovementioned theories, and some support the idea of *LFY* being the factor central to the origin of flowers (Baum and Hileman, 2006). Others, however, provided more complex explanations related to the floral quartet model (see Section 1.2.1 below) and its role in flower formation (Theissen and Melzer, 2007; Theissen et al., 2016). The two assumptions, in some way contradictory, gained support from the fossil record that gives enough evidence for those theories to be further explored (Doyle, 2008; Theissen and Melzer, 2007).

Despite the widespread use of molecular approaches in modern evolutionary research and the fast improvement of available bioinformatics methods, the origin of flowers still remains somewhat enigmatic. Theories based on the fossil record have nowadays more evidence in phylogenetic tree inferences (reviewed in: (Doyle, 2012)), however, there is still an open question whether the fossil interpretation and some inconclusive results regarding the phylogenetic analyses are in fact the best prediction on the formation of angiosperms.

1.1.2 Initiation of flower formation

In comparison to the evolutionary origin of flowers, the initiation of flower formation is a relatively well-understood process. *Arabidopsis* undergoes two main developmental transitions during its life cycle (Fig. 1.1), namely from embryogenesis to vegetative development and from vegetative to reproductive development. The latter transition is morphologically characterized by the conversion of the shoot apical meristem into a flower-producing inflorescence meristem (Hempel and Feldman, 1994). At the genetic level, a complex network of genes and pathways underlies this transition and tightly controls the time of flowering to ensure reproductive success (reviewed in: (Simpson and Dean, 2002)).

These specific pathways combine the physiological and environmental information to regulate the subset of floral pathway integrator genes (Abe et al., 2005). The activation of those genes allows for the floral transition, but only when the particular conditions are sufficient for the reproduction. The signaling pathways respond to endogenous and environmental stimuli, including gibberellic acid (GA), autonomous, photoperiodical and temperature cues. The GA pathway controls flowering in short-day conditions depending on the endogenous concentration of the phytohormone (Blazquez et al., 1998). The autonomous pathway accommodates endogenous stimuli from different developmental stages and controls the transition from vegetative to reproductive phases independently from environmental changes (Simpson, 2004). The vernalization pathway promotes flowering in response to cold exposure over a long period of time (Amasino, 2010). The photoperiodic pathway mediates the response to environmental signals such as day length (Kim et al., 2009; Kobayashi and Weigel, 2007; Komeda, 2004).

Amongst the many pathways that play a role during the time of transition, the most important event is the activation of *FLOWERING LOCUS T* (FT) in the leaf by the CONSTANS (CO) protein. This process arises together with the change in the temperature and light, promoting the signal that is consequently sensed by the shoot apical meristem (SAM) (reviewed in: (Parcy, 2005)). FT protein later translocates into the shoot apex where it interacts with *FLOWERING LOCUS D* (FD), a known positive regulator of flowering (Huang et al., 2005). This interaction causes activation of

SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (*SOC1*), which switches the vegetative state into the inflorescence meristem (IM). It has been shown that FT, together with FD act simultaneously in the shoot apical meristem in order to promote the floral transition and commence the development of a flower. They both activate the floral meristem identity gene *APETALA1* (*API*) (Abe et al., 2005; Wigge et al., 2005).

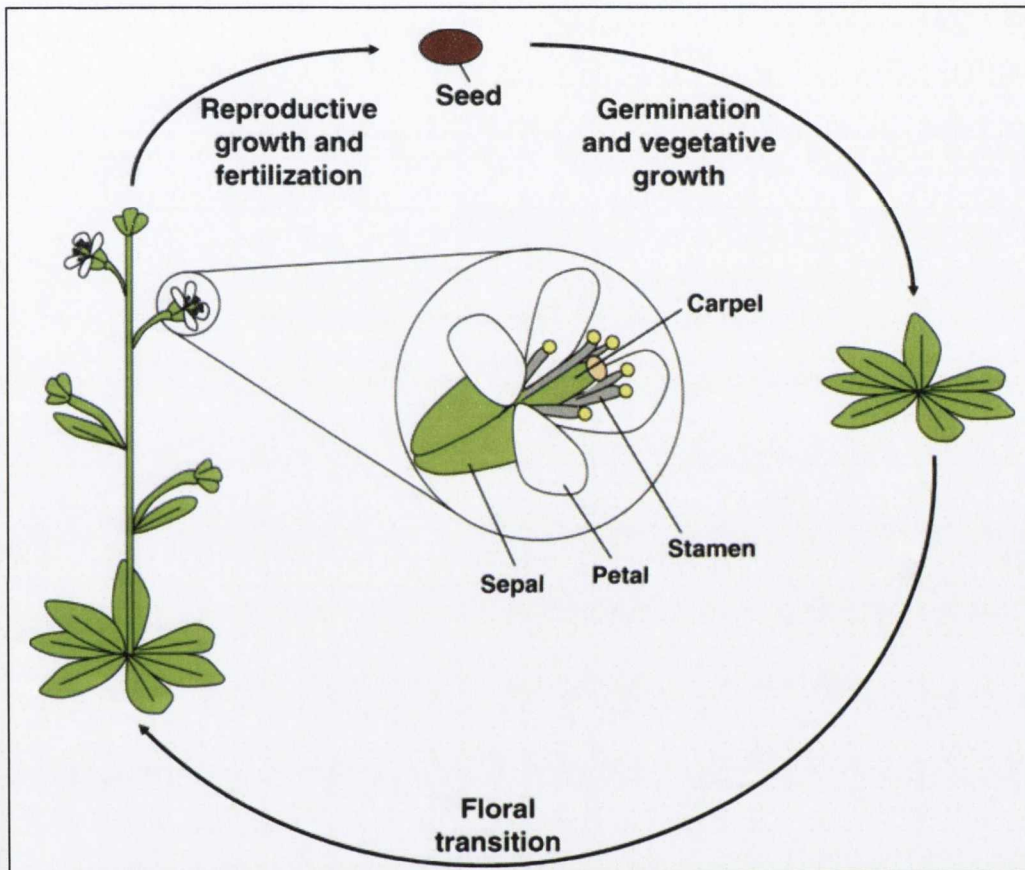


Figure 1.1 Life cycle and flower architecture of *Arabidopsis*. The change from the vegetative phase to reproductive phase (floral transition) is characterized by the transformation of the shoot apical meristem into an inflorescence meristem, which produces floral meristems. A schematic of a flower is shown in the center of the image. Figure adopted from (Liu et al., 2009).

Furthermore, *SOC1* and *AGAMOUS-LIKE 24* (*AGL24*) form a complex that directly activates another flower meristem identity gene *LFY* (Wang et al., 2009). The two genes, *LFY* and *API* specify the floral primordia that are initiated by the inflorescence

meristem (Simon et al., 1996). A mutation in any of the floral identity genes causes production of abnormally shaped flowers, whereas double mutant *lfy ap1* shows a conversion into shoots (Kempin et al., 1995; Weigel et al., 1992). Studies on these mutants indicated that the floral meristem identity genes activate the floral homeotic genes in specific regions of the developing flower (for review see (Liu et al., 2009)). After the floral homeotic genes have been activated they act in certain combinations to control the development of the different types of floral organs (Jack, 2004). Their roles in this process have been summarized in the so-called ABC model of floral organ identity specification that will be described in detail in the next section.

1.2 The ABC model of floral organ identity specification

A typical *Arabidopsis* flower consists of four types of floral organs, which are arranged in concentric circles or whorls. In the outermost whorl four sepals are formed. In the second whorl four petals develop. The third whorl is occupied by six stamens, the male reproductive organs. In the innermost (the 4th) whorl, two carpels fuse to form the gynoecium, the female reproductive organ (reviewed in: (Causier et al., 2010)). The ABC model provides a conceptional framework for the gene activities that specify these different organs types and has fundamentally changed the understanding of the complex genetic machinery underlying flower development. The model is based on the characterization of floral homeotic mutants of *Arabidopsis* and *Antirrhinum majus* (snapdragon), the two well described model organisms (Coen and Meyerowitz, 1991). The homeotic transformations observed in *Arabidopsis* flowers involve the conversion of one type of floral organ into another (reviewed in: (Krizek and Fletcher, 2005)). For instance, flowers of *apetala2* (*ap2*) mutants consist of carpels in the first whorl, stamens in the second whorl and carpels in the fourth whorl (Fig. 1.2 B[b]). Other homeotic transformations are present in *apetala3* (*ap3*) and *pistillata* (*pi*) flowers, where sepals are formed instead of petals, and carpels are produced in the place of stamens. *Agamous* (*ag*) mutant flowers form petals in place of stamens and carpels are replaced by a new flower so that reiterations of sepal-petal-petal whorls are observed (Fig. 1.2 B[d]). This “flower within a flower” phenotype of *ag* mutants is caused by indeterminacy of the floral meristem, indicating that AG, in addition to its function in the specification of the

reproductive floral organs, is also involved in terminating stem cell activity in the floral meristem (reviewed in: (Causier et al., 2010; Krizek and Fletcher, 2005)).

The major idea behind the ABC model is that three classes of genes (termed: A, B, and C) act in a combinatorial manner to specify the identity of the different types of floral organs (Coen and Meyerowitz, 1991) (Fig. 1.2 A). Specifically, *AP1* and *AP2* are genes belonging to class A, which determine sepal identity. Together with the B-class genes *AP3* and *PI*, they regulate the specification of petals (Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005). Furthermore, B-class genes together with the C-class gene *AG* govern the development of stamens, while the C-class acting alone specifies carpel identity (Yanofsky et al., 1990; Jofuku et al., 1994; Goto et al., 1994; Weigel et al., 1994). In addition, A and C class genes act in an antagonistic manner to generate non-overlapping domains of activity in the perianth whorls (A class) and the central two floral whorls (C class), respectively (reviewed in: (Causier et al., 2010)).

Although in the original ABC model, A function genes were proposed to specify sepals, further studies suggested that at least *AP2* is only indirectly involved in organ specification. In fact, it has been shown that *AP2* takes part in the patterning of developing floral primordia and controls the expression of floral organ identity genes (Krogan et al., 2012). With respect to the function of *AP1*, the analyses of *ap1* mutant flowers showed that they usually form bract-like organs in the first whorl whereas in the second whorl organs are often missing, and in their place secondary flowers sometimes develop (Litt, 2007). Moreover, whether A function genes together with B function genes specify petal development is still a matter of discussion. The production of petals in *ap1 ag* double mutants suggests that A function is not necessary for the formation of this organ type (Bowman et al., 1993).

Reverse genetics analyses led to the discovery of additional regulators involved in floral organ specification, namely *SEPALLATA1* (*SEP1*) through *SEP4*. Like most other floral organ identity genes, the *SEP* genes also encode MADS-domain transcription factors. Because these genes were shown to be broadly expressed in flowers and involved in the specification of all four floral organ types, they have been designated as ‘E-class’ genes (Pelaz et al., 2000). The detailed studies on SEP proteins gave more details how they interact with the ABC genes. The overexpression of *SEP* genes in combination with the genes of A and B functions, have caused transformation of rosette leaves into petaloid structures. Interestingly, co-expression of the B function genes alone was sufficient to prompt the petal phenotype in the early stages of plant

development (Honma and Goto, 2001; Pelaz et al., 2001). Moreover, *SEP* genes act redundantly and quadruple mutants (*sep1, 2, 3, 4*) produce flowers composed of leaf-like organs with some carpeloid features (Ditta et al., 2004).

Studies performed in petunia (*Petunia hybrida*) extended the ABC model in another direction (Colombo et al., 1995): D function was added, which is responsible for the specification of ovule identity. Further studies in *Arabidopsis* revealed that four closely related genes act together to determine the formation of ovules (Pinyopich et al., 2003). This clade of genes consists of *AG*, *SEEDSTICK (STK)* and *SHATTERPROOF1* and 2 (*SHP1* and *SHP2*) (Favaro et al., 2003). Homeotic transformations in mutants of *stk shp1 shp2* produce carpel or leaf-like organs in place of ovules (Colombo et al., 1995). On the other hand, ectopic expression of either *STK* or *SHP* is sufficient to turn sepals into organs reminiscent of carpels (Favaro et al., 2003).

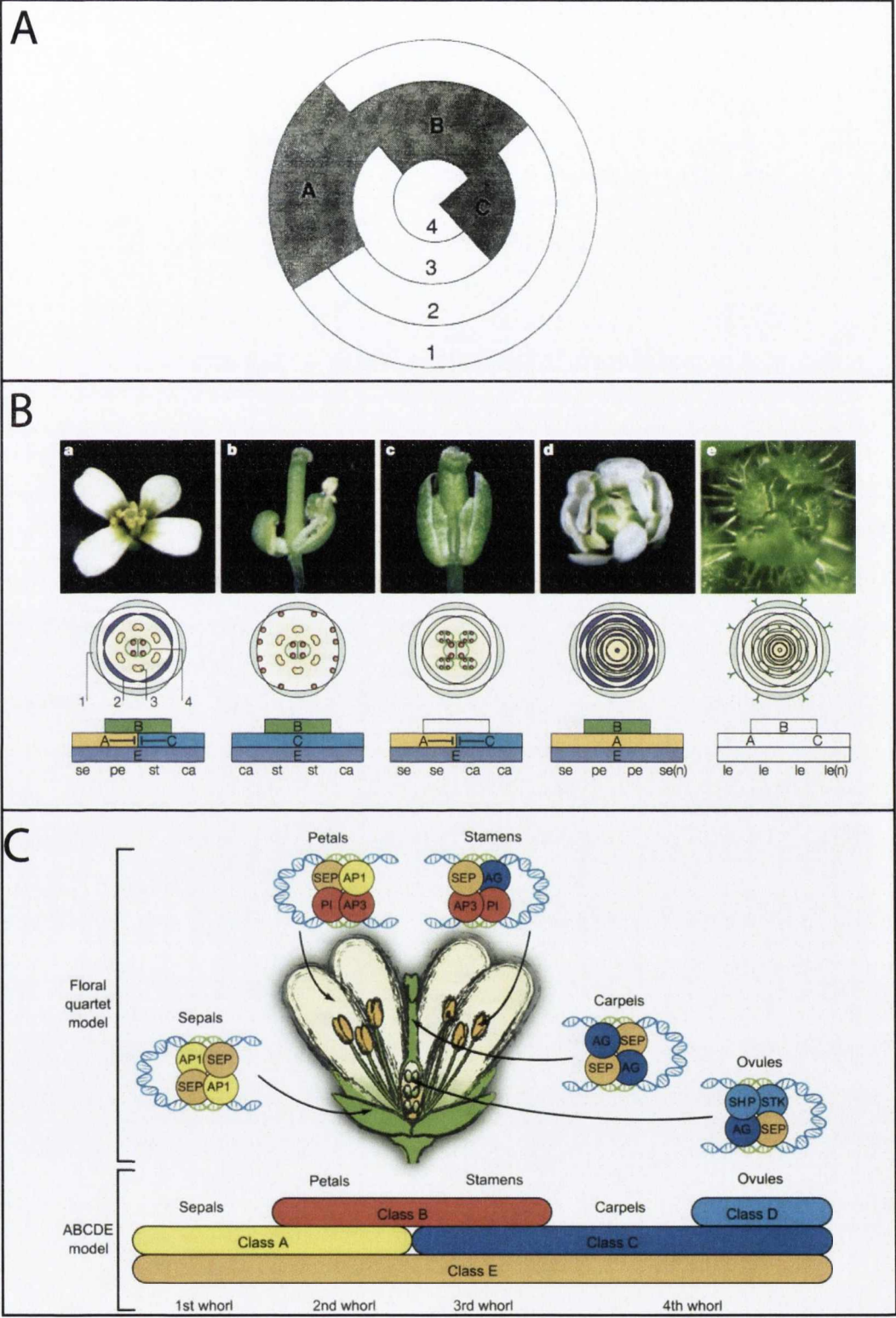


Figure 1.2 ‘Evolution’ of the ABC(DE) model over the past 25 years. (A) The original ABC model as proposed in 1991 by Coen and Meyerowitz. Three gene functions act together in

certain combinations to specify the four types of floral organs. **(B)** The ABCE model and phenotypes of floral homeotic mutants. Flowers of [a] wild-type plants; [b] *apetala2*; [c] *pistillata*; [d] *agamous*; [e] *sep1, 2, 3, 4* quadruple mutants. **(C)** The ABCDE model and the floral quartet model for floral organ identity specification. Tetrameric complexes of MADS-domain proteins bind to two genomic sites, leading to DNA looping it between them. Figure modified from (Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005; Theissen et al., 2016).

1.2.1 Floral organ identity factors and the quartet model

It has been shown in a large number of angiosperms that genes responsible for the control of floral organ specification encode MADS-domain transcription factors (reviewed in: (Gramzow et al., 2010). Studies showed that these proteins act in complexes to specify the given floral organ, and that is how the “quartet model” was proposed (Theissen, 2001). The presence of the specific complexes has been confirmed by elegant experiments (Smaczniak et al., 2012), however there might be additional co-factors playing similar functions in the specification of floral organs.

The floral organ identity factors belong to the MIKC-type sub-family of MADS-domain proteins and comprise highly conserved domains: MADS (M) domain, and intervening (I) domain, a keratin-like (K) domain and a C-terminal (C) domain (Kaufmann et al., 2005; Theissen et al., 1996). The main role of MADS domain is DNA binding, however it also takes part in dimerization and nuclear localization of MADS-domain transcription factors (Gramzow et al., 2010). On the contrary, the I domain is by comparison weakly conserved and plays a role in the formation of DNA dimers (Kaufmann et al., 2005). Next, the K domain, consist of hydrophobic residues that allows for formation of amphipathic helices which take part in formation of multimeric complexes (Puranik et al., 2014; Yang et al., 2003). Finally, the C domain, has a dual function: it takes part in transcription activation and also functions during the establishment of complexes (Kaufmann et al., 2005).

With regard to the quartet model, MADS-domain transcription factors act in complexes and it has been shown that they form those structures in a specific pattern. These proteins bind to the particular DNA sequences that are named CArG-boxes (5'-CC(A/T)₆GG-3'). In this case, two protein dimers of each tetramer can identify two distinct CArG-box sequences and bring them to closer distance by promoting DNA

looping between the exact CArG-boxes (Fig. 1.2 C) (Theissen, 2001; Theissen and Saedler, 2001). While the activities of MADS-domain transcription factors have been extensively studied and characterized in recent years, comparatively little is known about the functions of the target genes of these regulators (Dinnyen et al., 2004; Hiratsu et al., 2004; Sun et al., 2009; Takeda et al., 2004).

1.2.2 Identification of genes acting downstream of floral organ identity factors

Over the past 15 years, several studies aimed at identifying genes acting downstream of the floral organ identity factors. In one of the first, gene expression in *Arabidopsis* wild-type inflorescences was compared by microarray analysis to the gene expression profiles of inflorescences of the different floral homeotic mutants (Wellmer et al., 2004). The combined analysis of the resulting datasets allowed the identification of transcripts that were specifically or predominantly expressed in the different types of floral organs (Wellmer et al., 2004). In total, 1,453 organ-specific transcripts were identified of which most are expressed in the reproductive floral organs. This study gave initial insights into the genes that are regulated by the floral organ identity factors and thus, into the gene network that controls floral organ identity specification.

Even though the above-mentioned study revealed hundreds of genes that are preferentially expressed in the different organ types, the information obtained appeared to be valid mainly for later stages of flower development (Wellmer et al., 2004). However, a similar analysis for early floral stages was precluded by the fact that young *Arabidopsis* flowers are minute and extremely difficult to dissect. Furthermore, because flowers are initiated sequentially from the inflorescence meristem, each flower in an inflorescence is at a distinct developmental stage. Therefore, it is technically difficult to collect a sufficient amount of material to perform genome-wide analyses on early-stage floral buds. To circumvent this limitation, approaches that allow the collection of numerous floral buds of approximately the same stage of development were introduced. These methods use *ap1 cauliflower (cal)* double-mutant plants, which exhibit a delay in the onset of flower formation and instead undergo a dramatic over-proliferation of inflorescence-like meristems, (Ferrandiz et al., 2000; Ferrandiz et al., 1999) (for more details, see Section 1.4.2). In a study by (Gomez-Mena et al., 2005), the combination of

the *ap1 cal* double mutant was introduced into an *ag-1* mutant background. Moreover, the translational fusion of AG-GR was introduced to allow for synchronized flower development when inflorescences were treated with dexamethasone. This enabled for the formation of carpels and stamens at the same stage of development, which later allowed for collection of large numbers of flowers for the analysis. In total, around 150 genes were up-regulated and around 70 considered to be down-regulated. Interestingly, among them 26% were annotated as transcription factors (Gomez-Mena et al., 2005).

In another study that focused on broadening the knowledge on the genes acting downstream of the MADS-domain, a fusion between AP1 and the hormone-binding domain of a glucocorticoid receptor (GR) was introduced to the *ap1 cal* double mutant (Wellmer et al., 2006). This allowed for the collection of minute flowers from stage 1 to 5 after the treatment of plants with dexamethasone. Here, over 1,600 genes were found to be differentially expressed at different time-points. The majority of down-regulated genes were present in the early stages of flower development. Most of the up-regulated genes were present in later in the development. Among the 1,653 genes that were identified 222 genes coded for transcription factors. This number is an improvement compared with the study where only later stages of flower development were taken into account. This and the previous study suggest that the transcriptional programs of late and early flower development are distinct (Gomez-Mena et al., 2005; Wellmer et al., 2006; Wellmer et al., 2004).

More recent studies focused on genome-wide binding studies where chromatin immunoprecipitation (ChIP; see Section 1.4.4) in combination with ultra high-throughput sequencing (ChIP-seq) was used. One of these studies assayed the *SEP3* gene by using the wild-type and *ag-1* inflorescences (Kaufmann et al., 2009). Since *SEP3* was identified to form complexes with B and C class proteins (Honma and Goto, 2001) this suggested that the study would bring some information about these transcription factors as well. The ChIP-seq analysis revealed around 4,000 and 2,800 *SEP3* binding sites in the wild-type and *ag-1* background, respectively. Interestingly, not only high numbers of MADS-domain transcription factors were identified, but the analysis also identified families other than MADS-box proteins. This fact highlighted a chance of interaction of these proteins with the MADS-domain transcription factors in the process of flower formation (Kaufmann et al., 2009).

ChIP-seq was also used to characterize the genome-wide binding activities of AP1 (Kaufmann et al., 2010). Whereas in the previous study the whole inflorescence

was used for the experiments, here the use of synchronized flower development was applied. Around 1,900 genomic regions were identified to be bound by AP1 (2 days after dexamethasone treatment). These data were compared to datasets obtained from the microarray analysis, which was performed on floral buds 2, 4, 8 and 12 hours after dexamethasone treatment. It appeared that only 249 genes among the 1,900 genomic regions identified by ChIP were differentially expressed. This could be the effect of difference in the time-points of the two datasets that were compared.

In recent years, studies that combined all the methods discussed above were published (O'Maoileidigh et al., 2013; Wuest et al., 2012). Specifically, they employed gene perturbation assays, transcript profiling and genome-wide localization studies in combination with the floral induction system to allow a stage-specific analysis of flower development. First, the two closely related B function regulators AP3 and PI were analyzed (Wuest et al., 2012). Microarray experiments revealed more than 2,000 genes that were differentially expressed between wild-type and *ap3* or *pi* mutant flowers at early floral stages. To distinguish between direct and indirect target genes, AP3 and PI binding sites were identified by ChIP-seq. This analysis revealed over 1,500 genomic regions bound by AP3 and PI and the binding patterns for the two transcription factors showed a high degree of correlation in agreement with the notion that AP3 and PI act as oligate heterodimers. The datasets from the different genome-wide analyses were then compared to identify genes that are not only bound by the transcription factors but whose expression changes upon a perturbation of B function. This led to the identification of hundreds of candidates for genes directly targeted by AP3/PI. Also, a comparison of the binding data for these regulators with those for AP1 and SEP3 revealed a high correlation between the binding sites of AP3/PI and AP1 or SEP3. This is in agreement with the idea that AP1 and SEP3 interact with these regulators in order to form petals and stamens (Wuest et al., 2012).

Another MADS-domain transcription factor AG was characterized using a similar approach. ChIP-seq data identified 1,421 high-confidence binding sites for AG in the *Arabidopsis* genome (O'Maoileidigh et al., 2013). The obtained dataset correlated with the binding data available for the other MADS-domain transcription factors (Kaufmann et al., 2009; Kaufmann et al., 2010; Wuest et al., 2012). A transcriptomics experiment was also executed using the loss-of-function mutant in order to identify genes that might have been omitted in the studies of gain-of-function mutant. This experiment identified more than 1,000 genes that were differentially expressed. To

identify genes directly controlled by AG, these two datasets (ChIP and microarray) were compared and around 225 genes common for both datasets were identified. Interestingly, amongst these genes was *KNU*, which encodes the transcription factor that will be described in detail in the following sections.

1.3 Role of C2H2-type zinc finger transcription factors in flower development

The transcription factors mentioned above regulate numerous processes during floral organ formation. However, not only MADS-domain proteins play an important role in the regulation of flower development but the members of the Cys2-His2-type zinc finger-containing transcription factors (referred to C2H2), that include JAGGED (JAG), RABBIT EARS (RBE), SUPERMAN (SUP) and KNU as well (Dinny et al., 2004; Hiratsu et al., 2004; Sun et al., 2009; Takeda et al., 2004). These genes act downstream of the floral identity regulators. The structure and function of these transcription factors will be described briefly in the following paragraphs.

The C2H2 transcription factors contain two domains that are crucial for transcriptional regulation. One of them, a single zinc finger domain, displays a wide range of functions: binds DNA and RNA or may be involved in protein-protein interactions (Englbrecht et al., 2004). Another domain, containing an ethylene-response element binding factor-associated amphiphilic repression (EAR)-like domain, is the most predominant form of transcriptional repression motif so far identified in plants (Kagale et al., 2010). It has recently been shown that this domain is necessary for interaction with the transcriptional co-repressor of the TOPLESS (TPL) (Pauwels et al., 2010; Szemenyei et al., 2008), to recruit histone deacetylase (HDA) which results in epigenetic silencing of target genes (Long et al., 2006). In other words, members of the C2H2-type zinc finger protein family regulate the transcription of other genes by repressing their activity. Moreover, they play other roles in the flower formation process by acting with other regulators. They are responsible for the boundary formation between different organs.

This boundary formation is necessary for the normal development of the organs in flowers. JAG, together with ASYMETRIC LEAVES1 and 2 (AS1 and AS2), is involved in the repression of boundary-specific genes in the sepal and petal primordia

(Xu et al., 2008a). Besides this function, it also controls the outgrowth of lateral organs by suppressing the premature differentiation of the tissue. Moreover, the loss of JAG function causes organs to have serrated margins with leaves being most affected (Dinneny et al., 2004). Additionally, JAG is involved in fruit formation by controlling the valve margin pattern (Dinneny et al., 2005).

Similarly, RBE participates in the control of boundary formation between the second and the third whorls of the flower, which restricts *AG* expression to the 3rd and 4th whorl. Another type of boundary being maintained by RBE is one between the organ primordia within a whorl (Krizek et al., 2006). In addition, RBE is required for the development of petals, as mutations in the *RBE* gene lead to defects in the formation of these organs (Takeda et al., 2004). It has been shown that floral regulators such as AP1 or UNUSUAL FLORAL ORGANS (UFO) (Kaufmann et al., 2010; Krizek et al., 2006; Takeda et al., 2004) regulate *RBE* expression. More recent studies have shown that AP3 and PI directly regulate *RBE*, by binding to its regulatory elements. (Wuest et al., 2012).

Another target of floral regulators like LFY, AP3 and PI is *SUP*. A mutation in *SUP* results in the formation of supernumerary stamens and an increase or decrease of carpel tissue, depending on the allele used (Bowman et al., 1992). Its main function is to maintain the boundary between the 3rd and 4th whorl by repressing the function of B class genes (Sakai et al., 2000). Together with other regulators, it is thought to play a role in floral meristem determinacy (Sun and Ito, 2010).

The next transcription factor to be discussed is KNU, which is involved in stem cell maintenance. *KNU* is a direct target gene of *AG* and is believed to be a repressor of *WUSCHEL* (*WUS*) activity (Sun et al., 2009). In contrast to other floral regulators containing a C2H2 zinc-finger domain, KNU has not been implicated in the control of boundaries formation. The functions and further characterization of KNU will be described in detail below.

In conclusion, the proteins described in this section play an important role in flower development. However, despite the fact that they seem to act downstream of the floral organ identity factors, they do not seem to be directly involved in the specification of floral organs. Instead, they are usually involved in the formation or maintenance of boundaries between floral whorls, and some of them appear to be essential for controlling stem cell maintenance (Krizek et al., 2006; Sakai et al., 2000; Sun and Ito, 2010; Sun et al., 2009). The domain structure of the C2H2 transcription factors suggests that they may act as transcriptional repressors (Sun et al., 2009). Since

the target genes of this group of regulators are poorly characterized, one of the main aims of my project is to shed light on the functions of *KNU*. The mutant phenotype of the *KNU* and the further characterization of *KNU* will be described in detail in the following paragraph.

1.3.1 Function of KNUCKLES in flower development

As described in the previous section, *KNU* encodes a C2H2-zinc finger transcription factor. The protein has a molecular weight of ~25 kDa and consist of two distinct domains: a zinc finger domain, which in all probability plays a role in DNA binding, and a repressive EAR-like domain (see Section 1.3). A mutant allele for *KNU* (termed: *knu-1*) was discovered in a screen of ethyl methylsulfonate (EMS)-treated plants and exhibits defects in silique formation and impaired fertility (Fig. 1.3 A). *knu-1* contains a guanine to adenine transition such that a TGT codon is changed to TAT and results in the substitution of cysteine to tyrosine residue. (Payne et al., 2004). Phenotypic defects were first observed in the *knu-1* allele at stage 8, at the base of the gynoecium. At stage 9, ectopic floral meristems arise from the placental tissue that in wild-type plants is engulfed by two developing carpels (Fig. 1.3 E, F, G) (Payne et al., 2004). Consequently, mature *knu-1* plants display phenotypes that are the result of these abnormalities in floral stem cell control. Mutant siliques produce a mass of ectopic carpels and stamens that creates a bulged fruit (Fig. 1.3 B), which resembles a human knuckle (Payne et al., 2004). In addition, mutants for *KNU* form tricarpelloid siliques, pollen development is affected, and extended gynophores and exaggerated nectaries are also generated (Fig. 1.3 D) (Payne et al., 2004). Moreover, *knu* mutant plants flower much longer than wild-type plants, likely as a result of reduced fertility and seed set. Furthermore, *knu-1* is a temperature sensitive allele and only mutant plants grown at 25°C show the indeterminate phenotype with ectopic organs within the gynoecium (Payne et al., 2004). Additionally, plants are male sterile when grown under these conditions. However, this phenotype is partially rescued and anthers produce pollen when *knu* plants are grown in the lower temperature (16°C) (Fig. 1.3 C) (Payne et al., 2004).

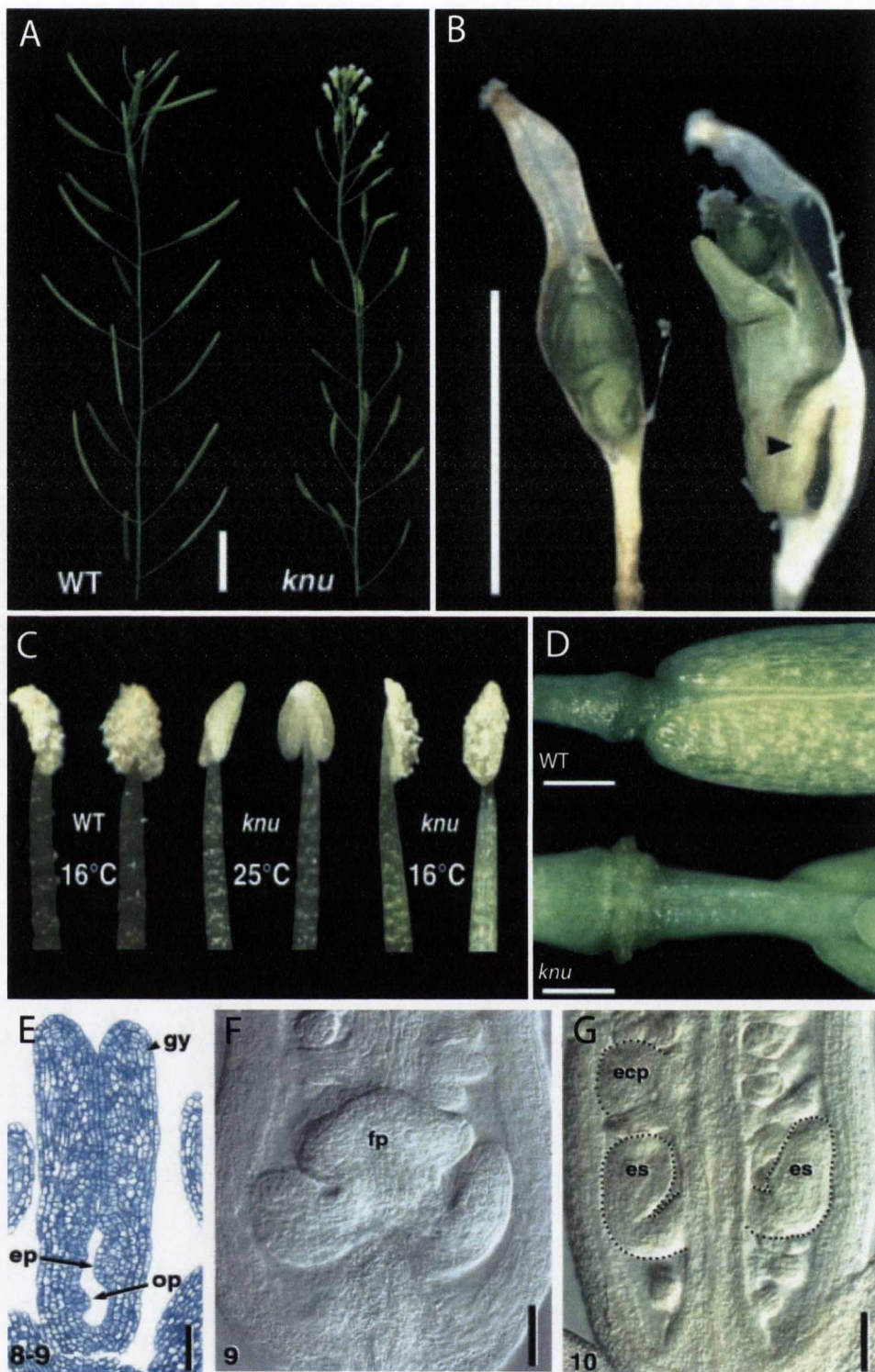


Figure 1.3 Defects of *knu-1* mutant flowers. (A) Comparison of wild-type (accession: Wassilewskija) and *knu-1* mutant plants. (B) Siliques of the *knu-1* mutant contain a mass of ectopic tissue. (C) Pollen production in flowers of wild-type and *knu-1* plants at different growth temperatures (as indicated). (D) Elongated gynophores and excessive production of

nectaries in *knu-1* mutant plants. **(E-G)** Ectopic structures arise from the placental tissue. **(E)** At stage 8, ectopic primordia (ep) form inside the gynoecium (gy). **(F)** Ectopic floral primordia (fp) arise at stage 9 of flower development. **(G)** Later in development, ectopic stamens (es) and carpels (ecp) appear inside of the *knu* gynoecium. Figure modified from (Payne et al., 2004). Scale bars: 10 mm in A; 200 μ m in B; 500 μ m in D; 50 μ m in E-G.

The examination of plants expressing a translational fusion between the entire *KNU* coding sequence and the β -glucuronidase (GUS) reporter (Payne et al., 2004) showed that the expression of *KNU* commences at the base of the developing gynoecium at stage 6 and persists until stage 13 (Payne et al., 2004). The figure below also shows, that at stage ~8, expression of *KNU* is displayed in the anthers of stamens (Fig. 1.4 E). At later stages of development, *KNU* expression decreases dramatically (Fig. 1.4 G, H) and is spatially confined to a small area at the base of the gynoecium. *KNU* is also expressed in the stigmatic tissue at the distal tip of the gynoecium at the stage 14 (Fig. 1.2 I). GUS activity has further been observed in the embryo sac and megaspore mother cell (not shown) (Payne et al., 2004), suggesting that *KNU* may also be involved in processes other than the control of floral meristem determinacy.

As mentioned above, it is believed that *KNU* might be an intermediate factor in the meristem determinacy pathway, playing the role of a repressor. Specifically, the feedback loop of *AG* and *WUS*, that is involved in floral meristem control, is in fact determined by *KNU* (Sun et al., 2009). *KNU* expression commences at stage 6 and coincides with a reduction in *WUS* expression (Sun et al., 2009). Genetic interactions in mutants presented by Sun and colleagues, suggest that *AG*, *WUS* and *KNU* function in the same pathway. Additionally, *WUS* expression is prolonged in *knu-1* mutant flowers, suggesting that *KNU* terminates expression of *WUS*. Furthermore, *AG* induces *KNU* activity at stage 6 of flower development thus leading to meristem determinacy (Sun et al., 2009).

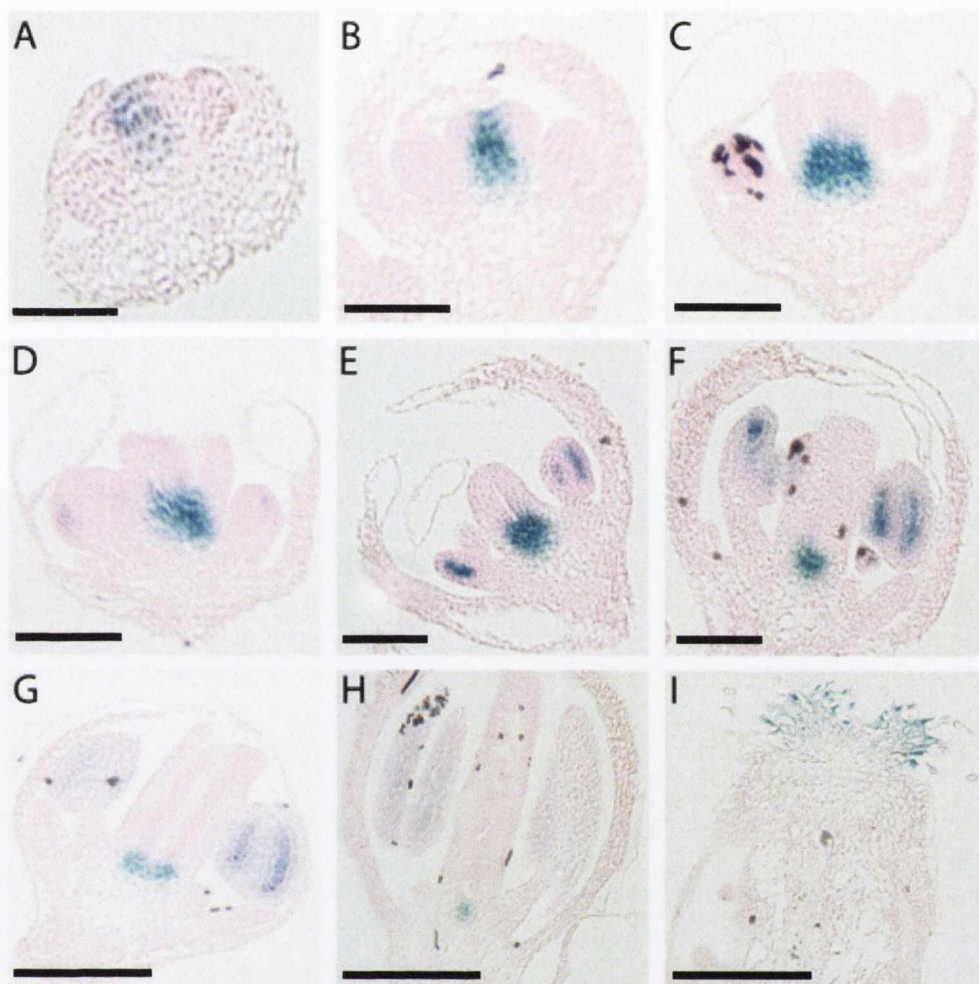


Figure 1.4 Expression pattern of *KNU* during flower development. A previously described reporter line, expressing a *KNU*-GUS fusion protein from the regulatory region of *KNU* (Sun et al., 2009), was analyzed as part of the present study. **(A-B)** *KNU* expression commences at stage 6 in the center of the developing gynoecium. **(C-E)** Later in development, expression occurs at the base of the gynoecium. **(E)** At stage 8, expression is also present in the anthers of stamens. **(F-I)** At later stages (~10-13), expression of *KNU* decreases. **(H)** GUS activity detected at the base of the developing gynoecium in stage 11 flowers. **(I)** GUS activity in the stigmatic tissue of mature flowers. Size bars: 50 μ m.

1.4 Strategies for analyzing the gene network controlling flower development

As described in Section 1.3.1 above, the KNU transcription factor is involved in controlling floral meristem determinacy most likely via direct or indirect repression of the stem cell regulator *WUS*. However, other genes that may act downstream of this regulator, and might be involved in some of the other functions that KNU controls, are currently unknown. To better understand the activities of this transcription factor during flower development, I attempted in this study to identify KNU target genes on a genome-wide scale. To this end, I employed a number of different experimental strategies that have been established, or found to be useful, for the analysis of gene regulatory networks in plant development. These approaches are outlined in the following sections.

1.4.1 Perturbation of floral regulators

It has been shown that the microRNA (miRNA) plays important role in the transcriptional and post-transcriptional regulation of genes expression, resulting in the gene silencing. Moreover, the microRNA is a significant component of processes occurring in plants, including flower development (Chen, 2004; Palatnik et al., 2003). The perturbation of a gene in the gene regulatory network of flower formation can reveal the processes in which the gene of interest is involved.

First, the pri-miRNA is transcribed by RNA polymerase II (Pol II) from the regions situated between the genes that code for proteins. The stabilization process begins when the RNA-binding protein DAWDLE (DDL) recognizes the harpin structure. Consequently, the pri-miRNA is converted into pre-miRNA in the nuclear processing centers called D-bodies (reviewed in: (Voinnet, 2009)). Here, the C2H2-zinc finger SERRATE (SE) protein interacts with the pre-mature miRNA and also HYPONASTIC LEAVES1 (HYL1) and Dicer-like 1 (DCL1) and nuclear cap-binding complex to form a mature miRNA (Mallory et al., 2008). In the cytoplasm, miRNAs are then methylated by HEN1 (Chen, 2005), which prevents its degradation by SMALL RNA DEGRADING NUCLEASE (SND). Next, the miRNA are exported to the

cytoplasm by HASTY, an exportin 5 ortholog. Here, the miRNA strand is incorporated into AGRONAUTE (AGO) protein to carry out the silencing reaction (Voinnet, 2009). Alternatively, the complex can target mRNA transcripts for degradation, translation repression or epigenetic modification (Mallory et al., 2008; Mi et al., 2008).

Among the numerous systems that can carry out a gene-of-interest perturbation (siRNA, null mutant), an artificial microRNA (amiRNA) seems to be one of the most useful. Through targeting the gene of interest with the amiRNA, the partial or full knockdown of the gene function can be accomplished (Schwab et al., 2006). This approach was used in order to investigate the function of KNU. As shown by the global microarray analysis, there was a minimal off target effects observed from ectopic expression of an amiRNA. Interestingly, it is possible to generate versions of gene of interest that is amiRNA-resistant by developing mutations in the regions bound by a given amiRNA, which may serve as an additional control. Moreover, the use of tissue-specific and inducible promoters in order to knock down the gene of interest is possible. The fact that the effect of the inducible promoter was temporary and allowed to resume the gene expression level to the normal conditions after the promoter was inactive, made a new quality to the study of gene function in plants (Schwab et al., 2006; Schwab et al., 2005)

The expression of the amiRNA under control of the constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter and 35S_{pro}:GR-LhG4/6xOp_{pro} system (Moore et al., 1998) can result in a full inactivation of the gene of interest. Upon the dexamethasone treatment of the tissue, the flowering process is activated. Further more, the other system that acts on the gene deactivation can be utilized. Namely, the inducible AlcA/AlcR promoter system that is responsive to ethanol treatment and allows reaching transcription levels similar to a constitutive 35S promoter (Roslan et al., 2001). These methods have been adopted and used successfully to perturb activity of transcriptional regulators (O'Maoileidigh et al., 2015; O'Maoileidigh et al., 2013; Wuest et al., 2012).

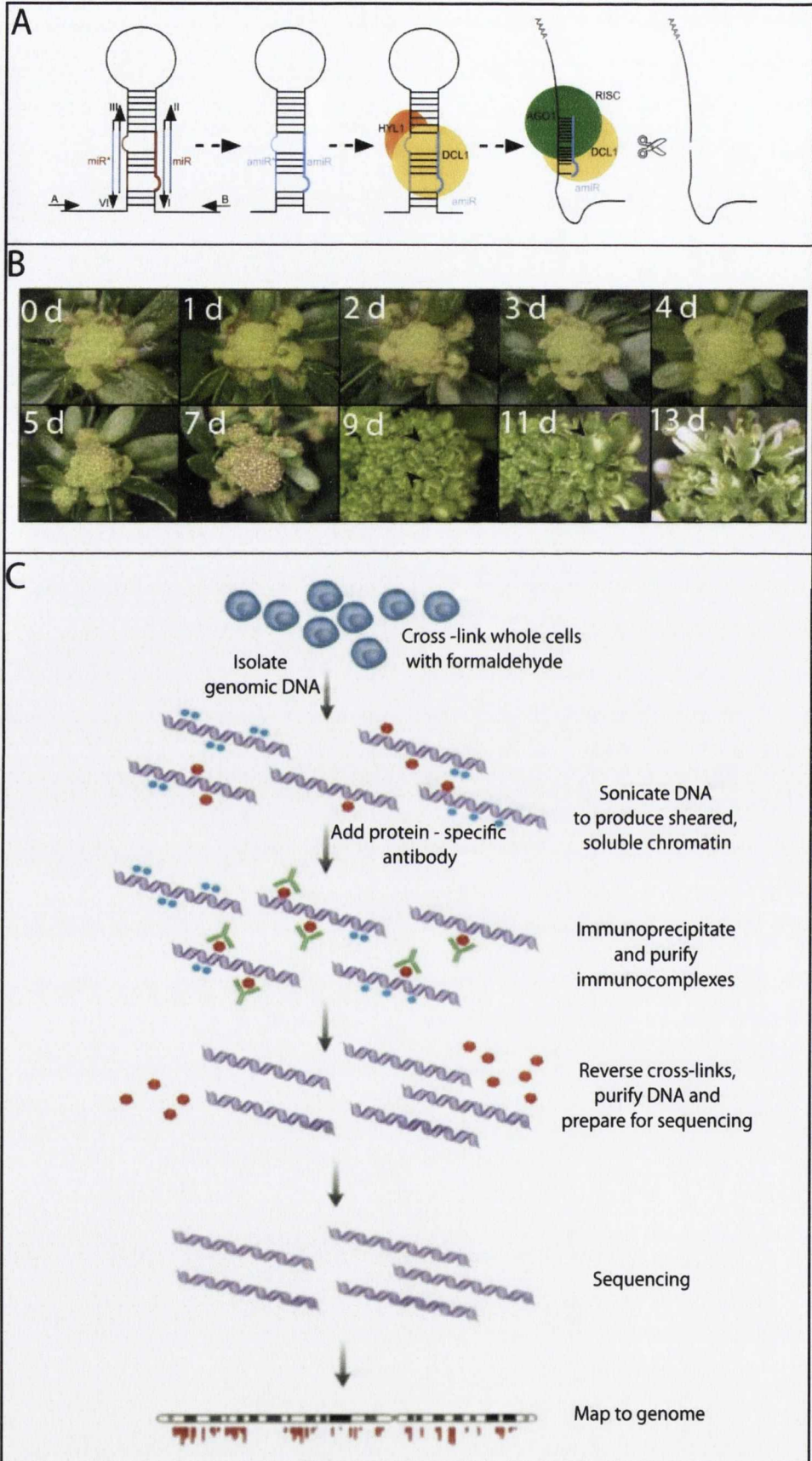


Figure 1.5 Experimental strategies used for investigating KNU function. (A) An artificial microRNA. The process of amiRNA construction is shown (see main text for details). **(B)** The flower induction system (FIS). Untreated inflorescence of an AP1_{pro}:AP1-GR *ap1-1 cal-1* plant (0 d) and of plants of this line at different times (from 1 to 13 d) after the treatment with a dexamethasone-containing solution. **(C)** Chromatin immunoprecipitation followed by ultra high-throughput sequencing. The different steps of the ChIP-seq procedure are shown. Images modified from: <http://wmd3.weigelworld.org>, (Mardis, 2007; Ryan et al., 2015).

1.4.2 A system for the synchronization of flower development

As described above (Section 1.2.2), one technical limitation for the study of early *Arabidopsis* flower development is that young floral buds are minute and difficult to dissect. The invention of the flower induction system (FIS) circumvented that problem and allowed the collection of a large number of floral buds at the same stage of the development (Wellmer et al., 2006). The system is based on the mutations in *AP1* and *CAL*, which result in an over-proliferation of inflorescence meristems (Kempin et al., 1995). Furthermore, flowering is (temporarily) blocked in *ap1 cal* double-mutant plants, leading to the over-proliferation of inflorescence-like meristems (Fig. 1.5 B) (Bowman et al., 1993). To permit the induction of flowering, a fusion between AP1 and GR was expressed in the mutant plants. The treatment of transgenic plants of AP1_{pro}:AP1-GR *ap1-1 cal-1* with the synthetic steroid hormone dexamethasone causes the AP1-GR fusion protein to change its location from cytoplasmic to nuclear. This induced nuclear import activates the synchronized development of flowers at the same specific stage (Fig. 1.5 B) (Wellmer et al., 2006). The examination of plants at later stages of development revealed that they resemble a lot the wild-type flowers (Fig. 1.5 B) (O'Maoileidigh and Wellmer, 2014).

The FIS has been used in a number of experimental approaches such as gene expression profiling or ChIP-seq analysis (Kaufmann et al., 2010; Smaczniak et al., 2012; Sun et al., 2009; Wellmer et al., 2006; Wuest et al., 2012). The introduction of amiRNA or a null mutant of the gene of interest into the FIS enables to track the changes of differentially expressed genes in the stage-specific manner. This method will be discussed in detail in the following section.

1.4.3. Gene expression profiling to identify downstream targets of floral regulators

As mentioned above, the identification of the processes depending upon the gene of interest can be accomplished by perturbing the activity of a selected gene. In order to identify the genes that are differentially expressed in response to the perturbation of the selected gene, the gene expression profiling can be performed. In order to monitor the gene expression upon specific gene inactivation, mRNA samples from inflorescences are isolated and reverse transcribed into cDNA with the addition of fluorescent dye. Next, the tested sample is combined with the reference sample and hybridized onto an microarray containing gene probes specific to the organism under study. The microarray slides are later scanned and fluorescence signals quantified.

As mentioned above (Section 1.2.2), this assay was used to study the changes that appeared in the plants upon knocking down the main players of flower development (Wellmer et al., 2004). Further studies on the specific transcription factors used similar approach, where the genes of interest were knockdown in an inducible manner and the stage-specific microarray experiment was performed (O'Maoileidigh et al., 2013; Wuest et al., 2012). Another stage specific genome-wide study was conducted with the use of FIS and microarray assay, which shed light on the dynamic expression changes by a large number of genes during flower development (Ryan et al., 2015). However, the genome-wide studies for the KNU transcription factor have not been conducted. To characterize KNU more in detail, I aimed at generating lines that could be used for the microarray analysis and consequently, to identify target genes of this regulator.

1.4.4 Genome-wide localization studies of floral regulators

Another method that aims at identifying genes that are targeted by a transcription factor is ChIP (Park, 2009). The purpose of a ChIP assay is the identification of genomic regions bound directly by the protein of interest (Graciet et al., 2014). In consequence, the direct interaction between the transcription factor and DNA is revealed. The ChIP technique is also used for the epigenome analyses and in particular for covalent histone modifications such as histone methylation. In this case the analyses concentrate on the

level of methylation in the particular area of the genome. In order to carry out ChIP experiments, the protein of interest needs to be tagged with the epitope-tag or a specific antiserum against that protein needs to be available (Lee et al., 2006). One of the most important aspects of this assay is the quality of the antiserum and the choice of the tag used. In a first step of the ChIP procedure, the DNA-binding proteins are cross-linked to the DNA. Next, 'free' chromatin and unbound proteins are washed away so that only chromatin-protein complexes remain. The chromatin is then sonicated in order to fragment the DNA (Fig. 1.5 C). Next, the DNA is precipitated by a specific antiserum and the precipitated DNA-protein complexes are isolated. Finally, the crosslinking step is reversed and the DNA can be sequenced and data analyzed (Graciet et al., 2014).

1.5 Aims of the project

In order to unravel the functions of KNU during *Arabidopsis* flower development, this study aimed at identifying genes controlled by the transcription factor on a genome-wide scale. To this end, I intended to use the above-mentioned experimental approaches for gene network analysis. When this study commenced, no genome-wide localization study or transcriptomics experiments on C2H2 zinc-finger transcription factors involved in flower development had been published (described in Section 1.3).

To identify the pathways and processes that are controlled by KNU during flower development a two-pronged experimental strategy was to be employed (Fig. 1.7). In brief, this strategy included genome-wide expression profiling after the perturbation of KNU activity and genome-wide localization experiments to globally identify KNU binding sites in the *Arabidopsis* genome. A combination of the datasets from these different experiments, as well as an advanced bioinformatics analysis, would then lead to the identification of genes that likely act directly or indirectly downstream of the KNU transcription factor.

To carry out this work, a number of reagents and complex transgenic lines would have to be established and characterized. Of particular importance for the work would be the identification of a functional amiRNA against *KNU* that could be used to perturb the gene at different stages of flower development. All technical approaches described above can be used in order to unravel mechanisms underlying developmental aspects of functions of KNU in the flower. In particular, the perturbation of a gene is

one of the methods allowing for identification of genes responsive to KNU, whereas ChIP-seq can be used to reveal the genomic regions bound by the transcription factor (Fig. 1.7). Moreover, the above-mentioned FIS should be employed to allow the identification of direct and indirect targets of KNU with stage-specific resolution.

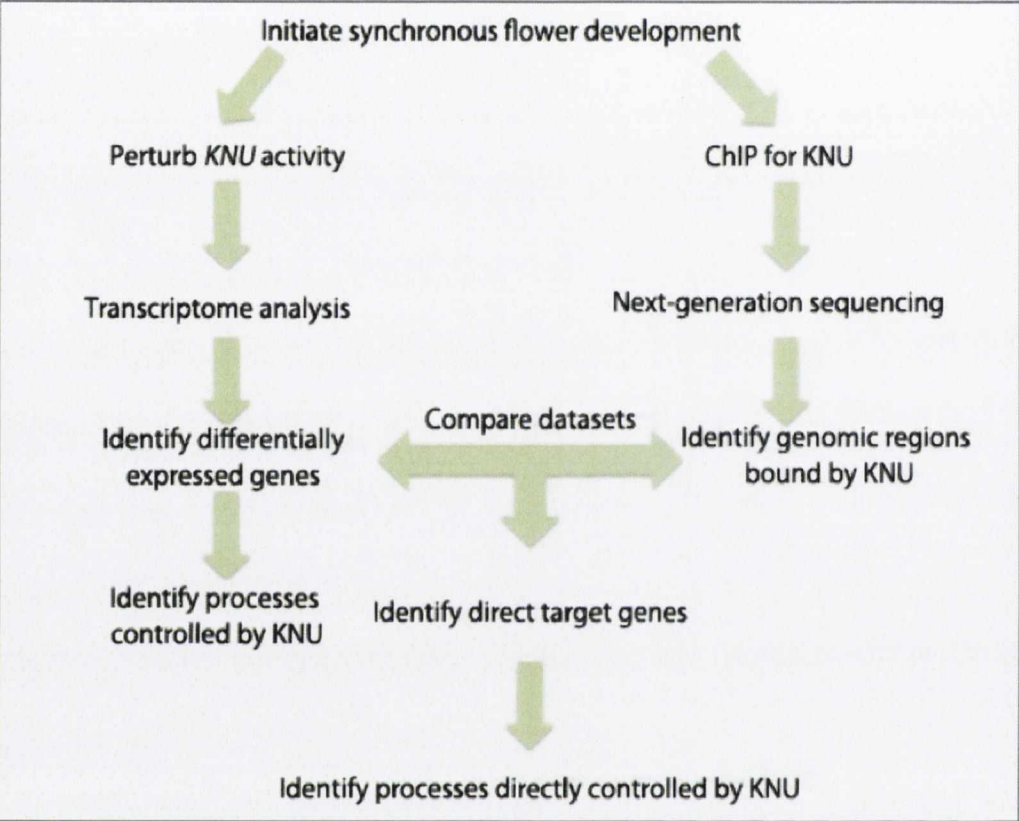


Figure 1.6 Experimental strategies to identify processes controlled by KNU. The FIS is used to synchronize flower development. This is followed by a perturbation of *KNU* activity through the use of an amiRNA targeting *KNU*. Subsequently, the effects of KNU perturbation on global gene expression are monitored by gene expression profiling. The results of the transcriptome analysis will be compared with the results of a ChIP-seq experiment for KNU.

As mentioned above, there are a number of ways to specifically perturb a gene of interest, which include the use of amiRNAs, an approach that has been extensively tested in the context of flower development (see Section 1.4.1). In addition to using amiRNAs for the induced perturbation of regulatory genes, they can also be employed

to test functional domains of activity. To determine where and when *KNU* expression is required during early flower development, I therefore sought to knock down *KNU* in different regions of the floral meristem. To this end, a *KNU*-amiRNA should be expressed under the control of meristem-specific promoters (Gross-Hardt et al., 2002; Lenhard and Laux, 2003; Schoof et al., 2000). Plants in which *KNU* function is impaired in different domains of the flower should then be analyzed for phenotypic abnormalities.

A second part of this study aimed at characterizing dynamic changes in the epigenome during early flower development. Epigenetic mechanisms are known to play an important role in plant development in general and the formation of flowers in particular (Calonje et al., 2008; Zhang et al., 2007a). However, relatively little is known about the genes that are regulated by epigenetic mechanisms during plant reproduction and the underlying molecular mechanisms are also not well understood. In particular, while the genome-wide localization of epigenetic marks has been characterized in seedlings and leaves (Lafos et al., 2011; Zhang et al., 2007b), the dynamic distribution of such marks during flower development remains unknown. To address this question, the AP1_{pro}:AP1-GR *ap1-1 cal-1* (FIS) line in combination with ChIP-seq assays was to be used to investigate the genome-wide distribution of two of the best characterized epigenetic marks, namely histone 3 lysine 27 trimethylation (H3K27me3) and histone 3 lysine 4 trimethylation (H3K4me3), during the early stages of flower development. These marks are commonly associated with genes that are being repressed and activated during development, respectively (Li et al., 2014; Liu et al., 2011; Smaczniak et al., 2012). It was hoped that the data obtained from these experiments would provide insights into the epigenetic regulation of flower development.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Strains and growth conditions

2.1.1 Plant lines

The previously published plant lines used in this study are:

Landsberg *erecta* (L-*er*) wild-type

35S_{pro}:AP1-GR *ap1-1 cal-1* (Wellmer et al., 2006)

AP1_{pro}:AP1-GR *ap1-1 cal-1* (O'Maoileidigh et al., 2013)

CLV1_{pro}:LhG4 (Schoof et al., 2000)

CLV3_{pro}:LhG4 (Lenhard and Laux, 2003)

WUS_{pro}:LhG4 (Gross-Hardt et al., 2002)

KNU_{pro}:KNU-GUS (Sun et al., 2009)

knu-1 (Payne et al., 2004)

clv3-1 (Clark et al., 1995)

clv3-3 (Feldmann and Marks, 1987)

crc-1 (Bowman and Smyth, 1999)

sup-5 (Gaiser et al., 1995)

2.1.2 Bacterial strains

The following bacteria strains have been used in the experiments:

- *Escherichia coli* strain XL1 blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIZ''M15 Tn10 (Tet)]) (Stratagene)
- *Escherichia coli* strain stb12 (F- endA1 glnV44 thi-1 recA1 gyrA96 relA1 Δ (lac-proAB) mcrA Δ (mcrBC-hsdRMS-mrr) λ- (Invitrogen)
- *Agrobacterium tumefaciens* strain C58 pGV2260 (McBride and Summerfelt, 1990)

2.1.3 Plant growth media

Plants were grown on a medium consisting of compost, perlite and vermiculite (NAD, Dublin, Ireland) in a ratio of 5:2:3 (V:V:V). When necessary, plants were grown on 0.5xMS agar plates (Murashige and Skoog medium) that consisted of 2.2 g/L MS salts, pH 5.7, 6 g/L agar.

2.1.4 Bacterial growth media

All bacteria used for preparation and propagation of vectors were grown on LB (Luria-Bertani medium): 10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl (15 g/L agar was added to make LB plates).

2.2 Cloning and genotyping

2.2.1 Plasmid DNA preparation

Plasmids used in this study were extracted from XL1-Blue and STBL2 *E. coli* cultures using the GeneJet™ plasmid miniprep kit (Fermentas) or Plasmid DNA Mini Kit (OMEGA).

2.2.2 cDNA synthesis

Total RNA was isolated from plant inflorescences using the Spectrum Plant Total RNA kit (Sigma-Aldrich) according to the manufacturer's instructions. RNA was reverse transcribed using oligo(dT)₁₈ primers (Fermentas) and the RevertAid H Minus reverse transcriptase (Fermentas) according to the manufacturer's instructions. cDNA obtained from this reaction was used for PCR or quantitative real-time PCR.

2.2.3 Preparation and transformation of competent *Escherichia coli*

There were two separate protocols that were used to generate competent *E. coli*. Competent XL1-Blue or STBL2 were generated as described in (Inoue et al., 1990). The TP16 *E. coli* competent cells were transformed by incubating for 10 min cells that

had been thawed at 4°C with the plasmid of interest. Afterwards the cells were incubated at 42°C for 1 min. The cells were then incubated at 37°C for 1 h with 1 mL of LB. After incubation an aliquot of the transformed cells were plated on an LB agar plate containing the appropriate antibiotic(s). If a low number of transformants was expected the cells were pelleted, the supernatant was removed and the pellet was resuspended in 200 µL LB before plating. Plates were incubated overnight at 37°C.

2.2.4 Preparation and transformation of competent *Agrobacterium tumefaciens*

In order to generate competent cells, a 5 mL culture of *A. tumefaciens* was grown overnight at 28°C. Then 250 mL of LB supplemented with 0.2 g/L MgSO₄ was inoculated with 0.5 mL of the 5 mL culture. This culture was grown at 28°C until an OD₆₀₀ between 1 and 2.5 was reached. Later, cells were pelleted by centrifugation and washed with ice-cold CaCl₂. Subsequently, the suspension was pelleted and resuspended in 4 mL ice-cold CaCl₂. 100 µL aliquots were made and frozen in liquid nitrogen. In order to transform the competent cells, an aliquot of cells was thawed and incubated with the plasmid of interest for 5 min in liquid nitrogen. The cell suspension was then thawed at room temperature and 1 mL of LB was added. Afterwards, the cell suspensions were incubated at 28°C for around 4 h. After incubation the cells were pelleted through centrifugation and the pellet was resuspended in 500 µL LB. An aliquot was then plated on an LB agar plate containing rifampicin (50 µg/µL), carbenicillin (100 µg/µL) and spectinomycin (100 µg/µL). Plates were incubated at 28°C for approximately 2-3 days.

2.2.5 Quick *Arabidopsis* genomic DNA preparation

This method resulted in crude genomic DNA preparations, which were used for genotyping assays. The procedure was performed as described in (Edwards et al., 1991).

2.3 Plant transformation and selection of transformants

2.3.1 Plant transformation and seed sterilization

Plants were transformed by the floral dip method as described in (Zhang et al., 2006). If selection of transformants was to be done on MS agar plates, seeds from transformed plants were sterilized using hydrochlorous acid generated by mixing 100 mL bleach (Domestos) with 3 mL concentrated hydrochloric acid. Sterilization was performed for 3 h in a sealed container under a chemical fume hood.

2.3.2 Selection of transgenic plants

In order to select for transformants that carry a T-DNA with a kanamycin resistance gene, sterilized seeds were plated on 0.5xMS agar containing 50 µg/µL kanamycin. The plates were placed in the dark at 4°C for 3 days and allowed to germinate under long day conditions. Plants that formed true leaves were transplanted onto soil and genotyped for the presence of the correct transgene.

In order to select for transformants carrying an ammonium-glufosinate (BASTA) resistance gene, seeds were germinated on soil and seedlings were sprayed three times in three-day intervals with a 0.15% (v/v) BASTA (Bayer) solution. The seedlings that formed true leaves were transplanted onto fresh soil and genotyped for the presence of the transgene.

2.4 Crosses and isolation of higher order mutants

In order to isolate higher order mutants, pollen from one parental line was used to fertilize emasculated flowers from the other parental line. F1 plants were genotyped (Section 2.4.1) for the presence of the parental mutations and seeds were collected. F2 plants were phenotyped and/or genotyped. Finally, lines homozygous for all parental mutations were isolated.

2.4.1 Genotyping assays for mutant alleles

To genotype for *knu-1* (which is a point mutation) the genotyping and restriction digestion (using *HypCH4III*) was performed using oligonucleotides KK-262/KK-263 as described in (Sun et al. 2009).

The *sup-5* mutant allele was genotyped by PCR using primers DS-50/DS-51 obtained from Dr Darragh Stewart.

The presence of the *crc-1* mutant allele was confirmed by using the *Derived Cleaved Amplified Polymorphic Sequences (dCAPS)* genotyping approach. First, the PCR reaction was performed with primers KK-280 and KK-281. The resulted PCR fragment was re-amplified using oligonucleotides KK-281/KK-282 and digested with *BanII*.

2.5 Microscopy techniques

2.5.1 Light microscopy

Dissection and tissue collection was done using an Olympus SZX7 zoom stereomicroscope. The photographs were taken using an Olympus SP-500UZ digital camera mounted directly to a microscope eyepiece using a camera-microscope adapter. The photographs of GUS stained tissue were taken using Olympus BX61 with the bright field.

2.5.2 Epifluorescence microscopy

Visualization of GFP was performed using an Olympus BX61 fluorescence microscope with excitation wavelengths of 460/480 nm and emission wavelengths of 495-540 nm.

2.6 GUS staining

The protocol was adapted from EMBO COURSE by J. Traas, F. Parcy and S. Brown (ftp.arabidopsis.org)

The plant tissue was harvested in 90% cold acetone and incubated for 15-20 min at the room temperature and vacuum infiltrate for 10 min. After removing the acetone, the staining solution containing X-gluc was added. Tissue was again vacuum infiltrate until it sunk into the bottom of the tube. Depending on the strength of the promoter, tissue was incubated at 37°C until the blue/purple color appeared. Next, tissue was dehydrated

with ethanol (20% - 90%) and treated with FAA for 30 min. This was followed by incubation in eosin 95% ethanol. Tissue was later cleared with TBA (25% and 50%) and incubated in 100% TBA overnight at 60°C. The following day, 50% of liquid paraplast was added and incubated for several hours at 60°C. The paraplast was then replaced few times and placed in a boat. Tissue in the paraplast was then sectioned at 8 μ m and placed on slides that were incubated overnight at 42°C. Next, paraffin was removed by HistoClear (National Diagnostics) washes and the staining could be visualized using bright-field microscopy as described in Section 2.4.1.

2.7 Tissue fixation and chloralhydrate clearings

Flowers were fixed in Farmer's fixative (3:1 ethanol: acetic acid) under 500 mbar vacuum for 10 min, two times and later washed three times with 1 mL of 100% ethanol (each wash lasted 10 min). Clearings were made using chloralhydrate: glycerol solution that consisted of 2.5 g chloral hydrate in 1 mL of 30% glycerol.

2.8 Treatments of lines encoding inducible promoters

2.8.1 Ethanol treatment of plants

Plants were placed in a sealable container with two 50 mL centrifuge tubes (Corning) containing 10 mL 100% ethanol each. Each tube was placed at opposite ends of the container. Mock-treated plants were placed in a similar container with the tubes containing the same amount of ddH₂O.

2.8.2 Treatment of plants with a dexamethasone-containing solution

Once plants had bolted > 1 cm, the inflorescence-like meristems of plants were treated with a solution containing 10 μ M dexamethasone (Sigma) and 0.015% Silwet-77 using a plastic Pasteur pipette.

2.8.3 Treatment of plants with a dihydrotestosterone-containing solution

Inflorescence of meristem-like plants that bolted were treated with a solution containing 2 μ M dihydrotestosterone (Sigma) and 0.015% (v/v) Silwett-77 using a plastic Pasteur pipette.

2.8.4 Collection of floral tissue

Following the induction, the wild type inflorescences were collected at stage 0 - 10 flower buds from the main treated inflorescence meristem and placed into the tube containing liquid nitrogen. The tissue of induced FIS inflorescences was collected from the main inflorescence meristem and placed in the tube with liquid nitrogen and placed in -80°C for the further experiments.

2.9 Generation and cloning of artificial miRNAs

The amiRNAs were designed using the Web microRNA WMD3 designer (<http://wmd3.weigelworld.org>). In brief, the Arabidopsis Genome Initiative (AGI) number of *KNUCKLES* (At5G14010) was submitted to the online database and a list of artificial microRNAs (amiRNAs) was presented which have the potential of targeting the mRNA of the gene of interest. These amiRNAs are ranked by different criteria such as amiRNA sequence composition; mismatch positions, hybridization energy when paired to intended targets, number of other genes that have 5 or less mismatches to the amiRNA, and hybridization energies of other genes with 5 or less mismatches to the amiRNA. The ranking system divides the potentially functional amiRNAs into four different color-coded classes. The amiRNAs predicted to perturb gene function most efficiently are colored in green, followed by intermediate ones in yellow and orange, and the remaining ones in red.

Once an amiRNA sequence was chosen, an Oligo Designer online application (<http://wmd3.weigelworld.org>) generated oligonucleotides that allowed mutagenesis to be performed on the backbone of pRS300 plasmid, which contains a copy of the

endogenous *Arabidopsis* miRNA, miR319a (At4g23713) (Palatnik et al. 2003). Overlapping PCR was used to generate the amiRNA precursor and the final PCR product was cut with *Cla*I and *Xba*I, and ligated to a vector (pBJ36) containing the CaMV 35S promoter, the AlcA ethanol-inducible promoter or the LhG4/pOP-inducible promoter. After verifying the sequence of the amiRNA, a fragment containing the promoter and the amiRNA was sub-cloned into either of the binary vectors pML-BART or pART-27 using *Not*I sites. This vector was then used to transform plants as described in Section 2.3.1.

2.10 Quantitative real-time PCR

For this analysis, primers were designed to have a T_m of $60 \pm 1^\circ\text{C}$. Each primer pair did not differ in annealing temperature of more than 1°C . In addition, primer pairs to amplify cDNA were designed to span exon-exon boundaries whenever possible and were as close to the 3' end of the coding sequence as possible. The annealing temperatures and primer dimerization potentials of these oligonucleotide primers were checked using the thermodynamic oligonucleotide software, Oligo Analyzer, available at the Integrated DNA Technologies website (<http://eu.idtdna.com>). The Lightcycler 480 (Roche) with SYBR green master 1 (Roche) was used to quantify relative enrichments of DNA. One reaction mix contained 5 μL of 2x SYBR green master 1, 1 μL (c)DNA, 1 μL of 10 μM primers and 3 μL of molecular biology grade water. An equivalent time of 60 s per 1 kb of DNA was given to generate the amplicon. Annealing temperatures between 58°C and 60°C were used depending on the primer pairs. The enrichment observed was calculated relative to the input sample. Therefore, for each locus analyzed, the enrichment was calculated as follows:

For ChIP: $(2^{\text{Cp}^{(\text{IN})} - \text{Cp}^{(\text{IP})}}) \times V(\text{IN})$

For cDNA: $(2^{\text{Cp}^{(\text{REF})} - \text{Cp}^{(\text{GENE})}})$

where Cp is the crossing point calculated by the Lightcycler 480, $\text{Cp}(\text{IN})$ is the Cp value for the input, $\text{Cp}(\text{IP})$ is the Cp value for the immunoprecipitated (IP) and $V(\text{IN})$ is percentage of the total volume of the IP used to generate the input sample. $\text{Cp}(\text{REF})$ is the Cp value for the chosen reference gene and $\text{Cp}(\text{GENE})$ is the Cp value for the

chosen target gene. The reference gene used for qRT-PCR was KK-29/KK-30 (At4g34270), which was chosen from (Czechowski et al., 2005).

2.11 Western blotting

For the extraction of nuclei from ~500 μ L of inflorescence tissue, the same procedure was carried out as described in Section 2.10. The exception was that formaldehyde and glycine were not added and that after washing the nuclei with the 1x Crosslinking Buffer (Diagenode) and they were resuspended in 50 μ L 2x SDS loading buffer (see Appendix 2 for the composition of all buffers used for SDS-PAGE electrophoresis and Western blotting) and incubated at 95°C for 5 min. Next, 15 μ L of this denatured protein extract was loaded onto a 12% SDS-PAGE gel and run for 2-3 h at 50 V. Proteins were transferred at 50 V for 2 hours (4°C) from the SDS-PAGE gel to an Immobilon-P membrane (Sigma-Aldrich) (Appendix 2). The membrane was then incubated in a blocking solution for 15 min at room temperature. A 2,000-fold dilution of the Ab290 antibody (Abcam) in blocking solution was then incubated overnight at 4°C with the membrane. Non-hybridized antibody was removed by rinsing the membrane with 1x PBS supplemented with 0.05% (v/v) Tween-20 (noted PBS-T) for 5 min. This wash was repeated twice more. The secondary antibody a-rabbit conjugated to horse radish peroxidase (Sigma)) was diluted 50,000-fold in the blocking solution and was added to the membrane. The latter was then incubated with the secondary antibody solution for 4 h at room temperature with mild shaking. The excess of secondary antibody was rinsed by washing the membrane 3 times 5 min with PBS-T. The presence of GFP was detected using the Amersham ECL Plus Western Blotting Detection Reagent (GE Healthcare) in the LAS- 3000 Intelligent Dark Box Image Reader (Fujifilm).

2.12 Chromatin immunoprecipitation

The chromatin immunoprecipitation protocol was adapted from the Diagenode Plant ChIP-seq kit manual instruction and optimized for the use with inflorescence tissue.

2.12.1 Crosslinking of plant tissue

An approximately 500 μ L (for KNU-GFP line ~800 μ L) inflorescence tissue was collected and frozen in liquid nitrogen. Tissue was fixed in 1 mL of 1x Crosslinking Buffer with 1% formaldehyde (37% (w/v) formaldehyde solution, Sigma-Aldrich) on ice under vacuum (800 mbar). The vacuum was released after 15 min, the tissue was mixed and the vacuum was applied 3 more times for another 15 min each (total fixation time was 60 min). The formaldehyde fixation was stopped by adding 50 μ L of a 2.5 M glycine solution. The tube was inverted several times, placed back on ice and vacuum (800 mbar) was applied for 5 min. The excess solution was removed and the tissue was washed in 1 mL 1x Crosslinking buffer. This wash step was repeated once more, after which the excess 1x Crosslinking buffer was removed carefully. The tissue was then snap frozen in liquid nitrogen.

2.12.2 Chromatin extraction from plant tissue and chromatin shearing

In order to extract the nuclei, 250 μ L E1 buffer (supplemented with Plant Protease Inhibitor Cocktail [Sigma] and B-mercaptoethanol at final volume of 5 mM [Sigma, stock solution at 14.3 M]) was added to the tissue, which was then ground thoroughly. More E1 buffer was then added until a final volume of 1.25 mL was reached. The resulting extract was then split in two and more E1 buffer was added, until the final volume of 2.6 mL was reached. In order to remove the debris, the cross-linked tissue was filtered twice through a layer of miracloth that had been pre-wet with E1 buffer. The filtered extraction was centrifuged for 20 min at 1,000 g, at 4°C. Next, the nuclei extract was resuspended in 1 mL of E2 buffer and spun for 10 min at 1,000 g, at 4°C. This step was repeated for four times more. The next wash step was performed by resuspending the nucleus pellet in 1 mL of E3 buffer, followed by centrifugation for 10 min at 1,000 g, 4°C. The nucleus pellet was finally resuspended in 300 μ L Sonication Buffer (supplemented with Plant Protease Inhibitor Cocktail from Sigma) and incubated on ice for 10 min in order to lyse the nuclei. Chromatin was sonicated using a Diagenode Bioruptor Pico at 4°C. The nucleus extract was transferred into sonication tubes and intense sonication cycles were applied, which consisted of 30 s sonication, followed by 30 s without sonication. 10 cycles of sonication were required in order to shear chromatin into fragments between 150 and 600 bp. The sonication efficiency was checked for each assay by precipitating 20 μ L aliquot of sheared DNA using glycogen, sodium acetate and 100% ethanol and analyzing it on a 1.5% agarose gel.

2.12.3 Immunoprecipitation

The protein A sepharose beads (Santa Cruz) were washed with 1x ChIP dilution buffer and blocked with a 0.5 mg/mL lipid-free bovine serum albumin solution. Next, the chromatin was pre-cleared by incubating it with 60 μ L of washed protein A sepharose beads for 90 min. A 100- μ L aliquot of this pre-cleared chromatin was taken as the input control sample. The remaining of the pre-cleared chromatin was pipetted out into a clean microtube and incubated with 10 μ L of GFP-specific antibody (Ab290; Abcam) or 4 μ L H3K27me3, H3K4me3 (Millipore) at 4°C for 1 h, with rotation. After incubation with the antibody, 60 μ L of washed protein-A sepharose beads were added, in order to purify the antibody/chromatin complexes. This mixture was incubated with rotation for 2 h at 4°C. Next, the protein A sepharose beads were washed two times with 1 mL of Wash Buffer1, 2 and 3 and one time with Wash Buffer 4.

2.12.4 Elution, de-crosslinking and DNA isolation

After removing last washing buffer, 400 μ L of Elution Buffer 1 was added to the sample and incubate for 30 min in a thermomixer at 65°C, 1300 rpm. The eluate was spun at the top speed for 2 min and transferred to a new tube without disintegrating a pellet. Next, 16 μ L of Elution Buffer 2 was added into isolated supernatant and incubated overnight at 65°C in a thermomixer (1,300 rpm). At this point, 300 μ L of Elution Buffer 1 and 16 μ L of Elution Buffer 2 was added to input sample and incubated overnight at 65°C in a thermomixer in order to reverse the cross-link. This step was followed by a phenol-chloroform extraction of the DNA. To this end, 500 μ L of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma) was added to the DNA/protein mixture. The latter was centrifuged in a phase-lock tube (5-prime) and the aqueous phase was transferred to a clean tube for DNA precipitation.

Finally, the DNA was precipitated by adding 40 μ L of DNA precipitation buffer, 5 μ L of DNA co-precipitant and 1000 μ L of 100% followed by overnight incubation at -20°C. After centrifugation at maximum speed for 15 min at 4°C, the DNA pellet was washed with 80% ethanol, dried and resuspended in Low TE buffer pH 8.

2.12.5 Quantification of genomic DNA

To obtain an accurate estimate of the amount of genomic DNA present in ChIP samples, the Quanti-T dsDNA HS assay (Invitrogen) was used according to the manufacturer's instructions.

2.12.6 Diagenode ChIP-seq library preparation

The preparation of Diagenode ChIP-seq libraries was performed as per manufacturer's instruction using MicroPlex Library Preparation kit v2. Briefly, the ChIP DNA at 5-10 ng in 10 μ L of final volume was used. First, the Template Preparation Buffer and Enzyme were added and placed in thermocycler for 25 min at 22°C followed by 20 min at 55°C. In this reaction the overhangs of the ChIP DNA were converted into phosphorylated blunt ends. Next, the Library Synthesis Buffer and Enzyme were added and sample incubated in thermocycler for 40 min at 22°C. Here, the adenine base was added to the 3' end of the blunt DNA fragments. The final step was to add Library Amplification Buffer and Enzyme as well as the Indexing Reagent. In this reaction the earlier blunted ends were ligated to adapters. The excess adapters were removed by using AMPure XP magnetic beads. These libraries were then analyzed on an Agilent 2200 TapeStation using the High Sensitivity DNA-1000 Screen Tape. The amount of DNA was measured as described in Section 2.15.5. Finally, these libraries were diluted to a final concentration of 50 pg/ μ L for qPCR analysis, and to a final concentration of 10 μ M for Illumina sequencing.

2.13 Microarray experiments

The preparation of the sample was performed as described in Agilent User Guide with some alterations.

2.13.1 Sample preparation

The amount of 500 ng of total RNA isolated (Section 2.2.3) from inflorescence tissue was subjected to reaction of reverse transcription in order to obtain cDNA. Next, in separate reaction, the samples were labeled with Cyanine dyes (Cy-5 for test sample and

Cy-3 for reference sample; the dye swaps were used in order to reduce dye bias). Purified and amplified samples were quantified using Nanodrop in order to measure the incorporation of the dye in the sample. Once, the sample incorporation reached < 8 pmol/ μ g it was later used in a fragmentation reaction that was followed by hybridization.

2.13.2 Hybridization

The same amount of the Cy-3 and Cy-5 labeled sample was added into the reaction and combined with 2x GE Hybridization Buffer (Agilent). This hybridization combination was added onto 4x 44 Agilent arrays and hybridized for 17 h at 65°C at rotation 10 (in the Agilent hybridization chamber).

2.13.3 Washes and slide scanning

The hybridized array was washed three two times: with Wash Buffer 1 (Agilent) at room temperature for 1 min and with Wash Buffer 2 (Agilent) at 37°C also for 1 min. Slides were left for 1 min to dry and scanned using Agilent's High Resolution G2505C Array Scanner followed by data and QC analysis with their Feature Extraction software.

2.14 Data analysis and bioinformatics

The analyses of data obtained from microarray experiments were performed by Dr Patrick Ryan using the *R* statistical software Version 2.14.0 and the listed libraries implemented in the Bioconductor project (www.bioconductor.org) Version 2.12, Low level data processing was performed using functionality provided by the Bioconductor package *limma* (Silver et al., 2009).

2.14.1 Microarray data processing and Gene Ontology analysis

Agilent median signals and background were read into R and background correction was performed using the *background Correct*-function (Ritchie et al., 2007) with

maximum likelihood estimation of the background (Silver et al., 2009) and an offset of 50. Between-channel normalization was performed using loess-normalization, while between-array normalization was carried out using *quantile* normalization (Smyth and Speed, 2003). Duplicated probes were averaged using the *avereps*-function provided in the *limma* package. Non-specific filtering was performed using the *genefilter* package, based on a signal cut-off at the median of all negative probe signals, so that only those probes with higher signals in at least three arrays were retained. To test for differential expression, separate-channel analysis (Smyth, 2005) was performed using linear models (Smyth, 2004) as described in the *limma* users guide.

Gene Ontology terms contained within the “biological process” class used for further analyses, and terms with less than 10 genes associated with them were removed. Parent-children relationship information as contained within the *GO.db* data package (Version 2.5.0) were used to remove redundancy between terms, and parent terms were removed if they had more than 95% percent gene-association overlaps with their daughter terms. Statistical significance of enrichment were calculated using fishers exact test using the *fisher.test* function in *R*. Adjustments for multiple hypothesis testing were performed using the *p.adjust* function in *R*, using Benjamini and Hochberg adjustments (Benjamini and Hochberg, 1995). Heat-maps were generated using the *gplots* package (Warnes et al., 2011).

2.14.2 Read alignment, quality control and general data handling for ChIP-seq

Dr Beibei Zheng analyzed the ChIP-seq data obtained from the ultra high-throughput sequencing, which was carried out by the Beijing Genomics Institute using an Illumina Genome Analyzer.

Sequences were aligned and mapped against the *TAIR10* assembly of the *Arabidopsis* genome, obtained from *Arabidopsis.org* using the *Burrows Wheeler Alignment* (BWA) tool. The resulting output file was converted into *.bam* format with *samtools* (Li and Durbin, 2010). Quality control of the sequenced libraries was performed using the *FastQC* software (www.bioinformatics.bbsrc.ac.uk/projects/fastqc/).

The peak calling was used to identify peaks in the mapped data. The *Model-Based Analysis for ChIP-seq (MACS)* (Zhang et al., 2008) uses a default setting for a *p*-value cut-off of <0.00001. Gene Ontology (GO) analysis was performed using the online software *PANTHER* (Thomas et al., 2006). The Bonferroni correction for multiple testing was used and a *p*-value cut-off of < 0.05 was employed. For the detection of motifs at putative binding sites, peak locations from a dataset were submitted to the browser-based *Hypergeometric Optimization of Motif EnRichment (HOMER)* (<http://homer.ucsd.edu/homer/>).

2.15 Generation of transgenic lines

2.15.1 Generation and expression of an amiRNA that targets the *KNU* mRNA

The description of how amiRNAs were designed, cloned and expressed in *Arabidopsis* is available in Section 2.9. The sequences of the primers used to generate the KNU-amiRNA are available in Appendix 1 (primers KK-31-KK-34 (DM-55-DM-58) were used to generate amiRNA1; primers KK-48-KK-51 were used to generate amiRNA-2; primers KK-52-KK-55 were used to generate amiRNA-3). In brief, the amiRNA was amplified as described in Section 2.9 and the resulting PCR fragment was cleaved with *ClaI* and *XbaI*. The resulting DNA fragment was ligated into pBJ36-based vectors that contained the CaMV 35S_{pro}, the OP_{pro} or the AlcA_{pro} promoter sequence which were also cleaved with *ClaI* and *XbaI*. Once the sequences of these vectors were verified, the 35S_{pro}:KNU-amiRNA cassette was excised from pBJ36 using the *NotI* restriction enzyme and ligated to the binary vector pML-BART that had also been cleaved using *NotI* and treated with alkaline phosphatase (Roche).

In order to generate lines for an ethanol-inducible expression of the KNU-amiRNA, a pML-BART vector that contained the 35S_{pro}:AlcR cassette was used for ligation in combination with the AlcA_{pro}:KNU-amiRNA cassette. Plants were transformed as described in Section 2.3.1. First generation transformants were identified by their herbicide resistance and by using PCR-based genotyping. Colony PCRs and genotyping assays were performed using primer KK-95 in combination with primer KK-35. All

generated constructs were sequenced using Sanger sequencing platform.

In order to generate the lines that were dexamethasone-inducible version of KNU-amiRNA, the pML-BART vector that contained 35S_{pro}:GR-LhG4 cassette was used for ligation in the combination with the 6xOP_{pro}:KNU-amiRNA cassette (in pBJ36 vector). Wild type plants were transformed by floral dip method (Section 2.3.1) and first generation transformants were identified by their herbicide resistance and by using PCR-based genotyping (KK-35/KK-95). All generated constructs were sequenced using Sanger sequencing platform.

2.15.2 Crossing the 35S_{pro}:KNU-amiRNA line into the floral induction system and isolation of homozygous line

Pollen from an AP1_{pro}:AP1-GR *ap1-1 cal-1* line was used to fertilize emasculated flowers from a 35S_{pro}:KNU-amiRNA plant. F1 plants were genotyped for the presence of the 35S_{pro}:KNU-amiRNA transgene and seeds were collected from plants homozygous for this allele (using primers DM-647/pMLBart-3). Next, F2 plants that exhibited an *ap1 cal* mutant phenotype were genotyped for the presence of the AP1_{pro}:AP1-GR transgene using primers DM-400/pMLBart, as well as for the presence of the 35S_{pro}:KNUamiRNA (KK-95/KK-35) transgene. Finally, F3 plants that were homozygous for all mutations and for the T-DNA were isolated (genotyped using primer pairs DM-647/DM-664 (absence of T-DNA) and DM-647 /pMLBart-3 (presence of T-DNA)). The sequences for the primers are available in the Appendix 1.

2.15.3 Generation of plants expressing the KNU-amiRNA in specific meristematic domains

The generation of plants containing the 6xOP_{pro}:KNU-amiRNA transgene was performed in the same manner as described in Section 2.15.1, except that the AlcA_{pro}:KNU-amiRNA-pBJ36 vector was used to excise the KNU-amiRNA sequence

and the primers DM-57/DM-31 were used for colony PCRs and genotyping assays. The vector containing the 6xOP_{pro}:KNU-amiRNA construct was transformed into *Agrobacterium* and plants containing constitutive promoters CLV1_{pro}:LhG4-GR, CLV3_{pro}:LhG4-GR and WUS_{pro}:LhG4-GR (Gross-Hardt et al., 2002; Lenhard and Laux, 2003; Schoof et al., 2000) were transformed. First generation transformants were selected on MS plates containing kanamycin and genotyped with primers KK-35/KK-95.

2.15.4 Over-expression of the *KNU* coding sequence

In order to generate a line that constitutively over-expressed *KNU*, the *KNU* coding sequence (486 bp) was amplified from cDNA generated from an mRNA population, which was extracted from *L-er* wild-type flowers, using primers DM-86/DM-87 that incorporated *Pst*I and *Acc*65I restriction sites, respectively. The resulting PCR product was cleaved and ligated to a pBJ36 vector containing the 35S promoter. Once the presence of the insertion had been verified by colony PCR, this vector was cleaved using *Not*I and ligated to the binary vector pML-BART that had also been cleaved with *Not*I and treated with alkaline phosphatase (Roche). The resulting vector was used for plant transformation. The sequence of the 35S_{pro}:KNU cassette was verified by sequencing. First generation transformants were identified by their herbicide resistance and were genotyped by PCR. Colony PCRs and genotyping assays were performed using primers DM-86 and DM-31.

2.15.5 Generation of and epitope-tagged over-expression line of *KNU*

In similar way, the *KNU* coding sequence together with the 5'UTR region was generated from cDNA (generated from mRNA of wild-type flowers) using primers KK-180/KK-198. These primers incorporated the *Eco*RI restriction sites. The resulting PCR product was cleaved to a pBJ36 vector containing *mGFP5* coding sequence. The fusion was confirmed using PCR-based genotyping assay with use of primers KK-35/KK-95. The *KNU-GFP* fusion was re-amplified using primers KK-180/KK-248 and cleaved with *Eco*RI enzyme. The resulting PCR product was digested with *Eco*RI and was ligated to pBJ36 containing 35S promoter, cleaved with the same enzyme. The insertion was verified by PCR and the vector cleaved with *Not*I and ligated to binary vector

pML-BART, as described above. Colony PCRs and genotyping assays were performed using primers KK-180/KK-35.

2.15.6 Generation of a line allowing for inducible over-expression of *KNU*

In order to generate a line allowing for inducible over-expression of *KNU*, the coding sequence of *KNU* that was generated in Section 2.15.5 was ligated to the *EcoRI*-cleaved pBJ36 containing the 6xOP promoter sequence. Once the presence of the insert has been verified by colony PCR, this vector was cleaved with *NotI* and ligated with the binary vector pML-BART, containing 35S_{pro}:GR-LhG4 cassette, that was also cleaved with *NotI* and treated with alkaline phosphatase (Roche). Colony PCR and genotyping assays were performed using primers KK-180/KK-148.

2.15.7 Generation of an epitope-tagged amiRNA-resistant version of *KNU*

The primer combinations DM-90/DM-93 and DM-91/DM-92 were used to generate two PCR fragments that were subsequently used in an overlapping PCR with primers DM-90 and DM-91. The primers DM-92/DM-93 introduced synonymous nucleotide substitutions into the region of the *KNU* coding sequence that the KNU-amiRNA targets. The primers DM-90/91 introduced the restriction sites *PstI* and *EcoRI* that were ~2.6 kb upstream of the translational start site and at the end of the *KNU* coding sequence, respectively. This PCR product was then ligated to a pBJ36-derived vector containing the *mGFP5*-coding sequence followed by the 3'OCS terminator sequence. Once the presence of the *KNU*-encoding fragment was confirmed by colony PCR, this vector was cleaved using *NotI* and ligated to the binary vector pART27 that had been cleaved with *NotI* and treated with alkaline phosphatase (Roche). Plants containing the 35S_{pro}:KNU-amiRNA transgene (line #2) were transformed with this construct. First generation transformants were identified based on kanamycin resistance and were genotyped using PCR. Colony PCR and genotyping assays were performed using the primers DM-59 and DM-31.

2.15.8 Crossing KNU_{pro}:rKNU-GFP, 35S_{pro}:KNUamiRNA into the FIS

Pollen from a AP1_{pro}:AP1-GR *ap1-1 cal-1*, 35S_{pro}:KNU-amiRNA line was used to fertilize emasculated flowers from a KNU_{pro}:rKNU-mGFP5 plant. F₁ plants were genotyped for the presence of the KNU-amiRNA insert, as described in Section 2.15.1, and seeds were collected from plants homozygous for this allele. F₂ plants that exhibited an *ap1 cal* mutant phenotype were genotyped for the presence of the AP1_{pro}:AP1-GR transgene using primers DM-400/pML-BART and for the presence of KNU_{pro}:rKNU-mGFP with primers DM-59/DM-31. Finally, F₃ plants that were homozygous for all mutations and for the T-DNA were isolated (genotyped using primer pairs DM-647/DM-664 (absence of T-DNA) and DM-647/pMLBart-3 (presence of T-DNA)). However, because no positional genotyping assay was available for the T-DNA encoding the KNU_{pro}:rKNU-mGFP fusion, a segregation analysis had to be carried out on the offspring of individual F₃ plants in order to isolate a line that was homozygous for the rescue transgene.

2.15.9 Isolation of AlcA_{pro}:KNU-amiRNA1 AP1_{pro}:AP1-GR *ap1-1 cal-1* plants

Pollen from FIS plants was used to fertilize emasculated flowers of a plant encoding the AlcA_{pro}:KNU-amiRNA (line #16). F₁ plants were genotyped for the presence of the AP1_{pro}:AP1-GR transgene using primers KK-59/KK-61. F₂ plants were checked for the presence of an *ap1-1 cal-1* double-mutant phenotype and treated with a dexamethasone-containing solution (see Section 2.8.2). Plants that responded to treatment with synchronous flowering (i.e. plants that had at least one copy of the AP1_{pro}:AP1-GR transgene) were then genotyped for the presence of the AlcA_{pro}:KNU-amiRNA transgene using primers KK-35/KK-95. These plants were then genotyped for the homozygous presence of the AP1_{pro}:AP1-GR-containing T-DNA using primer pairs DM-647/DM-664 (genomic locus in the absence of T-DNA) and DM-647/pMLBart-3 (presence of T-DNA at the expected genomic locus). Because the genomic location of the AlcA_{pro}:KNU-amiRNA transgene was unknown, a segregation analysis was carried out in the offspring of plants from the F₂ population to isolate plants that were homozygous for this T-DNA.

2.15.10 Cloning of a construct for over-expression of KNU-VP16

In order to generate the gain-of-function construct and translational fusion construct with constitutive activator domains, the *KNU* coding sequence was amplified using cDNA derived from *L-er* inflorescence. The *KNU* sequence was amplified with primers KK-180/KK-198 that introduced the *EcoRI* site. This PCR product was then ligated to a pBJ36-derived vector containing the 35S_{pro}:VP-16 activator cassette that was also cleaved with *EcoRI*. Once the presence of the *KNU*-encoding fragment was confirmed by colony PCR, this vector was cleaved using *NotI* and ligated to the binary vector pML-BART that had been cleaved with *NotI* and treated with alkaline phosphatase (Roche). Colony PCR and genotyping assays were performed using primers KK-180/KK-35.

To generate the mutated version of KNU (EAR-like repressive domain; mKNU), the *KNU* coding sequence was amplified using primers KK-180/KK-196 that introduced the mutation leucine (Leu) to alanine (Ala). Conserved leucines within the EAR-like motif of KNU were converted to alanines (**CTAGAGCTTTCTCTCCGTTTA** to **gcAGAGgcTTCTgcCCGTgcA**) by site-directed mutagenesis. This PCR product was then sequenced to ensure for the proper introduced mutations. The correct product was then ligated in the same way as described above.

CHAPTER 3 – IDENTIFICATION OF FUNCTIONS PLAYED BY KNUCKLES IN THE MERISTEMATIC CELLS OF THE FLOWER

3.1 Introduction

Key questions in developmental biology are how stem cells are maintained and how their differentiation is regulated to bring about cell type formation. In the floral meristems of *Arabidopsis*, stem cells are terminated after the production of a specific number of floral primordia; namely 4 sepals, 4 petals, 6 stamens and 2 carpels, which congenitally fuse to form the gynoecium. The homeodomain transcription factor WUSCHEL (WUS) is essential for the maintenance of stem cells in both shoot apical and floral meristems (Laux et al., 1996). *WUS* expression is confined to a small number of cells in the center of the meristem, termed the Organizing Center, by the inhibitory effects of the CLAVATA/CORYNE receptor/ligand signaling pathway. Determinacy of the floral meristem is established by the activities of other factors such as AG and KNU, which repress *WUS* through direct or indirect interactions (Sun et al., 2009). However, the fate of stem cells is controlled by numerous other factors (Cao et al., 2015; Sablowski, 2007) including epigenetic mechanisms (Ito and Sun, 2009; Sun et al., 2014).

The aim of this chapter is to identify processes during floral development that are regulated by KNU. As mentioned above, KNU is one of several proteins responsible for floral stem cell termination. Although a genetic framework has been established regarding the activities of KNU in the floral meristem (Sun et al., 2009), where exactly its activity is required is not well understood. Furthermore, the genome-wide activities of KNU are largely uncharacterized, as exemplified by the absence of known direct targets of this transcription factor. The work described in this chapter aimed at identifying the regions of the floral meristem where KNU activity is required.

3.1.1 Organization of the shoot apical meristem

In angiosperms, the root and shoot apical meristems (RAM and SAM, respectively), are established at the time of embryogenesis. The RAM is the source of cells required for the production of the entire root system while the SAM is required for the production of

all above-ground organs. In order for a plant to reach maturity and produce offspring, a balance between the loss of meristematic cells for the production of organs and their replenishment through division of stem cells must be achieved.

The SAM can be divided into three distinct radial domains (Steeves 1989). The central zone (CZ) contains stem cells in its apical layers (Fig. 3.1 A). These stem cells divide and their progeny, when displaced to the peripheral zone (PZ) of the meristem, undergo differentiation, leading to organ primordia formation on the flanks of the meristem (Esau, 1977). In the lower layers of the meristem, below the central and peripheral zones, cells of the rib meristem (RM) are located, which give rise to the pith of the stem.

The SAM can also be functionally sub-divided into cell layers (Poethig, 1987). The outermost layer of the so-called tunica consist of epidermal layer L1 and subepidermal layer L2 (Fig. 3.1 A). These layers are derived from anticlinal cell divisions and encompass the layer L3, or corpus, that consist of groups of cells dividing in all directions (Szymkowiak and Sussex, 1992). As expected, communication between neighboring cells as they divide and differentiate in the meristem is essential for proper vegetative and reproductive development (Poethig and Sussex, 1985a, b).

3.1.2 Regulation of meristem maintenance

As mentioned above, *WUS* is essential for the maintenance of shoot apical and floral meristems (Laux et al., 1996). The domain in which *WUS* is active is dependent on the activities of several receptor-ligand complexes. Receptors involved in this process include *CLAVATA1* (*CLV1*), *CLV2*, *CORYNE* (*CRN*), *RECEPTOR-LIKE PROTEIN KINASE2* (*RPK2*), *ERECTA* (*ER*), *BARELY ANY MERISTEM 1* (*BAM1*) and *BAM2*. These receptors are bound by several peptide ligands, of which the one encoded by the *CLV3* gene is arguably the most important. These receptor-ligand complexes initiate phosphorylation cascades that negatively impact on the expression of *WUS*. I will discuss the current state of knowledge regarding these receptor-ligand complexes in the following sections.

WUS acts in a regulatory feedback loop with *CLV* gene products to maintain the meristem. However, *CLV* proteins are not only responsible for the repression of *WUS* expression, but are also required to regulate the size of the stem cell reservoir in the SAM. The latter has been shown through the analysis of *clv* mutants, which produce

dramatically enlarged SAMs (Clark et al., 1993). This suggests that *CLV* genes control the rate of cell division in the SAM. In *clv* mutants, enlarged floral meristems lead to an increased number of floral organs (Clark et al., 1995). Furthermore, some plants of strong *clv* mutants accumulate undifferentiated cells inside of the pistil. This also confirms that *CLV* genes are involved in regulating the balance between meristem cell accumulation and differentiation. Genetic analyses have shown that the three *CLV* genes act together in the control of meristem size (Clark et al., 1995) and the subsequent molecular analysis has revealed that their protein products are part of the same signalling pathway.

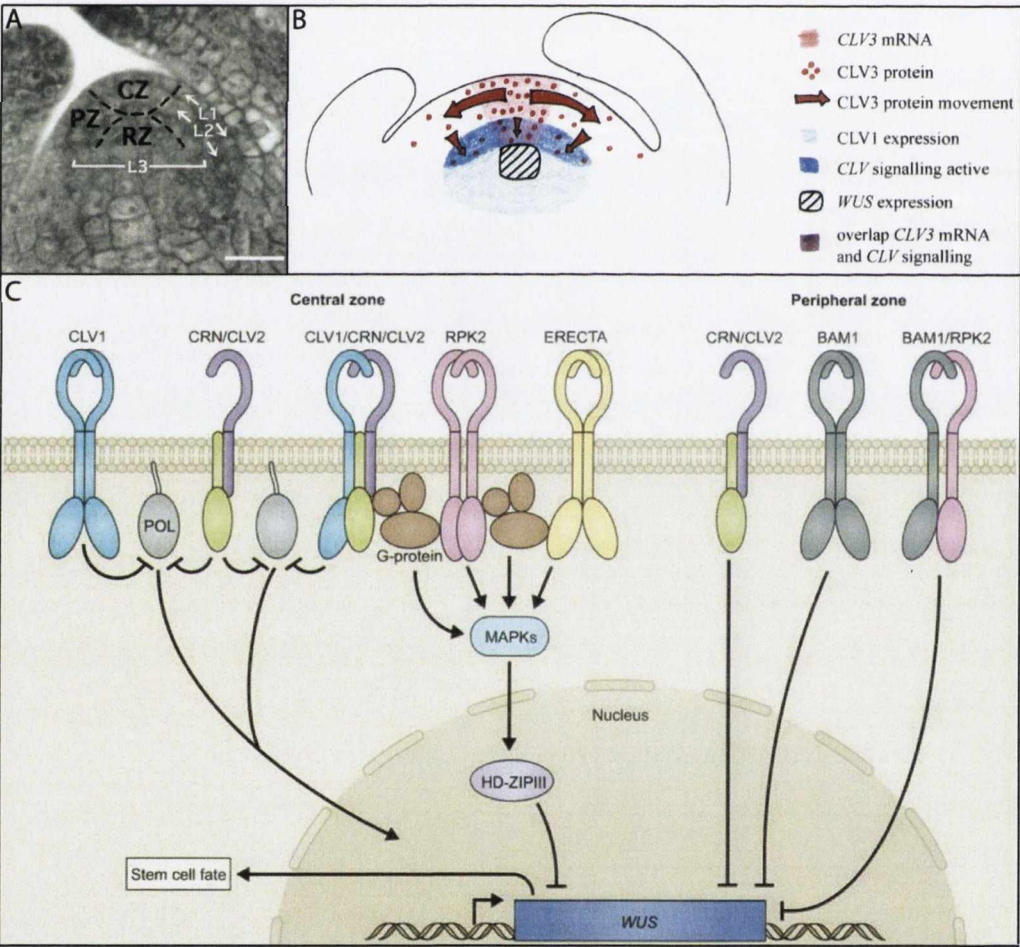


Figure 3.1 Mechanisms of stem cell regulation in *Arabidopsis*. (A) Section through a vegetative SAM showing cell layers and functional domains. CZ, central zone; PZ, peripheral zone; RZ, rib zone; L1, layer 1; L2, layer 2; L3, layer 3 (B) Regulation of stem cell maintenance in the SAM. The functional domains and mechanisms of activity of key stem cell regulators are

indicated. (C) Molecular components of the CLV-WUS signalling pathway. CLV1, CLAVATA1; CLV3, CLAVATA3; CLV2, CLAVATA2; WUS, WUSCHEL; CRN, CORYNE; RPK2, RECEPTOR-LIKE PROTEIN KINASE 2; BAM1, BARLEY ANY MERISTEM1; POL, POLTERGEIST; MAPKs, Mitogen-activated protein kinases. Modified from (Cao et al., 2015; Fletcher, 2002; Lenhard and Laux, 2003; Somssich et al., 2016; Sun and Ito, 2015).

3.1.3 CLV signal transduction

During the early stages of plant development, *CLV1* and *CLV3* are expressed together in the small group of cell in the embryo. As development proceeds the mRNA of *CLV3* expands towards the L1 and L2 cell layers in the SAM (Fletcher et al., 1999). *CLV3* expression is detectable in the central zone and in the apex of the floral meristem where the pluripotent stem cell population resides. On the other hand, *CLV1*, which is also expressed in floral meristems, is localized in the L3 layer of cells and is not present in either L1 or L2 layers (Clark et al., 1997). However, the expression patterns of these two genes partially overlap (Fig. 3.1 B). This phenomenon can be explained by the fact of cell communication via the CLV signaling pathway. The CLV3 protein spreads laterally from the center of the stem cell production (Fig. 3.1 B) and its action can be restricted by the CLV1 receptor, which is critical to prevent the inhibition of *WUS* expression by CLV3 (Lenhard and Laux, 2003).

As describe above, plants lacking functional alleles of the *CLV1*, *CLV2*, *CLV3* genes form enlarged and in some cases, extremely fasciated shoot and floral meristems. These phenotypes are caused by the ectopic expression of *WUS*, which is normally confined to the L3 of wild-type plants. In *clv1*, *clv2* and *clv3* mutants, the *WUS* expression domain is expanded and shifted up one cell layer.

CLV1 is normally expressed in the rib zone, in the L3 cell layer of the flower meristem. In comparison, *CLV2* is not only expressed in the shoot apices but also in siliques or rosette leaves (Jeong et al., 1999). *CLV3*, similarly to *CLV1*, is expressed in the flower meristem, however, in different cell layers (L1 and L2). CLV3 protein moves from the place were it is being produced and therefore acts non- cell autonomously (Fig. 3.1 B). A model that incorporated these three proteins proposed that CLV1 and CLV2 form a complex, which bind CLV3, leading to the phosphorylation of a downstream target. Although this model was attractive, the genetic evidence (Brand et al., 2002) suggested that there were additional components modulating the expression of *WUS*

that influenced the activities of the CLV proteins. For example, the phenotype of *clv2* mutants is much weaker than those of either *clv1* or *clv3*. Therefore, a suppressor screen in *CLV3* overexpressing plants was initiated and the *CRN* gene was identified. *CRN* encodes a membrane-associated receptor kinase that acts closely with *CLV2* (Muller et al., 2008).

Other components of this system have been identified by similar methods including *BAM1* and *BAM2*, which are expressed on the flanks of the meristem, and *RPK2*, that plays a role in anther and embryo development (Mizuno et al., 2007). These genes play redundant roles with the *CLV* receptors and the single mutant phenotypes are much weaker than *clv1* or *clv3*.

Studies on *clv1* and *clv3* mutant plants show that the expression domain of *CLV3* is highly enlarged. Taking into account the fact that *CLV3* is only expressed in stem cells, the presence of excess stem cells in the mutant plants is inferred (Fletcher et al., 1999). The similar enlargement of *CLV3* expression domain can be observed in the *clv2* mutant meristem. This suggests that three *CLV* genes serve to limit the number of stem cells expressing *CLV3*. The generation of transgenic plants overexpressing *CLV3* shown that the *CLV3* ligand is a crucial component involved in regulation of the size of the stem cell reservoir and therefore the number of lateral organs that is produced by the meristem (Brand et al., 2000). In conclusion, these data imply that the *CLV1*, -2 and -3 proteins act together in a stem cell-control pathway that functions with *WUS* as its major target (Brand et al., 2000; Schoof et al., 2000).

3.1.4 CLV receptor localization and interactions

As it was described above, the *CLV* genes play pivotal roles in the function of the meristem. *CLV1*, *CLV2* and *CLV3* encode for three units of the signal transduction pathway that is able to transmit the cell fate information between the cells on the border of shoot and floral meristem (for review see: (Fletcher, 2002)). Biochemical studies support binding of the *CLV* proteins in the plasma membrane to create a signaling complex (Trotochaud et al., 1999). Detailed characterization of mutants and genetic analysis shed more light on the identification of the components of the *CLV* signaling pathway. *CLV1* is a receptor-like kinase that contains extracellular Leu-rich repeats, a transmembrane domain and a cytoplasmic Ser/Thr kinase domain. *CLV2* acts as a receptor-like protein and is similar to *CLV1*, but lacks a kinase domain (Brand et al.,

2000; Schoof et al., 2000). The CLV3 peptide is processed from the larger precursor protein (Kondo et al., 2006) and belongs to the CLE protein family, consisting of a 12-amino acid long sequence, which is conserved within this group (Sharma et al., 2003). It was shown that CLE peptides, together with CLV3, play an essential role in the development of meristems as their misexpression can lead to an arrest of root meristem activity (Casamitjana-Martinez et al., 2003; Hobe et al., 2003).

The recent models of the receptor complex indicate that CLV3 as a binding component of receptor complex (consisting of CLV1 and CLV2 and CLV3 peptides) is binding the extracellular domain of CLV1 only (Ogawa et al., 2008). Yet, genetic screening identified additional important components of the receptor complex. The genetic interaction between weak *clv1* and *clv2* alleles and mutants for the *POLTERGEIST* (*POL*), which encodes a protein phosphatase, revealed suppression of the *clv* phenotypes and suggests that *POL* functions as a crucial regulator of meristem development. In other words, *POL* is negatively regulated by CLV3 signaling (Yu et al., 2000). Moreover, expression of *POL* activates the expression of *WUS* in the meristem (Song and Clark, 2005; Song et al., 2006; Yu et al., 2003).

As mentioned above, further studies on plants that overexpressed *CLV3* revealed the receptor kinase CRN that acts together with CLV2 and in parallel with CLV1 to perceive the CLV3 signal. The mutation in the gene leads to the overproliferation of stem cells. This is characteristic to *clv* mutants, which may indicate that the protein is a important component of the CLV3 pathway (Muller et al., 2008). It was later confirmed that CRN acts as a heterodimer with CLV2 and perceives the signal from CLV3 in the CLV1-independent manner (Bleckmann et al., 2010; Guo and Clark, 2010).

Another receptor-like kinase that works in parallel with CLV1 and CLV2/CRN complexes is RECEPTOR-LIKE PROTEIN KINASE2 (RPK2). It was identified in a screen for CLV-insensitive mutants where CLV3 peptide was used (Kinoshita et al., 2010). Using photoaffinity labeling, it was shown that RPK2 does not bind directly to CLV3 (Shinohara and Matsubayashi, 2015). However, the same study revealed that BAM1 can bind directly to the CLV3 peptide. BAM 1, 2 and 3 are CLV1-related receptor kinases that are expressed in different domains of the meristem (DeYoung et al., 2006). Their function seems to be redundant and partially complement CLV1 function in the organizing center when CLV1 is absent (Nimchuk et al., 2015).

Taken together, the CLV signalling pathway is complex and involves several proteins that contribute either directly or indirectly to perceiving the CLV3 signal originating from the stem cell population.

3.1.5 Regulation of floral stem cell termination

To ensure successful plant reproduction, floral meristems terminate at a specific stage of development when all floral organ primordia have been initiated, which in *Arabidopsis* takes place at approximately stage 6. Several genes have been implicated in the control of floral meristem determinacy, some of which will be described below.

AGAMOUS (*AG*) activity directs the development of the female and male reproductive organs while simultaneously establishing a determinate meristem (Bowman et al., 1989; Lohmann et al., 2001; Yanofsky et al., 1990). The expression of *WUS* persists in *ag* mutants and it has been proposed that *AG* directly negatively regulates *WUS* expression while *WUS* and the plant-specific transcription factor *LEAFY* (*LFY*) initially promote the expression of *AG* forming a negative feedback loop (Lenhard et al., 2001; Parcy et al., 2002). Given that the *WUS* expression domain is a subset of the *AG* expression domain and that *WUS* expression is maintained until stage 6 while *AG* expression commences at stage 3 of flower development, it was reasoned that additional factors were required for *AG*-mediated *WUS* expression termination (Bowman et al., 1989; Das et al., 2009; Sun et al., 2009). Additional support for this idea comes from conditional *AG* overexpression experiments, which failed to prematurely terminate the floral meristem (Mizukami and Ma, 1992).

The C2H2 transcription factor-coding gene *KNU* is also required for floral meristem determinacy. In *knu* mutants, ectopic stamens and carpels develop from the placenta inside of the developing gynoecium and as in *ag* mutants, *WUS* expression is prolonged in these mutants (Payne et al., 2004; Sun et al., 2009). *KNU* is expressed throughout the developing gynoecium from stage 6 onward and becomes restricted to a domain at the base of the gynoecium, which overlaps with the *WUS* expression domain (Lenhard et al., 2001; Payne et al., 2004). *KNU* is a direct target of *AG* (Sun et al., 2009) and it has been proposed that *KNU* and *AG* repress *WUS* expression in a step-wise manner (Liu et al., 2011). However, direct binding of *KNU* to the *WUS* promoter has not been demonstrated. The proposed mechanistic model involving *AG*, *KNU* and

WUS also includes a role for AG in removing trimethyl groups from lysine 27 of histone 3 to activate *KNU* expression (Sun et al., 2014; Sun et al., 2009).

As mentioned above, the C2H2 zinc-finger transcription factor *KNU* represses *WUS* activity (Sun et al., 2009). Consequently, *KNU* is necessary for controlling the determinacy of the stem cells. The double mutant *knu-1 wus-1* resembles the *wus-1* single mutant, indicating that the indeterminate phenotype of *knu* is dependent on the activity of *WUS*. Finally, it has been suggested that the phenotypes observed in the *knu* mutant plants are due to the prolonged expression of *WUS*, which leads to the sustained production of the stem cells. (Sun et al., 2009).

As described in the first chapter, *KNU* is expressed through the developing gynoecium and together with AG and *WUS*, plays a role in the maintenance of the meristem. *WUS* is expressed early in developing flower and at stage 3-4 it activates AG. Later in flower development, at stage 6, AG activates *KNU* expression (Fig. 3.1 C), which leads to *WUS* expression being suppressed, and consequently to termination of stem cell proliferation (Sun et al., 2009). *WUS* is the main player in the maintenance of flower meristems (Laux et al., 1996). The *wus* mutant fails to properly organize shoot and floral meristems whereas in the *wus ag* double mutant, *WUS* is responsible for establishment of excess organs in the center of the flower (Laux et al., 1996). Flowers of *knu* mutant produce indeterminate carpels inside the main carpel, resulting in a knuckle-like phenotype. The reiteration of carpels indicates that stem cell maintenance has been perturbed (Payne et al., 2004).

SUPERMAN, a C2H2 zinc finger transcription factor closely related to *KNU*, acts as a transcriptional repressor in flower development (Hiratsu et al., 2002). Mutant plants produce supernumerary stamens in the place of carpels, suggesting that *SUP* is playing a role in maintaining the boundary between the 3rd and 4th whorl (Sakai et al., 1995). *SUP* is also known to function in the meristem regulation in the parallel level to AG. The double mutant *ag-1 sup* produces enlarged floral meristems and generates flowers with petal reiterations (Bowman et al., 1992).

Another gene that is involved in flower meristem control is *CRABS CLAW* (*CRC*). The single mutant *crc-1* does not show any defects in the floral meristem, however in combination with other mutants ectopic floral organs are produced (Prunet et al., 2008; Zuniga-Mayo et al., 2012). It is a gene acting downstream of AG and is directly regulated by this transcription factor (Lee et al. 2005). Its expression starts at

stage 5 in the carpel primordia where it might regulate *WUS* in a non-cell autonomous manner (Bowman et al., 1999; Lee et al., 2005)

Other factors that also are involved in the regulation of floral stem meristem are REBELOTE (RBL) and SQUINT (SQN). These two genes strengthen the expression of *AG*, and in the *rbl sqn* double mutant flowers the *WUS* expression is prolonged. In addition double mutants produce supernumerary reproductive organs in the flowers (Prunet et al., 2008).

PERIANTHIA (PAN), a bZIP transcription factor, also plays a role in regulating stem cell activity. The levels of *AG* mRNA in *pan* mutants are reduced in short-day conditions, leading to an increased number of floral organs (Das et al., 2009; Running and Meyerowitz, 1996). Moreover, stronger meristem indeterminacy is observed in double mutants of *pan lfy* and *pan seu* (*seuss*) where ectopic floral organs grown inside the carpels. These findings also suggest that *LFY* and *SEU* have potential effect on the floral meristem regulation (Das et al., 2009; Wynn et al., 2014).

The receptor-kinase ERECTA (ER) that plays a role in the parallel pathway to the *WUS-CLV* in the SAM has been shown to regulate the expression of *WUS*. Double mutants of *er jba-1D/+* produce enlarged floral meristems and SAMs. The ectopic expression of *AG* was also detected in the double mutant. These plants produce ectopic carpels from the inflorescence meristem. This might indicate an indirect role of *ER* in the floral meristem identity control (Mandel et al., 2014).

Mutations in AUXIN RESPONSE FACTOR 3 (ARF3) have been reported to increase the severity of the *ag-10* phenotype. The double mutant *ag-10 arf3-29* produces flowers with ectopic floral organs arising from the unfused sepaloid carpels. This suggests a reinforcement of the stem cell determinacy through repression of *WUS* expression (Liu et al., 2014a). Moreover it was recently found that DNA topoisomerase *TOPOISOMERASE1a* gene enhances the *ag-10* phenotype. The double mutant *ag-10 top1a-2* produces an indeterminate floral meristem (Liu et al., 2014b).

In addition to the proteins described above, there are many microRNAs that are reported to be involved indirectly in floral meristem determinacy. *miRNA172* is known to promote termination of stem cells by lowering the expression of *AP2*. Plants expressing the ectopic amounts of *miRNA172* resistant *AP2*, produce indeterminate stamens and petals (Chen, 2004; Zhao et al., 2007).

miR165/166 target two class III HD-ZIP genes: *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*). Plants overexpressing both of the microRNAs in the background

of *ag-10* or *miR165/66* resistant *phb* and *phv* alleles can produce indeterminate growth of floral organs (Ji et al., 2011). Similarly, the triple mutant of *phb phv cna* generates additional carpels in the flowers. The *miRNA166a* regulates the expression levels of *PHB*, *PHV* and *CORONA* (*CNA*), which leads to enlarged shoot meristems in *Arabidopsis* (Prigge et al., 2005; Williams et al., 2005).

The process of stem cell termination is a well studied process hence more components of the process will be described in the near future. Regardless of the facts that are already known, further details are necessary to describe the termination of the meristem in more details. Some insights will be shown in the Results section below.

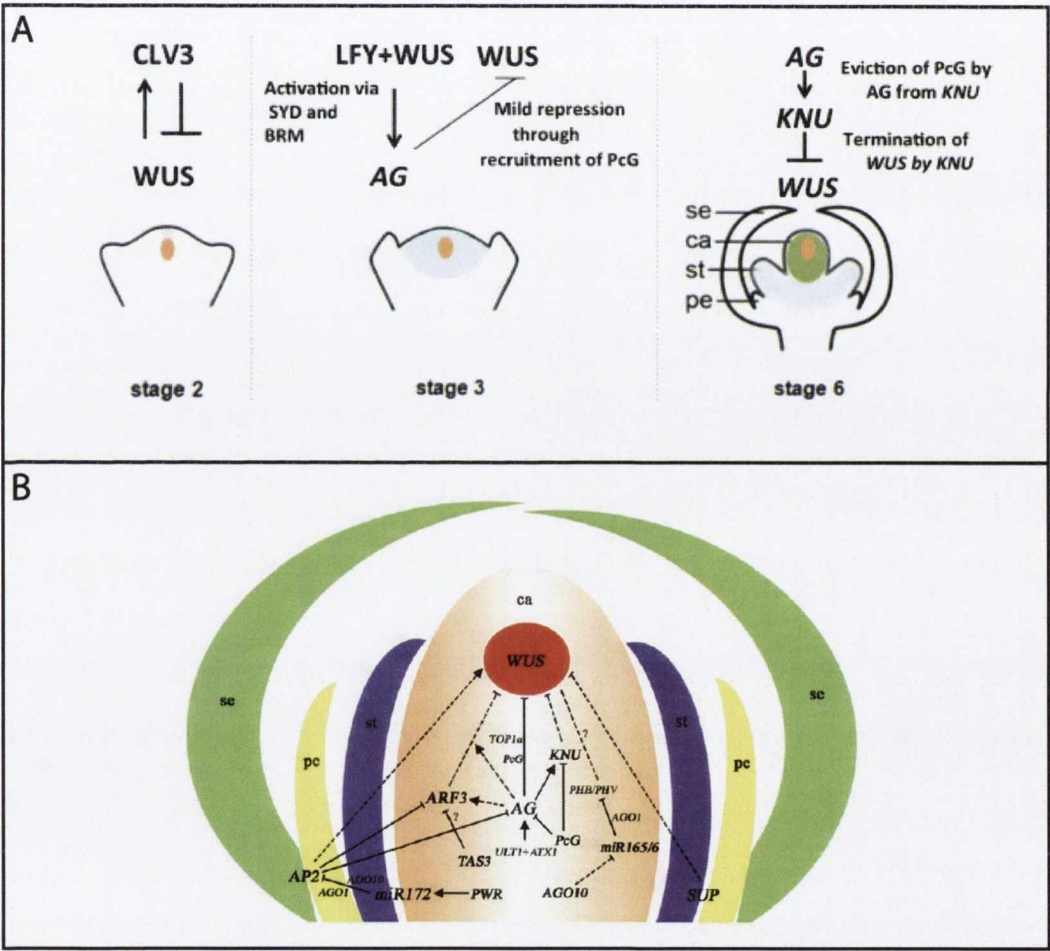


Figure 3.2 Mechanisms of floral stem cell termination in *Arabidopsis*. (A) Regulation of the timing of floral stem cell termination. At stage 2 (on the left), *WUS* is repressed by *CLV3* signalling. At stage 3 (center), activation of *AG* expression in the center of the floral meristem leads to repression of *WUS*. At stage 6 (on the right), *AG* activates *KNU* expression, leading to an inactivation of *WUS* and stem cell termination. (B) *WUS* expression is regulated by many

different genetic pathways and epigenetic mechanisms. PcG, Polycomb Group; SYD, SPLAYED; BRM, BRAHMA; AGO1, AGRONAUTE1; AGO10, AGRONAUTE10; PWR, POWERDRESS; ARF3, AUXIN RESPONSE FACTOR3; TAS3, TRANS-ACTING SIRNA 3. Modified from (Cao et al., 2015; Sun and Ito, 2015).

3.1.6 Epigenetic mechanisms that control meristem regulation

A growing number of studies show that the meristem fate is controlled by a large number of epigenetic mechanisms, including deposition of repressive mark H3 lysine 27 tri-methylation (H3K27me3) on the locus of *WUS*. This repressive mark is associated with Polycomb Repressive Complex2 (PRC2), which is composed of Polycomb Group proteins (PcG). These proteins act as repressors of expression. In contrast, trithorax group proteins (trxG) are activators of gene expression and by accumulating the H3 lysine 4 tri-methylation mark, initiate on the process of gene regulation.

Epigenetic regulation of *WUS* in floral meristems was reported by Liu et al. (2011). It was shown that the MADS-domain transcription factor AG binds directly to *WUS* and represses its expression. Through an ethyl methanesulfonate mutagenesis (EMS) screen of the weak *ag-10* allele a mutant allele of *CURLY LEAF (CLF)* *clf-47* was identified. This suggests that CLF plays a role in floral meristem termination (Liu et al., 2011). CLF is one of the components of mentioned above PRC2. This finding might indicate that *WUS* is repressed by AG by the deposition of the H3K27me3 repressive mark. *Ag-10 clf-47* double mutants produce ectopic organs inside enlarged carpels. This phenotype is similar to the one observed in the double mutant *ag-10 tfl2-2*. The *TERMINAL FLOWER 2 (TFL2)* allele, which is a PRC1 factor, also enhances the indeterminate phenotype of *ag-10*. These results indicate that PcG targets *WUS*, and AG is responsible for recruiting these proteins onto the *WUS* locus (Liu et al., 2011).

While AG represses *WUS* weakly only after stage 3 of flower development, KNU terminates floral stem cell at stage 6 by repressing its expression (Payne et al., 2004; Sun et al., 2009). Similarly to *WUS*, PcG-mediated H3K27me3 also regulates KNU while AG is involved in the removal of the repressive mark. Level of the H3K27me3 mark on the *KNU* locus reduces during the 2 days that takes to induce its expression. This might indicate the relationship between the transcriptional activation of

KNU by AG and the possible removal of the H3K27m3 mark from *KNU* locus in an AG-dependent manner (Sun et al., 2009).

Further studies proposed that AG removes the PcG to activate *KNU*. Two components of PcG, FIE (FERTILIZATION-INDEPENDENT ENDOSPERM) and EMF2 (EMRYONIC FLOWER 2) are bound to the specific promoter regions of *KNU*, which include the binding sites for AG. Moreover, these regions include the Polycomb response element (PRE) that play important role in PcG-mediated silencing of a ubiquitous promoter (Sun et al., 2014). This competitive binding between AG and PcG on the *KNU* locus was mimicked by the introduction of an artificial protein (LacI-GR) that contained the lactose operon operator DNA binding domain and glucocorticoid hormone binding domain, but no detectable transactivation activity (Sun et al., 2014). Furthermore, two of three AG binding sequences on the *KNU* promoter were replaced with by two lac operator sequences and the third one was mutated in order to prevent AG binding. A clear induction of KNU expression was detected 3 days after the dexamethasone treatment. These results indicate that binding of the chimeric protein on the two operon sequences can block the PcG complex binding and enable the expression of KNU through the removal of the repressive marks, which mimics the endogenous function of AG (Sun et al., 2014).

Another possibility would be that the H3K27me3 mark could be erased by the JmjC-domain-containing histone demethylases REF6, JMJ30 or JMJ32 (Gan et al., 2014; Lu et al., 2011). It has been stated that AG, REF6 and other MADS-domain proteins can form protein complexes with unknown functions (Smaczniak et al., 2012). This suggests that AG might recruit REF6 to remove actively the repressive mark and induce *KNU*.

ULTRAPETALA1 (ULT1), a SAND-domain-containing protein, which activates expression of *AG* in the meristematic cells of the flower (Carles and Fletcher, 2009) appears to act in similar manner. This process is independent from the activation by LFY (LEAFY), which together with WUS, activates *AG* at stage 3 of flower development (Engelhorn et al., 2014). *ult1* mutant flowers exhibit enlarged meristems and prolonged activity of WUS is observed. The enlarged floral meristems give rise to supernumerary petals, explaining the name of the *ult* mutant (Fletcher, 2001). It has been reported that ULT1 may negatively act on floral stem cell proliferation. Studies indicate that WUS expression domain in flower buds is repressed by ULT1 (Carles et al., 2004). This SAND domain containing protein turned out to be a trithorax group

protein (trxG) that possibly interacts with other similar trxG protein, ATX1 (THRITORAX-LIKE PROTEIN1), which is a H3K4me3 methyltransferase (Alvarez-Venegas et al., 2003; Carles and Fletcher, 2009). In this way, the direct binding of ULT1 to *AG* proclaims of the recruitment of ATX1 in order to harmonize the methylation status on the *AG* locus.

In summary, the studies on epigenetic regulation of floral stem cell regulation described above are just the beginning of revealing the many processes underlying production, sustaining and termination of the stem cell fate. The sections below summarize the results of experiments that aimed at clarifying some of the processes necessary for the promotion of stem cell termination.

3.2 Results

3.2.1 Perturbation of *KNU* function

As described in Chapter 1, the perturbation of a regulatory gene followed by the global analysis of gene expression is a powerful technique to understand gene functions and to obtain insights into the hierarchy of gene regulatory networks. To apply this approach to *KNU*, I sought to identify an amiRNA that would efficiently perturb *KNU* activity and that could be combined with an inducible promoter system to specifically knock down *KNU* activity at different stages of flower development (see Section 4.2.1). In addition, such an amiRNA could be used to perform genetic analysis by expressing it from tissue-specific promoters to perturb *KNU* activity in subsets of its expression domain. In the sections below, I describe the identification of an amiRNA that efficiently perturbs *KNU* activity. Furthermore, I will outline the results of experiments that aimed at understanding where in floral meristems *KNU* activity is required.

3.2.2 Identification of an amiRNA that perturbs *KNU* activity

An amiRNA designed to target the *KNU* mRNA was previously generated by Dr Diarmuid O'Maoileidigh as described in Section 2.13.1. This amiRNA (referred to as KNU-amiRNA1; Fig. 3.3 B) was placed under the control of the constitutive

Cauliflower Mosaic Virus (CaMV) 35S promoter (Fig. 3.3 A). The resulting construct was transformed into wild-type plants using the floral dip method. Sixty-three independent transformants were obtained of which 40 displayed *knu*-like phenotypes such as bulged siliques. To quantify the phenotypes of these transformants, the number of bulged siliques produced by each line was determined. Additionally, the levels of *KNU* transcripts in these lines were measured by qRT-PCR. Importantly, there appeared to be a correlation between the strength of the mutant phenotype in a given line and the reduction in *KNU* mRNA levels in that line (Fig. 3.4 A). The line, which displayed the strongest phenotypes (Line #28, ~14% of bulged siliques) and in which the levels of *KNU* mRNA were the lowest (~25% of wild-type levels), was crossed into the FIS as described in Section 2.9.1. This line was later used for the gene expression profiling experiments described in Section 4.2.3.

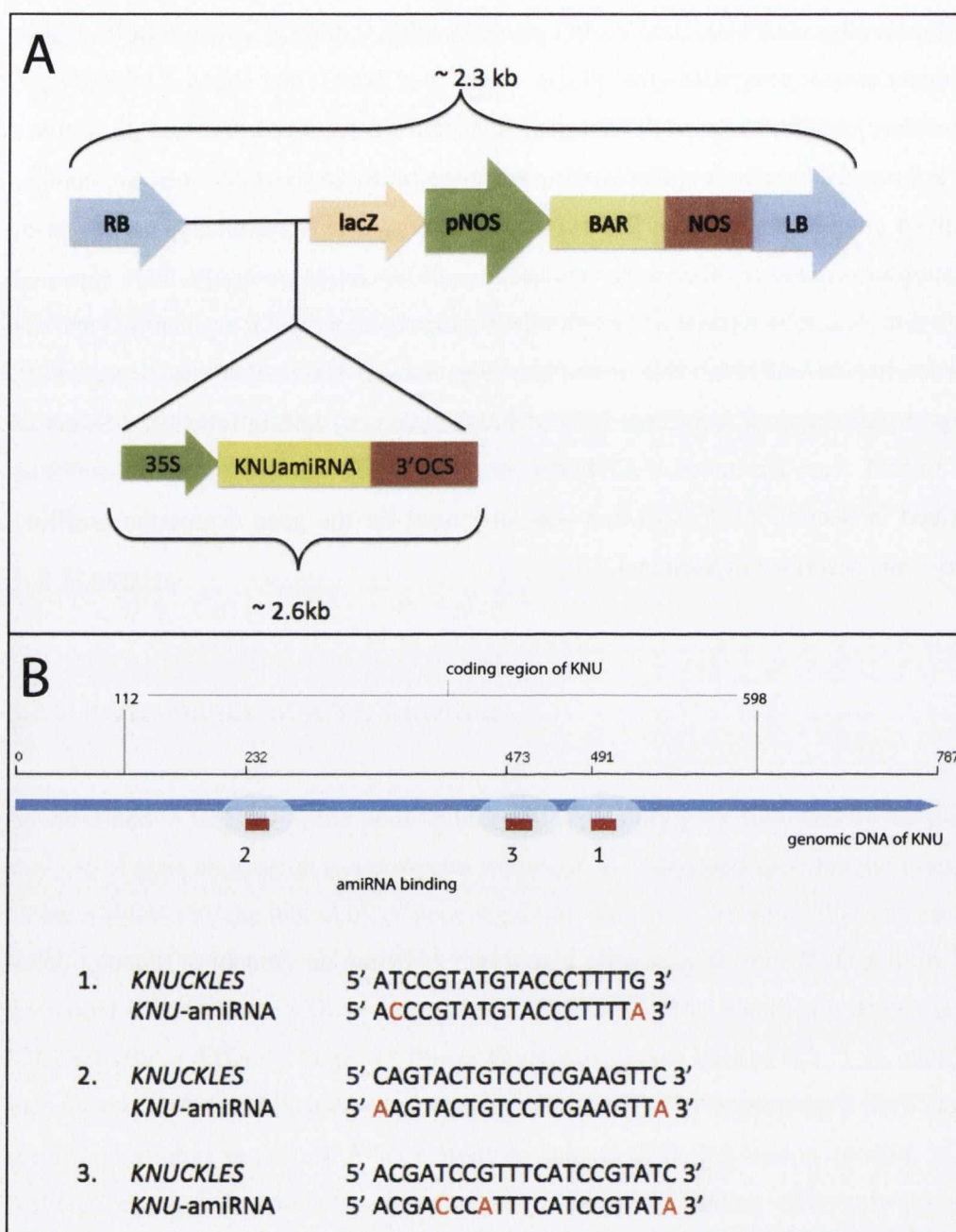


Figure 3.3 Schematic representation of the amiRNAs designed to target *KNU*. (A) A schematic of the T-DNAs mediating the expression of the different *KNU*-amiRNAs under control of the 35S CaMV promoter. (B) A diagram illustrating complementarity of each amiRNA to the genomic region of *KNU*. Sequences of the three amiRNAs designed using the online program *WMD3* (Web MicroRNA Designer, <http://wmd3.weigelworld.org>) are shown. 3'OCS, octopine synthase terminator sequence; RB, right border of T-DNA; LB, left border of T-DNA; lacZ, a modified version of the β -galactosidase-coding gene from *E. coli*; pNOS,

nopaline synthase promoter sequence; BAR, BASTA® resistance gene; MCS, multi cloning site; NOS, nopaline synthase terminator sequence.

To better characterize the activity of the KNU-amiRNA1, I analyzed T2 plants of 22 independent 35S_{pro}:KNU-amiRNA1 lines and determined the percentage of bulged siliques (Fig. 3.4 B). Five of these lines displayed strong phenotypes (10-17% of bulged siliques) (Fig. 3.4 B). Furthermore, these siliques contained ectopic organs as often observed in *knu* mutants (Fig. 3.5 C, D).

Although the appearance of *knu*-like phenotypes and the reduction of *KNU* mRNA levels indicated that the KNU-amiRNA1 was functional, the phenotypes were not as strong as what is found in the *knu-1* allele. Therefore, two additional *KNU*-specific amiRNAs (KNU-amiRNA2 and KNU-amiRNA3) were designed (Fig. 3.3 B) and expressed in wild-type plants from the CaMV 35S promoter. Eighty-two independent transformants were isolated for KNU-amiRNA2 of which 57 displayed *knu*-like phenotypes. Twenty-two independent transformants were isolated for the 35S_{pro}:KNU-amiRNA3 construct of which none displayed *knu*-like phenotypes. These lines were further characterized in the second (T2) generation by determining the percentage of bulged siliques relative to non-bulged siliques. The average percentage of bulged siliques among the KNU-amiRNA2 lines varied but in most cases less than 5% of siliques produced bulges, which contained ectopic carpels and stamens (Fig. 3.5 E, F). However, 8 lines displayed higher percentages of “knuckled” siliques (ranging from 6-27%), indicating that this amiRNA was in principle suitable to efficiently knock down *KNU* activity (Fig. 3.4 C).

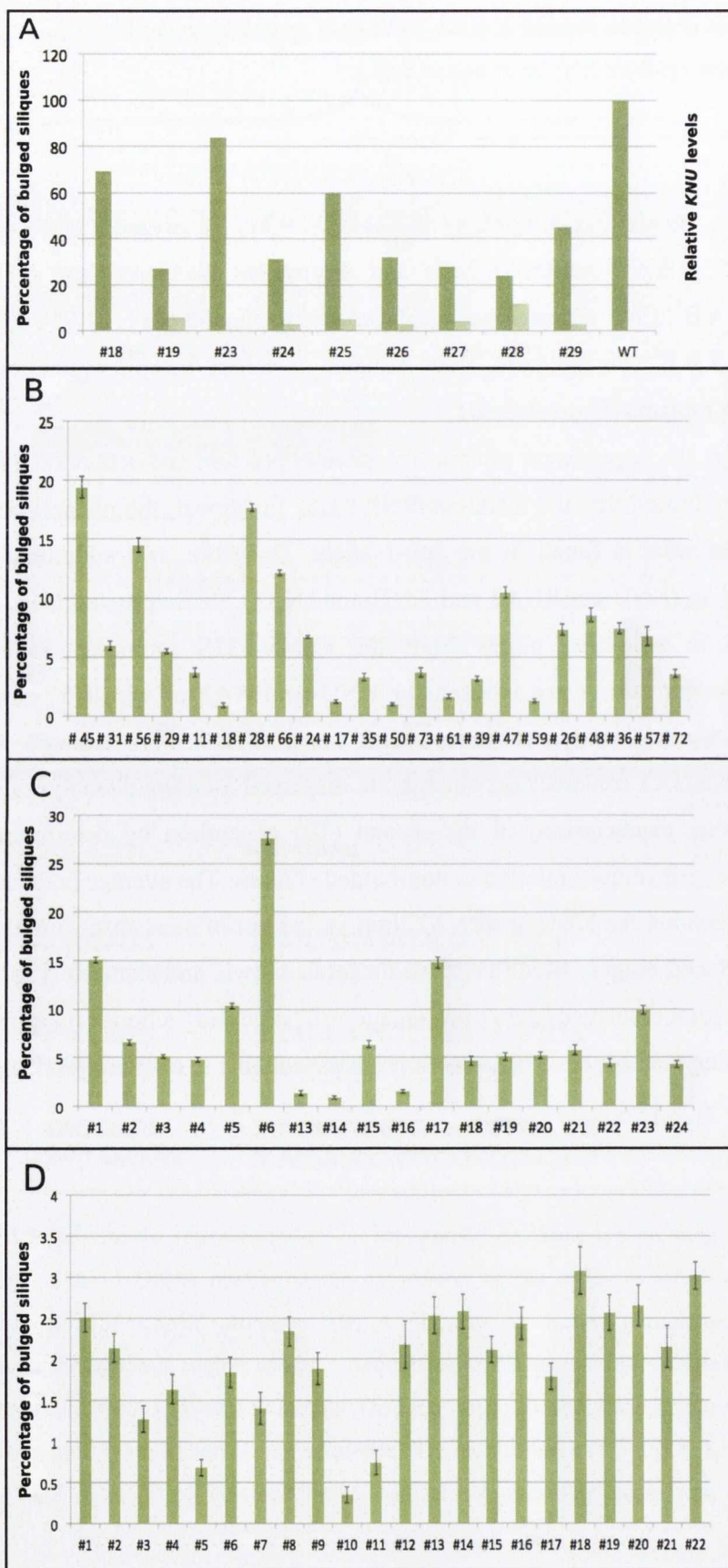


Figure 3.4 Effects of KNU-amiRNA over-expression. (A) Levels of *KNU* transcripts (dark green) in selected 35S_{pro}:KNU-amiRNA1 lines (T1 generation) as determined by qRT-PCR. The percentage of bulged siliques relative to non-bulged siliques present on each plant is shown by light green bars (data adapted from O'Maoileidigh 2012). (B-D) Mean percentage of bulged siliques relative to non-bulged siliques present among T2 plants (homo- or heterozygous for the 35S_{pro}:KNU-amiRNA transgenes). Error bars indicate s.e.m. The siliques of 15 plants were counted per line, and at least 10 siliques per plant. (B) Mean percentage of bulged siliques in selected KNU-amiRNA1 lines. Five lines exhibited relatively strong phenotypes (>10% of bulged siliques). (C) Average percentage of bulged siliques in selected KNU-amiRNA2 lines. One line (no. 6) displayed a much higher percentage of bulged siliques than the others. (D) Mean percentage of bulged siliques in selected KNU-amiRNA3 lines. Phenotypes observed were overall weaker than in the KNU-amiRNA1 and 2 expressing lines.

Further characterization of 22 T2 lines carrying the 35S_{pro}:KNU-amiRNA3 construct showed that the average percentage of bulged siliques was similar in all lines but was also very low (typically around or less than 2%; Fig. 3.4 D). In addition, these siliques did not show the characteristic *knu* phenotype and did not form ectopic stamens or carpels (Fig. 3.5 H). Instead, many of the siliques were tricarpelloid (Fig. 3.5 G). Taken together, these results indicate that the KNU-amiRNA3 was not as efficient in knocking down *KNU* activity as the other two KNU-amiRNAs tested. Furthermore, of the two functional KNU-amiRNAs, the analysis of the T2 plants described above showed that the KNU-amiRNA1 was overall more effective than the KNU-amiRNA2. Consequently, KNU-amiRNA1 was selected for all experiments that involved an amiRNA-mediated knockdown of *KNU* activity.



Figure 3.5 Phenotypes of 35S_{pro}:KNU-amiRNA plants. (A) The comparison of wild-type and *knu-1* mutant siliques. (B) *knu-1* siliques are short and contain ectopic carpels and stamens. In addition, the number of seeds is reduced (indicated by arrows). (C, D) Siliques of plants expressing KNU-amiRNA1 were shorter than wild-type siliques (C) and contained ectopic organs (D). (E, F) Siliques of plants expressing KNU-amiRNA2 were bulged and most of them contained ectopic carpels and stamens. (F) Ectopic carpels produced inside siliques were smaller than the ones produced by *knu-1* mutant plants. (G, H) The “knuckled” phenotype did not occur in plants expressing KNU-amiRNA3 and no ectopic organ formation was observed within these siliques. However, tricarpeoid gynoecia were often observed (G). Size bars: 1 mm.

3.2.3 Effects of *KNU* perturbation in different domains of the floral meristem

As mentioned in the introduction of this chapter, *KNU* has been implicated in the control of floral meristem determinacy. However, the spatial domain in which *KNU* activity is required for this function is not well defined. In particular, it remains to be

tested whether *KNU* activity is required in stem cells or, for example, in the organizing center, which is pivotal for stem cell maintenance. Given that *KNU* has been shown to regulate expression of the stem cell regulator *WUS* (Payne et al., 2004; Sun et al., 2009), which is expressed in the organizing center (Laux et al., 1996), it can be hypothesized that *KNU* activity is of particular importance in this domain. To test this, I generated a set of transgenic plants that expressed the *KNU*-amiRNA1 from the promoters of genes that are active in specific domains of the floral meristem. For this work, I took advantage of previously described LhG4 transactivation driver lines for the promoters of *CLV3*, *WUS* and *CLV1* (Gross-Hardt et al., 2002; Lenhard and Laux, 2003; Schoof et al., 2000). In order to disrupt the activity of *KNU* in specific domains of the meristem, the *KNU*-amiRNA1 was placed under the control of six repeats of the synthetic OP promoter sequences, which can be bound the chimeric LhG4 transcription factor. This work was originally started by Dr Diarmuid O'Maoileidigh in the laboratory and then continued by myself. The results of this analysis are presented in the next section of this chapter.

3.2.3.1 Expression of the *KNU*-amiRNA in the *CLV1* domain

Thirty-one independent *CLV1*_{pro}:LhG4/6xOP_{pro}:*KNU*-amiRNA transformants (denoted *CLV1*_{pro}>>*KNU*-amiRNA hereafter) were obtained by Dr Diarmuid O'Maoileidigh and I analyzed 16 of these lines in the T2 generation for the occurrence of *knu*-like phenotypes. Most of the plants produced only weak mutant phenotypes (at most, 3.5% of siliques were bulged; Fig. 3.6 A), and bulged siliques did not contain any ectopic carpels or stamens (Fig. 3.6 B, C). However, elongated gynophores were often visible, which is a characteristic phenotype of the *knu-1* allele. The number of ovules was also reduced, especially in those plants that showed relatively strong mutant phenotypes. Two lines with the strongest phenotypes (#25 and #27), did produce bulged siliques with ectopic carpels and stamens organs (Fig. 3.6 D). Taken together, these results show that an amiRNA-mediated knockdown of *KNU* from the *CLV1* domain led to only weak phenotypic effects when compared to plants that ectopically express the *KNU*-amiRNA from the 35S promoter (see Fig. 3.5 B, C).

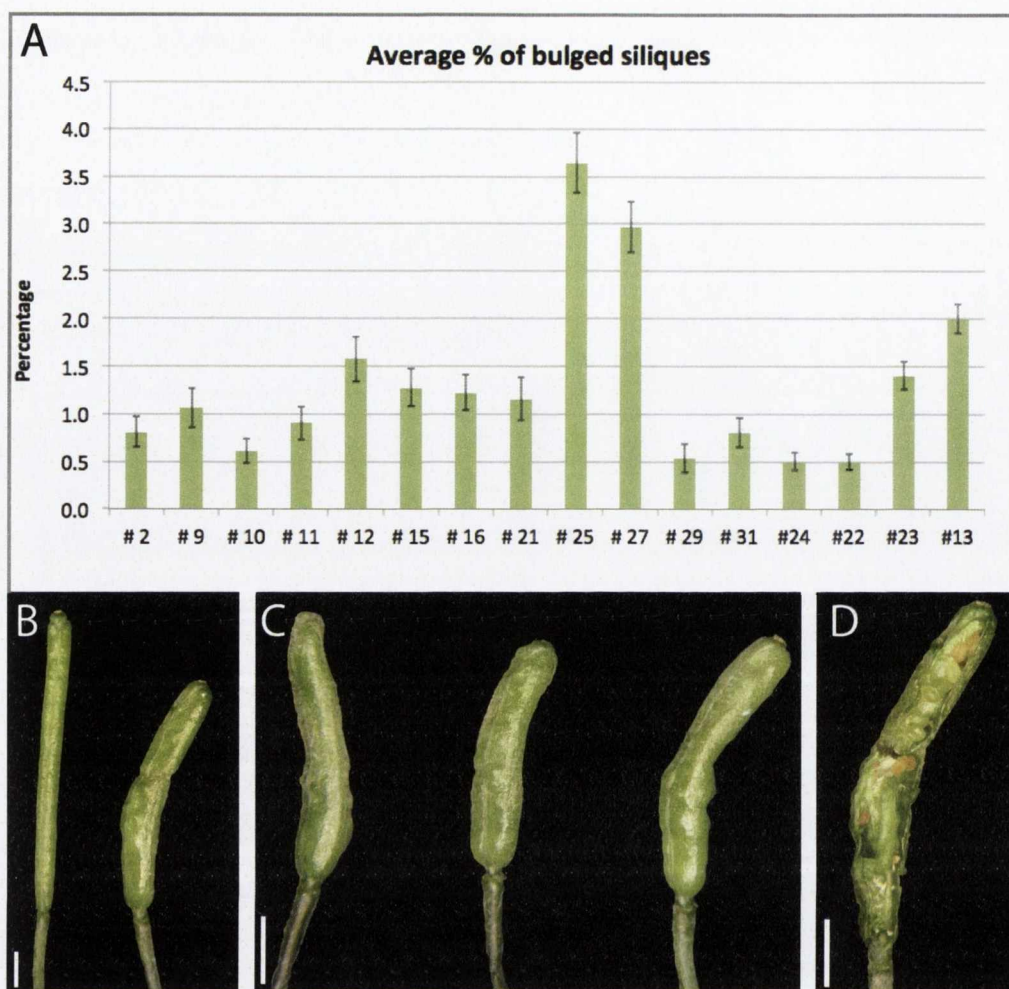


Figure 3.6 Effects of expressing KNU-amiRNA1 in the *CLV1* domain. (A) Average percentage of bulged siliques in selected lines that contained the *CLV1*_{pro}>>KNU-amiRNA1 transgene. Error bars indicate s.e.m. The siliques of 15 plants were counted for each line, and at least 15 siliques per plant. (B) Siliques of a plant expressing KNU-amiRNA1 under control of the *CLV1* promoter and of a wild-type plant. Siliques of *CLV1*_{pro}>>KNU-amiRNA1 plants were sometimes shorter than those of the wild type. (C) Slightly bulged siliques from plants containing the *CLV1*_{pro}>>KNU-amiRNA1 transgene. These siliques did not form ectopic carpels and stamens. (D) Bulged silique of line #27 that contained ectopic carpels and stamens (indicated by arrows). Size bars: 1mm.

3.2.3.2 Expression of the KNU-amiRNA1 in the *WUS* domain

As mentioned above, it has been proposed that KNU is a repressor of *WUS* at stage 6 of flower development (Ito and Sun, 2009). To understand the consequence of removing *KNU* activity specifically in the *WUS* expression domain, a 6xOP_{pro}:KNU-amiRNA1

transgene was introduced into the WUS_{pro}:LhG4 driver line *via* the floral dip method. Seeds from twenty-two independent transformants (denoted WUS_{pro}>>KNU-amiRNA hereafter) were grown and characterized in the T2 generation. Similar to CLV1_{pro}>>KNU-amiRNA plants, the percentage of bulged siliques observed was low and, with the exception of line #12, did not exceed 2% (Fig. 3.7 A). Siliques of the WUS_{pro}>>KNU-amiRNA plants were in most cases shorter than those of the wild type but did not contain ectopic carpels or stamens. The most severe phenotype observed in these lines was a reduced number of seeds (Fig. 3.7 C). Moreover, problems with fertilization and premature ovule abortion were observed. Also, some of the plants formed tricarpeloid siliques (not shown). Line #12 showed the strongest phenotypes (more than 6% of bulged siliques) and produced ectopic carpel and stamen-like organ in some of the siliques (Fig. 3.7 D). This line was later confirmed to be doubly homozygous for both transgenes, and the progeny was used for a cross with KNU_{pro}:KNU-GUS plants (see Section 3.2.5 below).

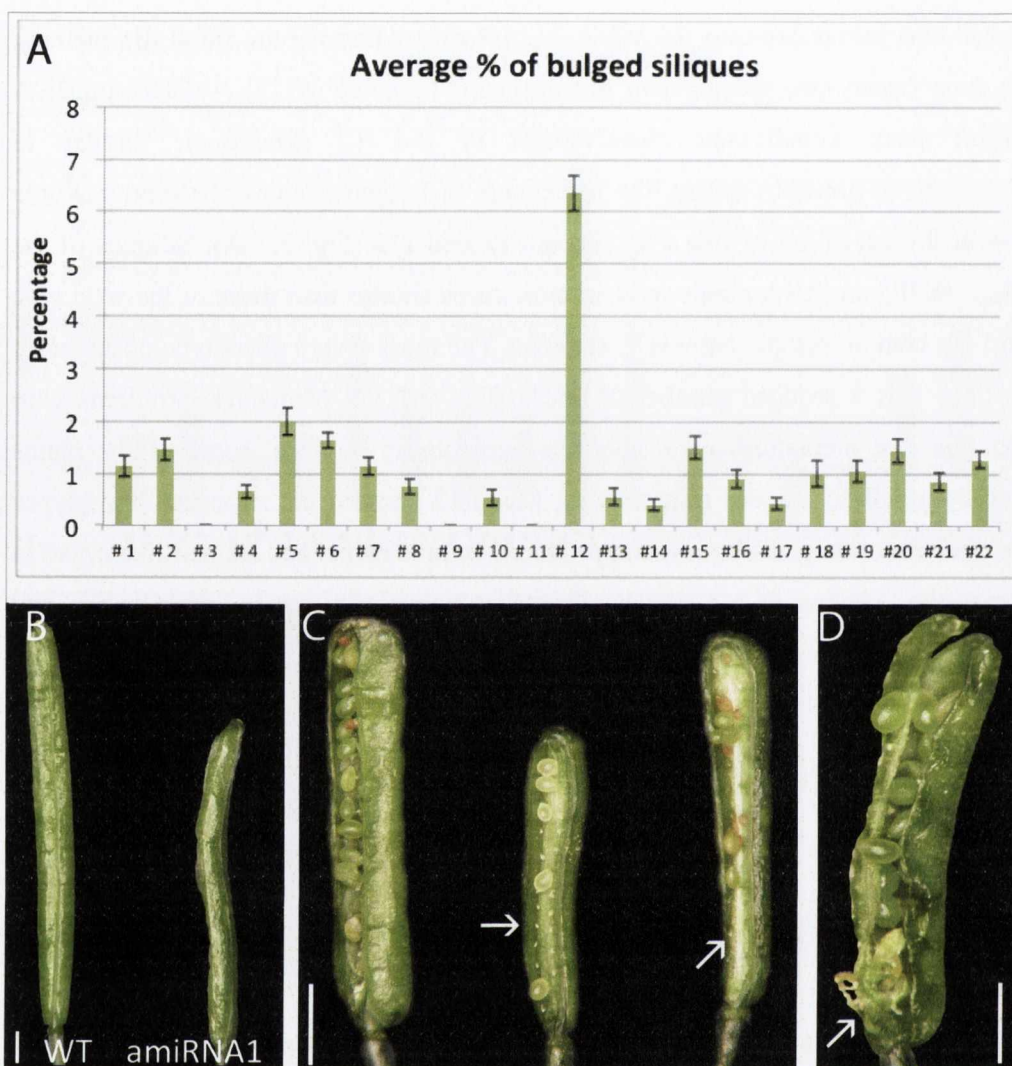


Figure 3.7 Effects of expressing KNU-amiRNA1 in the *WUS* domain. (A) The average percentage of bulged siliques in plants containing the *WUS*_{pro}>>KNU-amiRNA1 transgene. Error bars indicate s.e.m. At least 15 siliques from a minimum of 15 plants were analyzed. (B) Siliques of a plant expressing KNU-amiRNA1 from the *WUS* promoter and of a wild-type plant. (C) Most siliques formed by plants carrying the *WUS*_{pro}>>KNU-amiRNA1 transgene were shorter than wild-type siliques but did not contain ectopic organs. However, they exhibited a reduced number of ovules and produced fewer seeds (indicated by arrows). (D) Only one line (#12) produced ectopic organs within siliques. Size bars: 1 mm.

3.2.3.3 Expression of the KNU-amiRNA in *CLV3* domain

As described above, floral phenotypes were relatively weak in plants that expressed the KNU-amiRNA1 from the *WUS* and *CLV1* promoters, respectively. To test the effect of a

KNU knockdown specifically in the stem cell domain of the floral meristem, I generated plants that expressed the *KNU*-amiRNA1 from the *CLV3* promoter (denoted *CLV1_{pro}*>>*KNU*-amiRNA hereafter). Fifty-five independent transformants were identified of which I characterized 20 in detail. To this end, I analyzed plants of the T2 generation and found that the average percentage of bulged siliques varied in these lines (Fig. 3.8 A). However, they were phenotypically overall much stronger than the *CLV1_{pro}*>>*KNU*-amiRNA and *WUS_{pro}*>>*KNU*-amiRNA lines described above. Seven of the 20 lines displayed relatively strong phenotypes with the percentage of bulged siliques being equal or higher than 10%. Moreover, all of the bulged siliques from these lines contained ectopic stamens and carpels (Fig. 3.8 D). Plants that did not contain ectopic organs inside siliques formed tricarpeloid gynoecia instead (Fig. 3.8 C, on the right). Moreover, the plants with the strongest phenotypes flowered noticeably longer than the wild type, likely as a consequence of a reduced seed set. This phenotype is similar to what is observed in *knu-1* mutant plants. For one line (#10), homozygosity for all transgenes was confirmed and this line was then crossed into a *KNU_{pro}*:*KNU*-GUS background for further characterization (see Section 3.2.5).

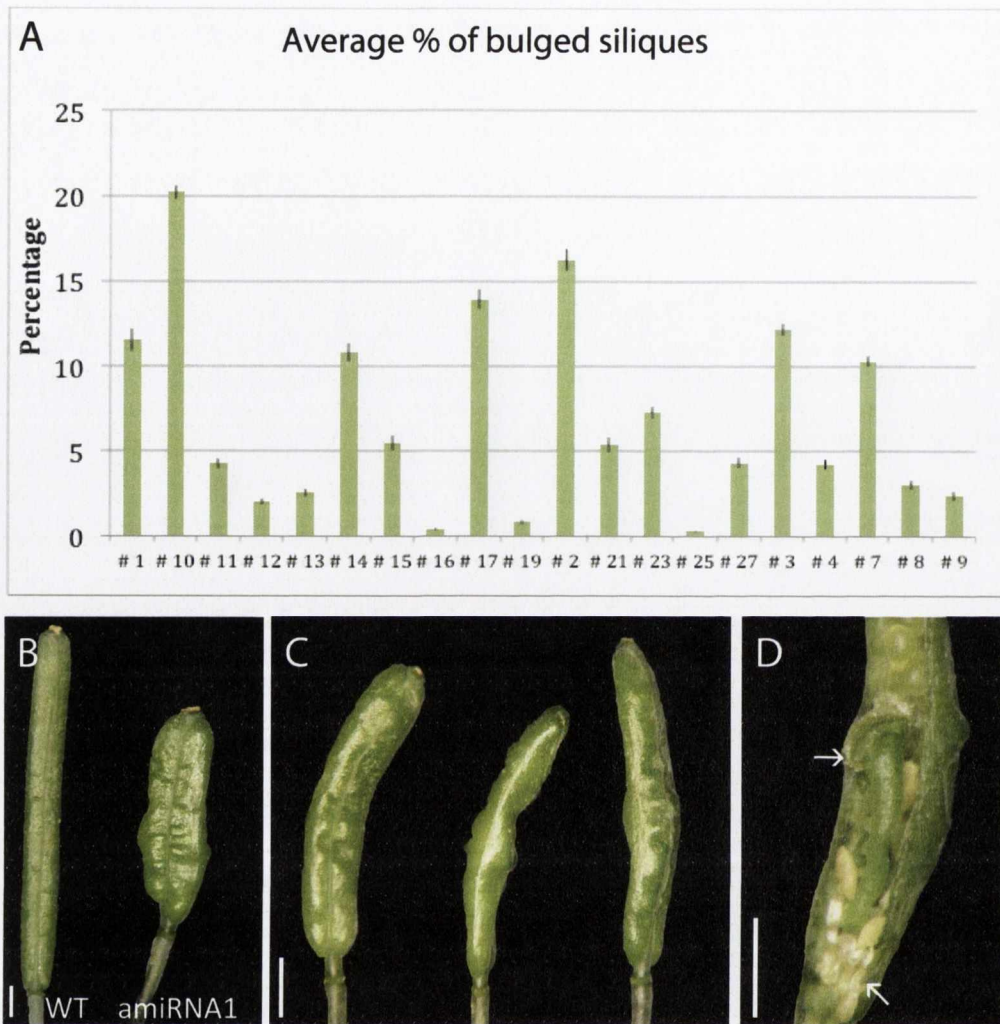


Figure 3.8 Effects of expressing KNU-amiRNA1 in the *CLV3* domain. (A) Percentage of bulged siliques in selected lines that expressed the KNU-amiRNA1 from the *CLV3* promoter. Error bars indicate s.e.m. At least 15 siliques per plant were analyzed from a minimum of 15 plants per line. (B) Siliques of a plant expressing KNU-amiRNA1 from the *CLV3* promoter and of a wild-type plant. The former siliques were much shorter than wild-type siliques. (C) Most of the siliques from *CLV3*_{pro}>>KNU-amiRNA1 plants that were bulged contained ectopic carpels and stamens and some produced tricarpelloid siliques. (D) The plants with the strongest phenotype produced multiple stamens and carpel-like organs with stigmatic tissue (indicated by arrows). Size bars: 1 mm.

3.2.4 Validation of the meristem-specific LhG4 driver lines

The results outlined above suggest that a knockdown of *KNU* in different regions of the floral meristem has different consequences for floral meristem determinacy.

Specifically, the relatively strong phenotypes observed with the $CLV3_{pro}>>KNU$ -amiRNA1 construct suggest that KNU activity in floral stem cells may be of particular importance for meristem control. However, it is also possible that the effects observed are a consequence of driver lines that do not accurately reproduce the expression patterns of the different meristem regulators they are derived from. To exclude the latter possibility and to validate the driver lines, I characterized the expression patterns mediated by the $CLV1_{pro}$ -LhG4, $CLV3_{pro}$ -LhG4 and WUS_{pro} -LhG4 lines, respectively. To this end, I introduced a $6xOP_{pro}$:GUS transgene into each of the driver lines *via* the floral dip method. T1 plants were selected for kanamycin resistance as described in Section 2.3.2 and genotyped for the presence of the GUS coding sequence (with KK-233 and KK-234 primers). The progeny of a number of independent transformants, which were homozygous for both transgenes, was characterized in the T3 generation (denoted: $CLV1_{pro}>>GUS$, $CLV3_{pro}>>GUS$ and $WUS_{pro}>>GUS$, respectively). The inflorescences of these plants were dissected and stained using X-gluc solution for one hour (Section 2.12). Under these conditions, GUS staining was not detected in inflorescences of the $CLV1_{pro}>>GUS$ and $CLV3_{pro}>>GUS$ lines, while a weak and patchy GUS signal was observed in $WUS_{pro}>>GUS$ flowers (Fig. 3.9 E, left panel).

To optimize the staining procedure, all lines were incubated with the GUS substrate X-gluc for two hours and then analyzed. This prolonged incubation time dramatically improved the detection of GUS activity so that signals were obtained in all cases (Fig. 3.9 B, D, F, G). In the case of $CLV1_{pro}>>GUS$, staining in the expected expression domain in the central zone underneath the stem cells was detected: blue color was visible in the center of the developing meristem, below the L1 and L2 cell layer (Fig. 3.9 B). Tissue from $CLV3_{pro}>>GUS$ plants (Fig. 3.9 D) displayed staining in the L1 and L2 layers of very young floral buds as well as in the top layers of the central zone in slightly older flowers, similar to what has been reported for endogenous *CLV3* (Fletcher et al., 1999). After two hours of GUS staining, tissue from $WUS_{pro}>>GUS$ plants displayed the strongest signal in the central zone underneath the region where stem cells reside (Fig. 3.9 F). However, weaker staining was also observed in cells of the L1 and L2 layers, where *WUS* is not thought to be expressed (Laux et al., 1996). In these experiments, the KNU_{pro} :KNU-GUS line served as a positive control. After two hours of staining, a faint signal was detected at the base of the developing gynoecium (Fig. 3.9 G).

Taken together, the results of the experiments outlined above by and large confirmed the validity of the driver lines used. The only exception may be the WUS_{pro} driver line, which appeared to result in a broader domain of expression as previously described for endogenous *WUS*. Importantly, though, the strength of the three driver lines that were used appeared to be very similar so that the differences in the phenotypic effects of a knockdown of *KNU* activity in distinct domains of the meristem could be correlated with differences in the expression patterns of *KNU*-amiRNA1 in those lines.

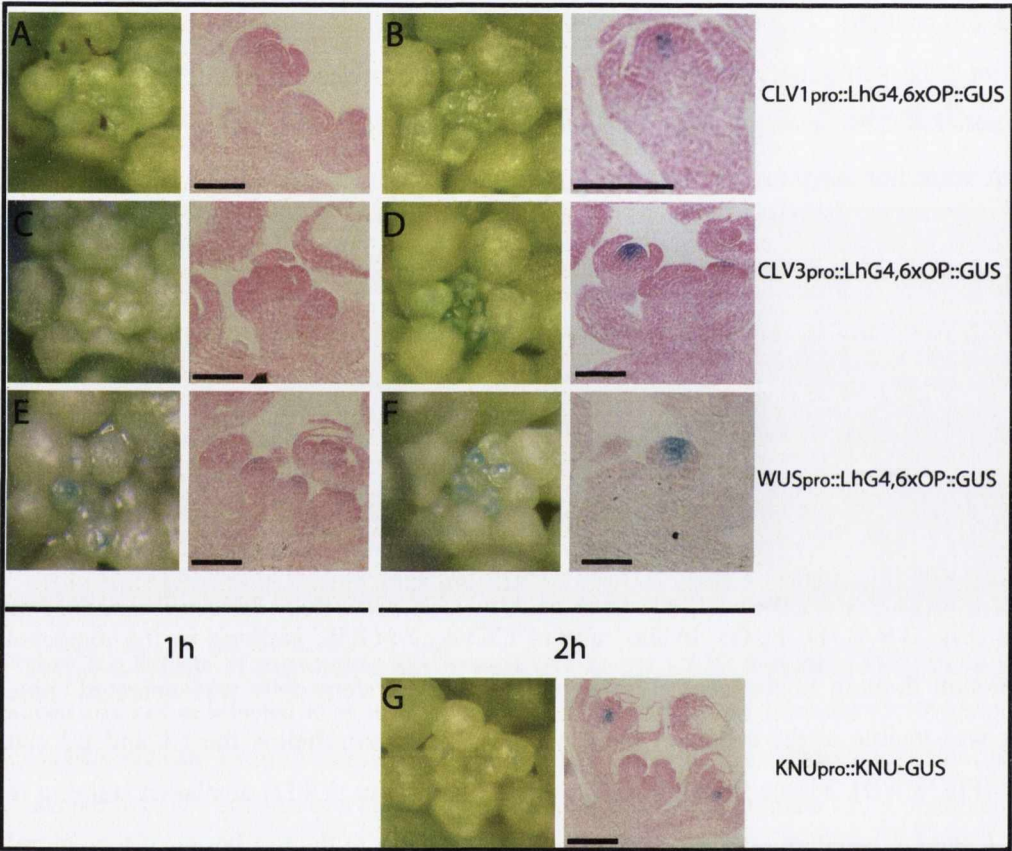


Figure 3.9 Characterization of meristem-specific driver lines. GUS activity was monitored in the $CLV1_{pro}$, $CLV3_{pro}$ and WUS_{pro} driver lines (as indicated). (A, C, E) Incubation for one hour resulted in no or only weak staining in all lines tested. (B, D, F, G) Incubation for a duration of two hours resulted in staining in all of the examined lines. (B) Staining in the center of a floral meristem (~stage 4) from a $CLV1_{pro}>>GUS$ plant. The staining pattern resembles that of endogenous *CLV1*. (C, D) GUS staining in $CLV1_{pro}>>GUS$ plants at early stages of flower development. (E, F) Elevated levels of GUS staining were observed in lines containing the $WUS_{pro}>>GUS$ transgene. (G) $KNU_{pro}:KNU-GUS$ served as a positive control in this experiment and was staining with a lower intensity than the other lines. Size bars: 50 μm.

3.2.5 Analysis of *KNU* expression in lines expressing KNU-amiRNA1 in specific domains of the floral meristem

To directly investigate the effects of KNU-amiRNA1 on *KNU* expression in the different LhG4 driver lines (i.e., CLV1_{pro}, WUS_{pro} and CLV3_{pro}), the lines with the most severe *knu*-like phenotypes were crossed with the previously published KNU_{pro}:KNU-GUS reporter line, respectively (a gift from Dr Toshiro Ito; denoted: KNU-GUS). In the resulting lines, the KNU-amiRNA1 should reduce the levels of KNU-GUS in the domain where the promoter-driver constructs are active. Plants of the F2 generation were genotyped for the presence of the KNU-amiRNA1 and GUS transgenes (using primer pairs KK-32/33 and KK-233/234, respectively). Inflorescences of plants positive for both transgenes were stained with X-gluc solution over night. Tissue was later fixed, embedded in paraplast and sectioned into 8 µm thick ribbons. These sections were then analyzed by light microscopy (Section 2.5.2).

From 17 plants carrying both the KNU-GUS and CLV1_{pro}>>KNU-amiRNA1 transgenes, 15 showed strong GUS staining (Fig. 3.10 D-F) after the overnight incubation, and two showed a weaker staining (not shown). The patterns of GUS staining were similar in lines containing the CLV1_{pro}>>KNU-amiRNA1 transgene and those lacking KNU-amiRNA expression (Fig. 3.10 A-C). Staining was detected in the center of flowers from stage ~5 onward (Fig. 3.10 D). Later in development, GUS activity was also detected in anthers (not shown). Taken together, these results show that *KNU* expression in the CLV1_{pro}>>KNU-amiRNA1 lines is not or only slightly altered, possibly explaining the weak *knu*-like phenotypes observed.

Similar results were obtained when 22 plants containing both the KNU-GUS and WUS_{pro}>>KNU-amiRNA1 transgenes were examined. The pattern of GUS staining appeared unchanged relatively to plants that expressed KNU-GUS but not the KNU-amiRNA1 (Fig. 3.11 A-C). However, in this case, the GUS signal observed was consistently lower in KNU-GUS plants that expressed the KNU-amiRNA1 than in plants that did not (Fig. 3.11 D-H). In spite of the lower level of detectable KNU-GUS staining, none of these plants displayed clear *knu*-like phenotypes (described in Section 3.2.4).

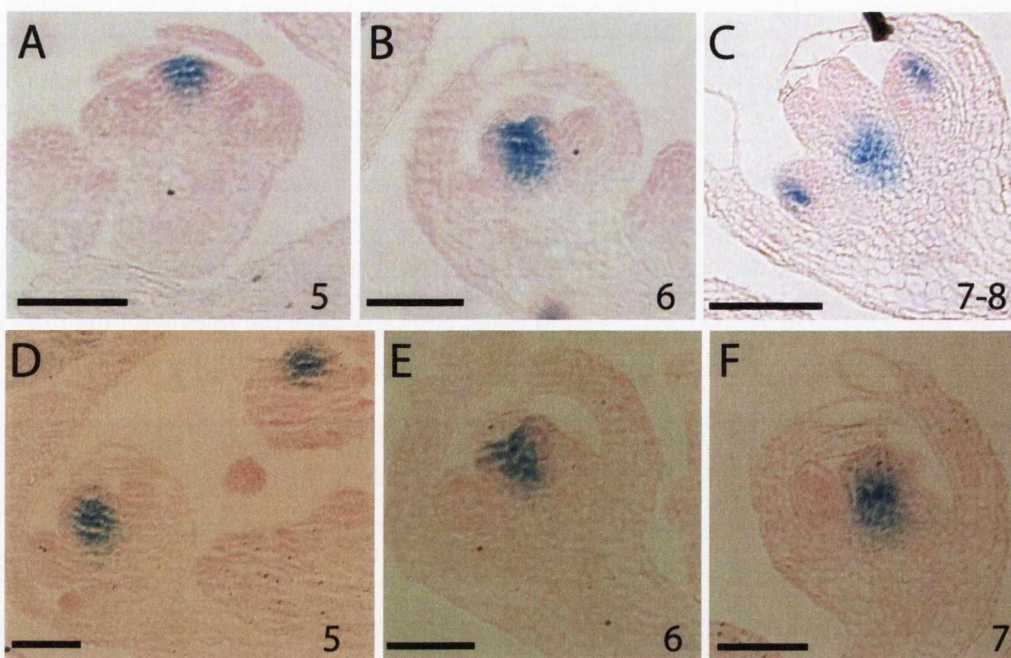


Figure 3.10 Effect of $CLV1_{pro}>>KNU\text{-}amiRNA1$ expression on KNU-GUS activity. (A-C) GUS staining in flowers of $KNU_{pro}:KNU\text{-}GUS$ plants. (D-F) KNU-GUS activity in flowers of plants that contain the $CLV1_{pro}>>KNU\text{-}amiRNA1$ transgene. GUS staining in these lines was similar in pattern and intensity to that in $KNU_{pro}:KNU\text{-}GUS$ plants. Approximate floral stages are indicated by numbers in panels. Size bars: 50 μm .

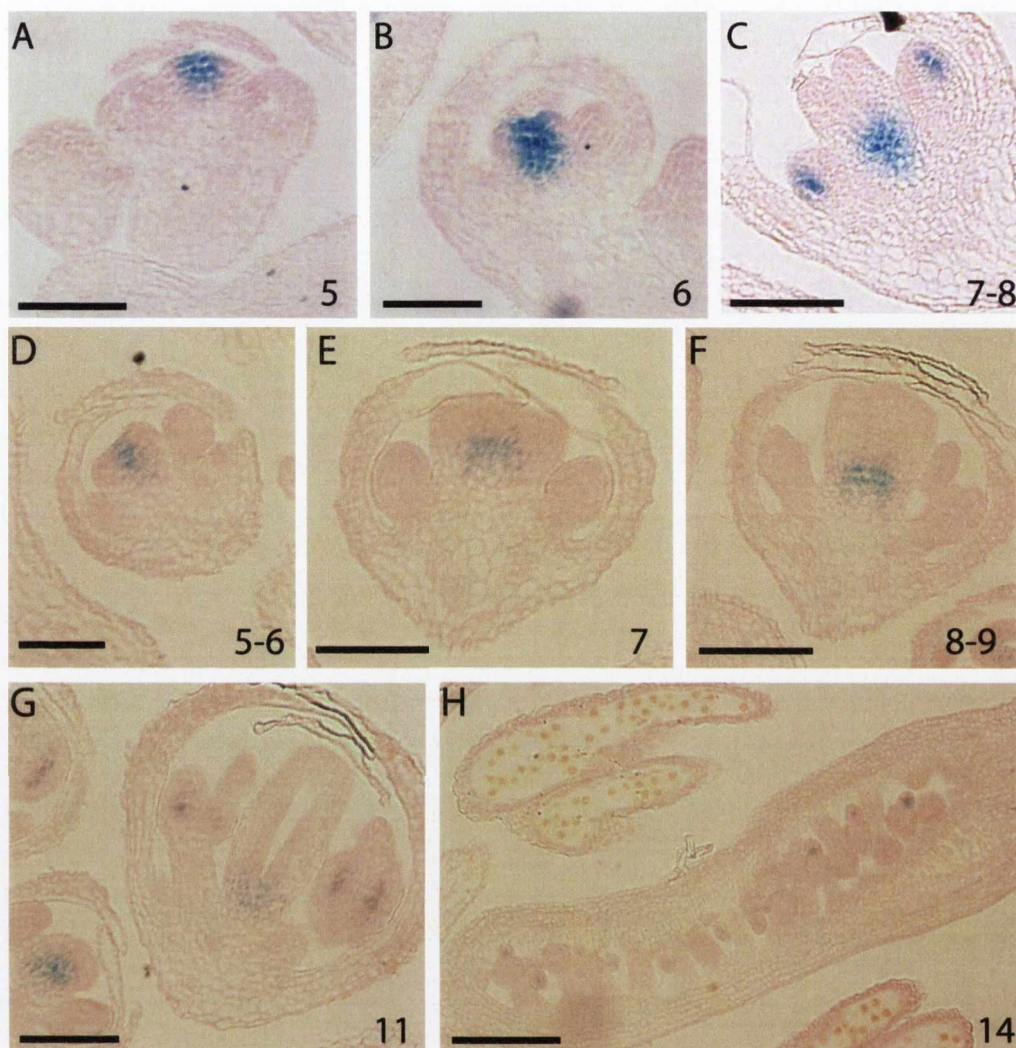


Figure 3.11: Effect of $WUS_{pro}>>KNU\text{-}amiRNA1$ expression on $KNU\text{-}GUS$ activity. (A-C) GUS staining in flowers of $KNU_{pro}:KNU\text{-}GUS$ plants. (D-H) Expression of $KNU\text{-}GUS$ in plants containing the $WUS_{pro}>>KNU\text{-}amiRNA1$ transgene. A reduction of GUS activity relative to $KNU_{pro}:KNU\text{-}GUS$ plants was detected throughout flower development. Approximate floral stages are indicated by numbers in panels. Size bars: 50 μm .

To analyze the effects in the $CLV3_{pro}$ driver line, 18 plants carrying both the $KNU\text{-}GUS$ and $CLV3_{pro}>>KNU\text{-}amiRNA1$ transgenes were isolated and inflorescences were subjected to GUS staining (as a positive control, $KNU_{pro}:KNU\text{-}GUS$ were stained in parallel; Fig. 3.12 A-C). Seventeen of those 18 plants showed no GUS staining in the floral meristem (Fig. 3.12 D). Also, no staining was detected in the regions of KNU expression at the base of the developing gynoecium at any stage (Fig. 3.12 D).

However, staining was found in anthers at later stages of development (Fig. 3.12 E-G), indicating that the staining procedure had worked. Further characterization of the KNU-GUS; CLV3_{pro}>>KNU-amiRNA1 plants revealed a partial rescue of the *knu*-like phenotype that I observed in CLV3_{pro}>>KNU-amiRNA1 plants (Fig. 3.12 H). The partially rescued siliques of these plants were shorter than those of wild-type plants but they were not bulged and did not contain ectopic carpels or stamens inside the gynoecium. Furthermore, the number of seeds was increased when compared with CLV3_{pro}>>KNU-amiRNA1 plants (not shown). However, plants still exhibited phyllotaxis defects, though at a much lower frequency than *knu-1* or CLV3_{pro}>>KNU-amiRNA1 plants.

Taken together, the results of the experiments outlined above show that the expression of the KNU-amiRNA1 from the *CLV3* promoter led to the suppression of *KNU* expression in the floral meristem as well as at the base of developing carpels. Similar effects were not observed with the *CLV1* and *WUS* promoters whose expression is also thought to overlap with that of *KNU*. Thus, the stem cell domain appears to be crucial for the onset and/or maintenance of *KNU* expression in the floral meristem.

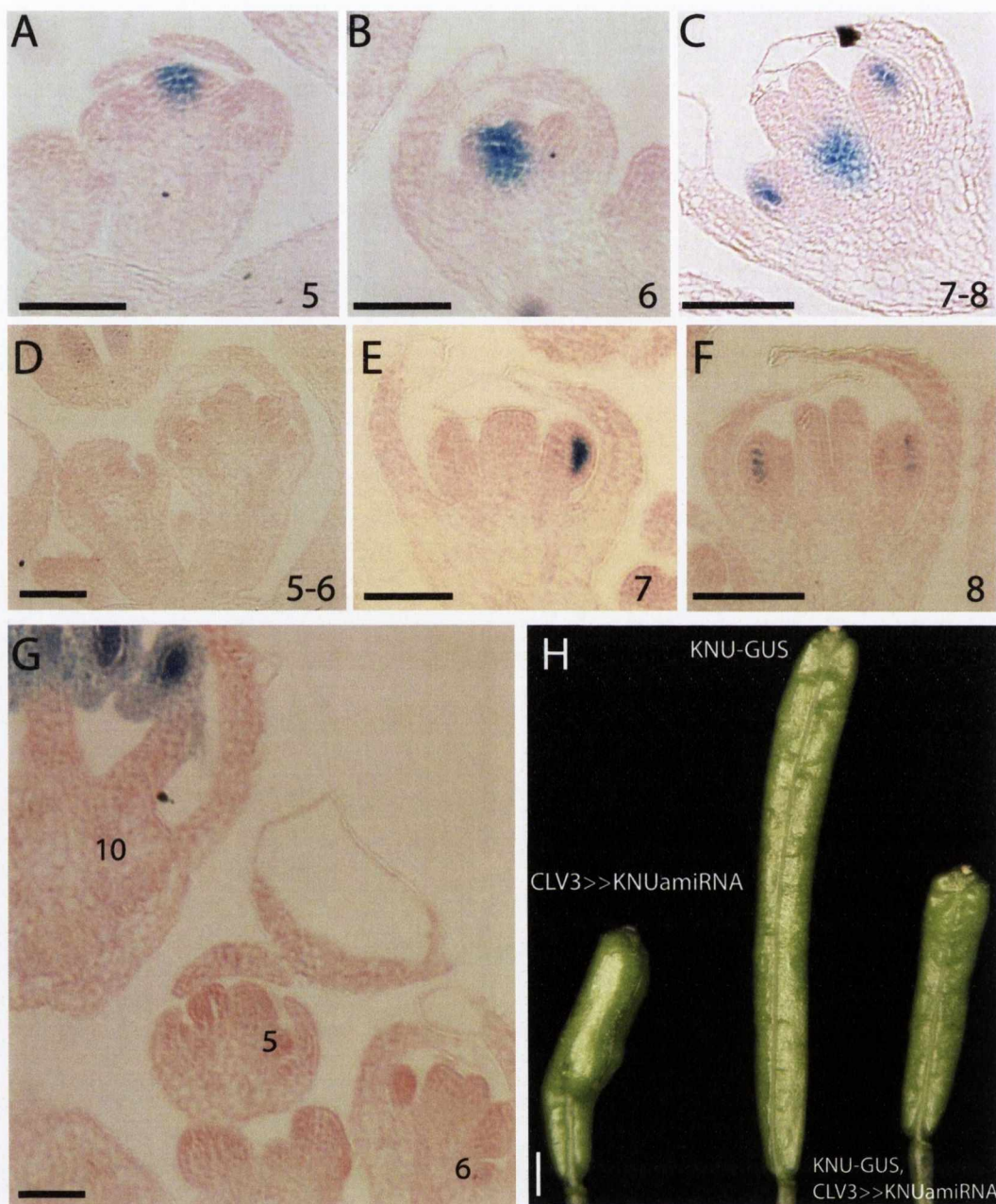


Figure 3.12: Effect of CLV3_{pro}>>KNU-amiRNA1 expression on KNU-GUS activity. (A-C) GUS staining in flowers of KNU_{pro}:KNU-GUS plants. (D-G) GUS staining in flowers of KNU_{pro}:KNU-GUS, CLV3_{pro}>>KNU-amiRNA plants. No GUS staining was detected in the center of the floral meristem (D). However, staining was visible in the anthers of stamens at later stages of development (E-G). (H) Comparison of siliques from CLV3_{pro}>>KNU-amiRNA (left), KNU_{pro}:KNU-GUS (middle) and CLV3_{pro}>>KNU-amiRNA, KNU_{pro}:KNU-GUS (right) plants. The double transgenic line lacks the characteristic *knu-1* phenotype, but siliques are

much shorter than in the wild type. Approximate floral stages are indicated by numbers in panels. Size bars: 50 μ m in A-G, 1 mm in H.

3.2.6 Genetic interactions between *KNU* and *CLV3*

Given the results described in the section above, I decided to further characterize the role of the stem cell domain in *KNU* function using a genetic approach. To this end, the *knu-1* mutant was crossed with two different *clv3* alleles, in which the stem cell domain in both shoot and floral meristems is enlarged (Fletcher et al., 1999). First, the *clv3-1* allele (Clark et al., 1995) was introgressed into *knu-1* (see Section 2.4). Plants of the F2 generation were phenotyped and genotyped (as described in Section 2.17.2). As described in the introduction of this chapter, *knu-1* plants have bulged siliques that contain ectopic carpels and stamens (Fig. 3.13 A, B). Also, the number of carpels is often higher than in the wild type. In contrast, the number of the other organ types is largely unchanged (Fig. 3.13 G).

The phenotypically intermediate *clv3-1* allele was identified in an EMS screen of wild-type plants (accession: L-*er*) and, in addition to defects in the shoot apical meristem, exhibits an over-proliferation of the floral meristem. Flowers of these plants were reported to contain on average 5 sepals, 5 petals, 9 stamens, and 5 carpels (Clark et al., 1995). Under our conditions, organ numbers were slightly lower but overall similar to the previous study (Fig. 3.13 G). Flowers of *clv3-1 knu-1* double-mutant plants produced fewer floral organs than *clv3-1* single mutants so that organ numbers were similar to that of the wild type (Fig. 3.13 G). The only exception was the number of carpels, which at ~ 3.5 was in-between those of *clv3-1* single mutants and wild-type flowers (Fig. 3.13 G). In addition, filamentous structures instead of stamens were occasionally observed (Fig. 3.13 E). Taken together, these results suggest that the *knu-1* allele can partially rescue the floral meristem over-proliferation phenotype of *clv3-1* mutants.

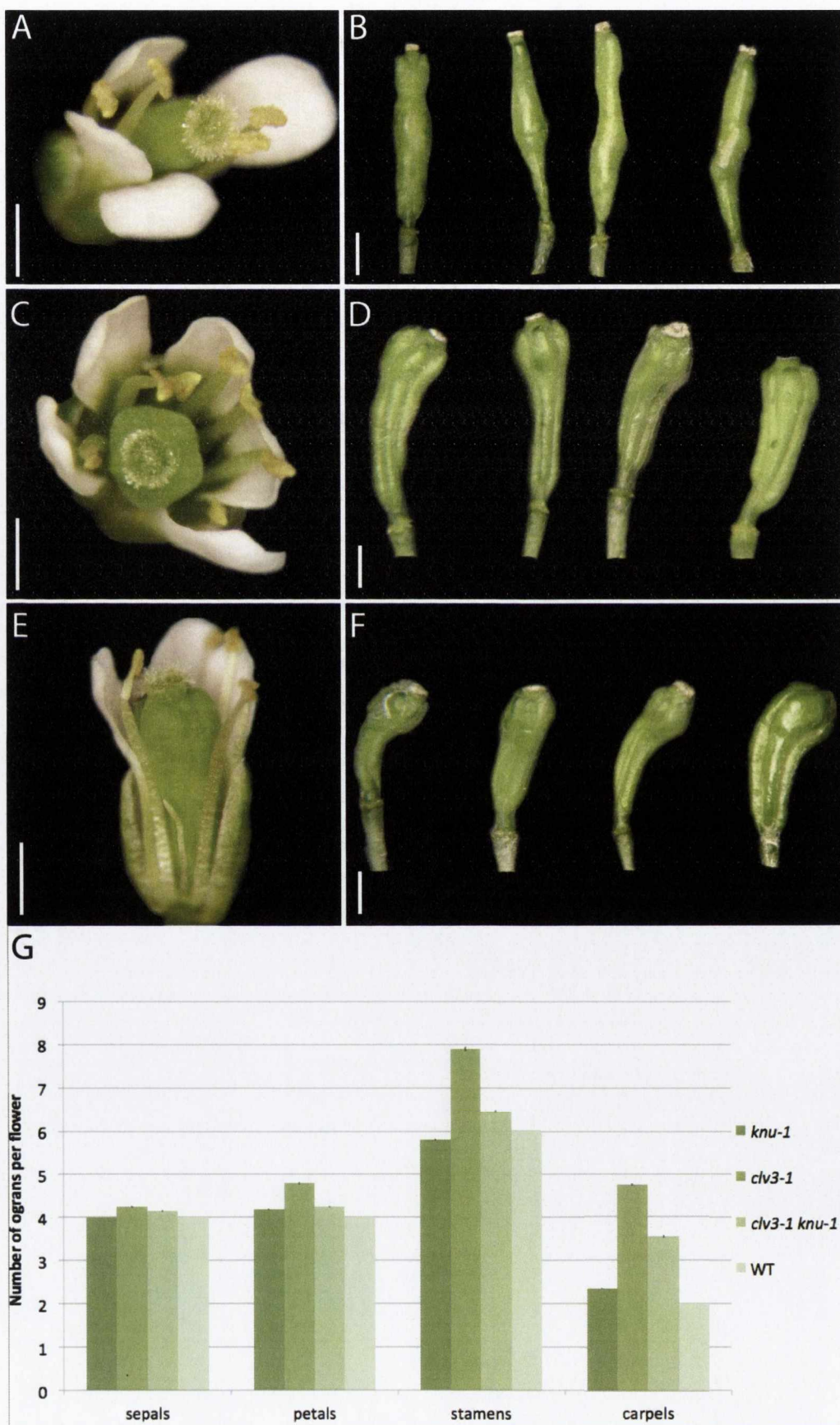


Figure 3.13 Genetic interactions between *KNU* and *CLV3*. (A-B) Phenotype of *knu-1* in an *L-er* background. Bulged siliques (B) and a flower (A) are shown. (C-D) Phenotype of *clv3-1* plants. Flowers contain more petals and reproductive organs (C). Siliques are multicarpelled (D). (E-F) Phenotypes of *clv3-1 knu-1* double-mutant flowers. Flowers no longer formed supernumerary petals (here one petal was removed for better visibility) as in *clv3-1* flowers, and instead of extra stamens, filaments occasionally formed (E). Siliques are shorter and are composed of fewer carpels than *clv3-1* single mutant siliques (F). (G) Number of sepals, petals, stamens and carpels in wild type, *knu-1*, *clv3-1* and *clv3-1 knu-1* plants. Error bars indicate s.e.m. At least 20 flowers were analyzed per genotype. Size bars: 1 mm.

Next, I tested the genetic interaction between *knu-1* and the weak *clv3-3* allele (Fletcher et al., 1999). Flowers of the latter mutant form more carpels than the wild type but the numbers of the other floral organs are largely unchanged (Fig. 3.14 A-C). Flowers of *clv3-3 knu-1* double-mutant plants resembled *knu-1* flowers. Flowers did not produce pollen (Fig. 3.14 D) (as in the case of the *knu-1* single mutant) and gynoecia typically contained a bulge with ectopic carpel tissue that seemed to be shifted upwards in the double mutant relative to *knu-1* single mutants (Fig. 3.14 D, F, H). *Clv3-3 knu-1* double-mutant plants were much smaller than either *clv3-3* or *knu-1* single mutants and their phyllotaxis was more severely affected. In addition, the double-mutant plants produced fewer siliques, which were much smaller than those of *clv3-3* or *knu-1* single-mutant plants (Fig. 3.14 G).



Figure 3.14: Characterization of *clv3-3 knu-1* double-mutant plants. (A-C) Flowers of a *clv3-3* single-mutant plant. Tetracarpelloid gynoecium (A) and anthers that produce pollen (B) are shown. Siliques with normally developed ovules (C). (D-F) Flowers of a *clv3-3 knu-1* double-mutant plant. Bulged gynoecium of a sterile flower (D). Gynoecium with a bulge (E) and ectopic carpel tissue on the inside (F). (G) *clv3-3* and *clv3-3 knu-1* plants (as indicated) with defects in phyllotaxis. (H) Clearing of a silique from a *clv3-3 knu-1* plant with ectopic carpel (ecp) arising from placental tissue. Size bars: 1 mm in A-F, 10 mm in G, 500 μ m in H.

3.3 Discussion

KNU is an important developmental regulator involved in the control of floral meristem determinacy (Sun et al., 2009). In order to clarify if KNU plays additional roles in flower development, several transgenic lines were generated which can be used to identify genes controlled by KNU activity.

3.3.1 Identification of a functional amiRNA against *KNU*

The over-expression of the three different KNU-amiRNAs revealed that two of them could efficiently perturb *KNU* activity while the third amiRNA appeared non-functional. Phenotypes consistent with those of *knu-1* were observed in the amiRNA-expressing lines, however, the penetrance of these phenotypes was lower than what has been described for loss-of-function mutants (Payne et al., 2004). This could be a consequence of an incomplete knockdown of *KNU* through amiRNA expression. Alternatively, the different accessions that provide the background of the known *knu* alleles and the KNU-amiRNA lines may influence the strength of the phenotypes. Specifically, the *knu-1* allele was identified in accession Wassilewskija (Ws) (Payne et al., 2004) while the amiRNAs against *KNU* described in Section 3.2.2 were transformed into the *Arabidopsis* accession L-*er*. Furthermore, the same 35S_{pro}::KNU-amiRNA1 plasmid was used to transform plants of the accession Colombia (Col). The results of these experiments are not described because the penetrance of mutant phenotypes was very low and most plants did not contain any of the characteristic features of *knu-1* mutants (crossed into L-*er*) or of KNU-amiRNA1 lines in an L-*er* background. These differences in the phenotype could be explained by the fact that mutations in *ERECTA* (ER) may play a role in the strength of the phenotype (Mandel et al., 2014). Specifically, it has been shown that ER regulates floral meristem identity in a different

pathway than the CLV-dependent pathway. It also plays a role in the regulation of *WUS* transcription and the control of phyllotaxis (Mandel et al., 2014). In fact, the double mutant *jba-1D/+ er-20* forms enormous meristems with many more cells than the wild type. These data suggest that ER may enhance the meristem-related phenotypes of *knu* mutant plants possibly by effecting a parallel pathway for floral meristem control.

Although the phenotypes of the amiRNA lines I generated might have been enhanced in *L-er* plants, the penetrance of the phenotype was not as strong as described for the *knu-1* allele in either a *Ws* or a *L-er* background. Yet because of the *knu*-like phenotypes observed, line #28 obtained with the KNU-amiRNA1 construct, which showed the strongest phenotype (~14% of bulged siliques) and in which the level of *KNU* mRNA was the lowest (~25%) was crossed into the FIS. Homozygous plants were then isolated (as described in Section 2.13.8) and used in further experiments that aimed at identifying genes that act downstream of KNU.

3.3.2 Effects of KNU-amiRNA expression in the *CLV1*, *CLV3* and *WUS* domains

KNU is expressed in the center of the floral meristem from approximately stage 6 onward (Payne et al., 2004; Sun et al., 2014). However, as described below (Fig. 3.15), its expression may actually commence slightly earlier than previously thought. In order to test in which domain of the floral meristem development KNU function is required, I expressed the KNU-amiRNA1 from promoters with distinct domains of activity in the meristem. While all lines expressing the KNU-amiRNA developed a *knu-1* phenotype, the penetrance and strength of the phenotype was not consistent across all the lines. The strongest phenotype was displayed in plants that contained the *CLV3_{pro}*>>KNU-amiRNA transgene and the weakest in plants that contained the *WUS_{pro}*>>KNU-amiRNA transgene.

The mRNAs of *CLV1*, *CLV3* and *WUS* have been shown to be present until stage 5 of flower development when their expression ceases. Because the expression of *KNU* has been reported to commence at stage 6 of flower development (Payne et al., 2004), no phenotypes should be observed in those lines. There are several ways to explain this: 1) The rate of KNU-amiRNA decay may be low so that the levels of the amiRNA are sufficiently high to perturb *KNU* expression and function at and beyond

stage 6 of flower development; 2) The activity of the *CLV1*, *CLV3* and *WUS* promoters continues beyond stage 5; 3) *KNU* might be expressed earlier than stage 6 of flower development. Despite the above-mentioned caveats, the experiment presented here suggests that expression of a *KNU* amiRNA in the three different driver lines is sufficient to cause the *knu*-like phenotypes.

The driver lines used for this study were previously found to accurately reflect the expression patterns of the endogenous genes (Gross-Hardt et al., 2002; Lenhard and Laux, 2003; Schoof et al., 2000). In agreement with this notion, GUS activity was detected in inflorescences of *CLV1*_{pro}:LhG4,6xOP_{pro}:GUS plants in the precise area of *CLV1* expression. GUS signal was confined to cells of the L3 layer in the center of the meristem, but was not detected in cells of the L1 or L2 layers. For plants containing the *CLV3*_{pro}:LhG4,6xOP_{pro}:GUS transgene, GUS activity was detected in the L1 and L2 layers at early stages of flower meristem development and was later visible in the central zone. This is consistent with the results obtained by another group using the same promoter sequence (Fletcher et al., 1999). In the *WUS*_{pro}:LhG4, 6xOP_{pro}:GUS line, a low level of the GUS activity was detected after one hour of tissue staining. Two hours of GUS staining improved the signal, however, the resulting expression pattern appeared broader than that of endogenous *WUS*. This result could be due to over-staining and leakage of the GUS staining product. This appears likely because in contrast to the other driver lines, a one hour staining of *WUS*_{pro}:LhG4, 6xOP_{pro}:GUS plants resulted in some visible staining, suggesting that this line may be 'stronger' than the others.

Further characterization of these lines was performed to identify the regions in which *KNU* activity is required in floral meristems. By using the *KNU*-GUS reporter driven by the *KNU* promoter in combination with the *KNU*-amiRNA1 expressed in different regions of developing meristems, the effect of a *KNU* knockdown in the domains of *CLV1*, *CLV3* and *WUS* expression could be determined. As described in Section 3.2.5, expression of the *KNU*-amiRNA1 in the *CLV1* domain did not cause any difference in the GUS staining compared with plants containing only *KNU*-GUS. This result is in agreement with the weak phenotypic effects observed in lines in which *KNU* was knocked down in the *CLV1* domain (see Section 3.2.4). Specifically, expression of the *KNU*-amiRNA1 from the *CLV1* promoter did not substantially reduce *KNU* expression, resulting only in a weak reduction of its activity.

In contrast to the former line, KNU-GUS activity in plants expressing the KNU-amiRNA1 from the *WUS* promoter was much lower when compared with KNU_{pro}:KNU-GUS plants. Still, this reduction appeared to have little effect on the overall function of KNU, since *knu*-like phenotypes were infrequently observed in these plants. This result was surprising given that it has been previously shown (Sun et al., 2009) that KNU represses *WUS* at stage 6 of flower development to stop further meristem growth. However, there is no description of this interaction during even earlier stages in the meristem. As described in a detailed bioinformatics analysis of gene expression during the course of flower development (Ryan et al., 2015), *KNU* appears to be expressed earlier than at the stage 5-6 (Fig. 3.15 A).

An analysis of plants containing both the KNU_{pro}:KNU-GUS and CLV3_{pro}:KNU-amiRNA transgenes showed that expression of KNU-GUS vanishes in the center of the developing gynoecium, but, as expected, is still detected in anthers. This result suggests that the stem cell domain is crucial for the onset or maintenance of KNU expression. Alternatively, it could be argued that the *CLV3* driver is simply ‘stronger’ than the other driver lines used, leading to a more efficient knockdown of *KNU* expression and activity. However, this appears unlikely because the results of the experiments in which KNU-GUS was driven by different drivers did not indicate any substantial differences in strength of expression. If anything, it appears that the *WUS*_{pro} driver line may be somewhat stronger than those that utilize the *CLV1* and *CLV3* promoters, respectively (see above). Taken together, these results suggest that the stem cell domain is crucial for KNU function, which is surprising given that KNU is supposed to act on *WUS*, which is not expressed in stem cells but in the Organizing Center underneath the stem cell domain.

3.3.3 Genetic interactions between *KNU* and *CLV3*

In order to better understand the effect of stem cells and of *CLV3* and KNU function *clv3-1 knu-1* and *clv3-3 knu-1* double mutants were generated and validated by genotyping.

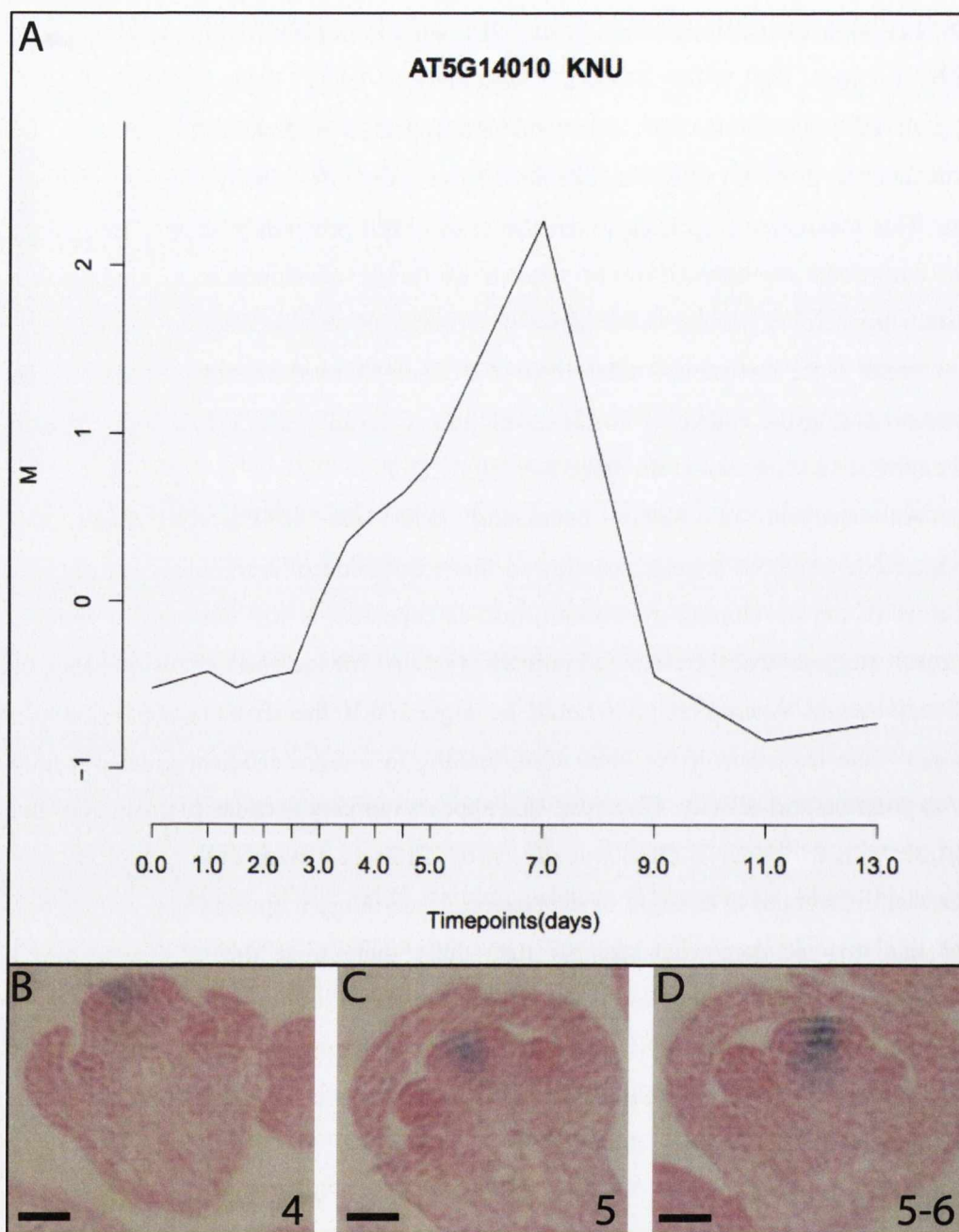


Figure 3.15 *KNU* is expressed earlier in flower development than previously thought. (A) Expression of *KNU* at different time-points after activation of flower development in the AP1_{pro}:AP1-GR *ap1 cal* floral induction system. Data were taken from (Ryan et al., 2015). (B, C, D) GUS activity in early-stage flowers of KNU_{pro}:KNU-GUS plants. Staining was detected not only in the center of the developing gynoecium (C, D) but also in the L1, L2 and L3 layers of the floral meristem (~stage 4) (B). Approximate floral stages are indicated by numbers in panels. Size bars: 30 μ m.

In case of the *clv3-1 knu-1* double mutant, the number of stamens was reduced relative to *clv3-1* mutants and closer to the that of *knu-1* or wild-type flowers. Similarly, the number of petals in the *clv3-1* mutant was also higher than in flowers of double mutant plants. Together with the finding that *clv3-3 knu-1* mutants resemble, by and large, *knu-1* single mutants, these results suggest that the expression of the *clv3* phenotype may dependent, at least to some extent, on the presence of functional KNU. Given that I found that the stem cell domain is important for *KNU* expression, a working model for the interaction between CLV3 and KNU could be as follow: CLV3 promotes *KNU* expression, and the KNU transcription factor then represses not only *WUS* but also *CLV3* to terminate floral meristems (Fig. 3.16). In a weak to intermediate *clv3* mutant, lower levels of *KNU* than in the wild type would be reached, resulting in a reduced repression of *CLV3* and consequently in a partial restoration of its function.

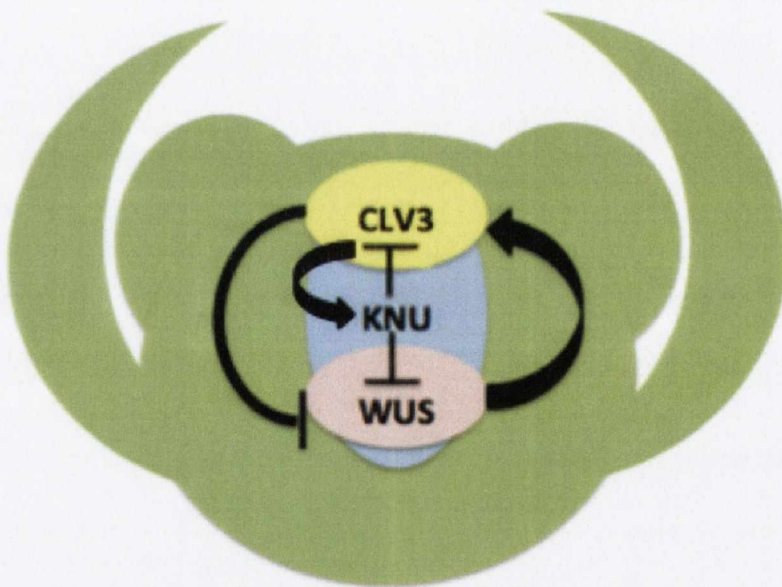


Figure 3.16: A working model for the interactions between CLV3, KNU and WUS.

CLV3 promotes expression of *KNU* at early stages of flower development. KNU transcription factor represses not only *WUS* but also *CLV3* to terminate the floral meristem.

CHAPTER 4 - CHARACTERIZATION OF THE TRANSCRIPTIONAL NETWORK CONTROLLED BY KNUCKLES

4.1 Introduction

As mentioned in Chapter 1, KNU is thought to regulate floral meristem determinacy by repressing *WUS* expression. In Chapter 3, I showed through genetic analysis that KNU antagonizes *CLV3* expression and is likely to interact with *CLV3*, which could influence the feedback loop between *CLV3* and *WUS* in floral meristems. However, KNU may have additional functions in the control of floral meristems and indeed, in the regulation of other processes during flower development. This is suggested by the relatively broad and prolonged expression of the *KNU* gene in floral primordia, as well as by the floral phenotypes of *knu* mutants (see Fig. 1.3 and 1.4).

As described in Section 1.4.4, the aim of this thesis was to identify genes that are directly or indirectly regulated by KNU on a genome-wide scale by using approaches such as microarray analysis and chromatin immunoprecipitation combined with ultra high-throughput sequencing. To make this work possible, several transgenic lines were generated that allow a perturbation of *KNU* activity (including a dexamethasone-inducible line to perturb *KNU* activity at distinct stages of flower development) (Section 4.3.1.2) as well as the identification of genomic regions that are bound by KNU (Section 4.3.4). These transgenic lines were used in combination with the abovementioned floral induction system, which allows the collection of large numbers of flowers at similar stages of development. The generation of these lines is described below. In addition, the results of experiments are outlined where these lines were used for gene expression profiling experiments (Section 4.3.7) and genome-wide localization studies (Section 4.3.6).

4.2 Identification of genes acting downstream of C2H2 zinc finger transcription factors

Genome-wide approaches have led to the identification of large number of genes that are differentially expressed during flower development and may play important roles in this process (e.g., (Ryan et al., 2015)). Furthermore, several genome-wide studies have been published describing the activities of MADS-domain transcription factors and genes that act downstream of these key regulators of flower development (Kaufmann et al., 2009; Kaufmann et al., 2010; O'Maoileidigh et al., 2013; Wuest et al., 2012). Despite this progress, the activities of many other floral regulators are currently not well understood. These include the different C2H2 zinc finger transcription factors that are known to play key roles in the control of flower development.

Recently, a study described the activities of a related transcriptional repressor JAGGED (JAG) has been published (Schiessl et al., 2014) by using ChIP-seq in combination with transcriptome analysis to identify target genes. In order to reveal the JAG binding sites, a GFP tagged JAG protein was expressed in the background of *jag-2* inflorescence from its own regulatory elements. In total, 1,634 genes were identified as putative targets of JAG. For the gene expression examination, the transcriptome of the *jag-1* mutant and a conditional overexpression of *JAG* were compared with control plants, respectively, which identified 1,242 differentially expressed genes. The overlap between the ChIP-seq and array analysis was ~400 genes in which they identified 235 direct, transcriptionally responsive, target genes (Schiessl et al., 2014).

Most of the genes are involved in control and hormone response as well as regulation of growth and organ identity. Some genes that were identified by this study were responsible for floral patterning (*CLV1* and *HANABA TARANU (HAN)*). Other genes that played a role downstream of JAG were floral identity genes like *LFY* and its direct targets *LATE MERISTEM IDENTITY 1* and *2 (LMI 1* and *2)*. In brief, the combination of the two approaches gave some interesting information regarding JAG. They showed that the C2H2 transcription factor is involved in controlling organ identity and organ patterning, and also represses genes that are required to execute tissue growth (Schiessl et al., 2014).

In conclusion, the combination of different approaches of molecular biology allow for detailed study of the transcription factors controlling flowering. Furthermore, the applications of the experimental techniques give an opportunity to dissect the gene regulatory network (GRN) underlying flower development.

It is possible that the regions of the genome bound by JAG and those bound by KNU might be similar since they have similar DNA binding domains. It has been

shown that the overlap of the number of putative direct targets was high for MADS-domain transcription factors, AP1 and AG (O'Maoileidigh et al., 2013). Similar work was done in this thesis in order to gain information regarding KNU and the results are described in Sections below.

4.3 Results

4.3.1 amiRNA-mediated perturbation of *KNU*

As mentioned above, it is possible to identify genes that are directly or indirectly regulated by a transcription factor by perturbing its activity and by subsequently monitoring, through transcriptomics approaches, the effects that the perturbation has on global gene expression. In order to identify the most efficient system for a perturbation of *KNU* activity, I used the KNU-amiRNA1 that was described in Section 3.2.2 in combination with two chemically inducible promoter systems: the GR-LhG4/6xOP_{pro} dexamethasone-responsive system and the AlcR/AlcA_{pro} ethanol-responsive system. The results obtained with these different approaches are described in the sections below.

4.3.1.1 Characterization of an ethanol-inducible KNU-amiRNA line

In Section 3.2.2 it was demonstrated that the expression of the KNU-amiRNA1 under the constitutive 35S promoter led to an efficient knockdown of *KNU* mRNA levels as well as to a disruption of KNU activity. To generate lines that would allow an inducible knockdown of *KNU* at distinct stages of flower development, the amiRNA1 against *KNU* was expressed under the control of 35S_{pro}:AlcR/AlcA_{pro} ethanol-inducible promoter system (Roslan et al., 2001) in wild-type plants (Fig. 4.1 A). In total, 13 independent transformants were isolated and genotyped to verify the presence of the transgene. The progeny of these plants were treated continuously with ethanol vapor for 48 h and then screened for the presence of *knu*-like phenotypes. Ten of the lines did not display any discernable mutant phenotypes and an additional line displayed a severe loss of the apical dominance, a phenotype that is not observed in *knu* mutant plants. The remaining two lines (#1 and 2), showed mild *knu*-like phenotypes (Figure 4.1 B)

(O'Maoileidigh, 2012). The siliques of these plants were typically shorter than siliques of wild-type plants. Moreover, the characteristic bulged silique phenotype appeared sometimes, but the bulges did not contain ectopic carpels or stamens. However, most of the siliques were tricarpelloid (Fig. 4.1 C), in agreement with the idea of a mild floral meristem indeterminacy defect in these plants. The phenotypically strongest line (#1.16) was further characterized to find out whether it could be used for *KNU* perturbation experiments followed by transcript profiling. To this end, plants of this line were treated with ethanol vapor for 3, 6, 9 or 24 hours or with water vapor as a control. Subsequently, RNA was isolated from ~20 inflorescences (containing stage 0-10 floral buds) per sample and used in qRT-PCR assays to determine *KNU* expression levels.

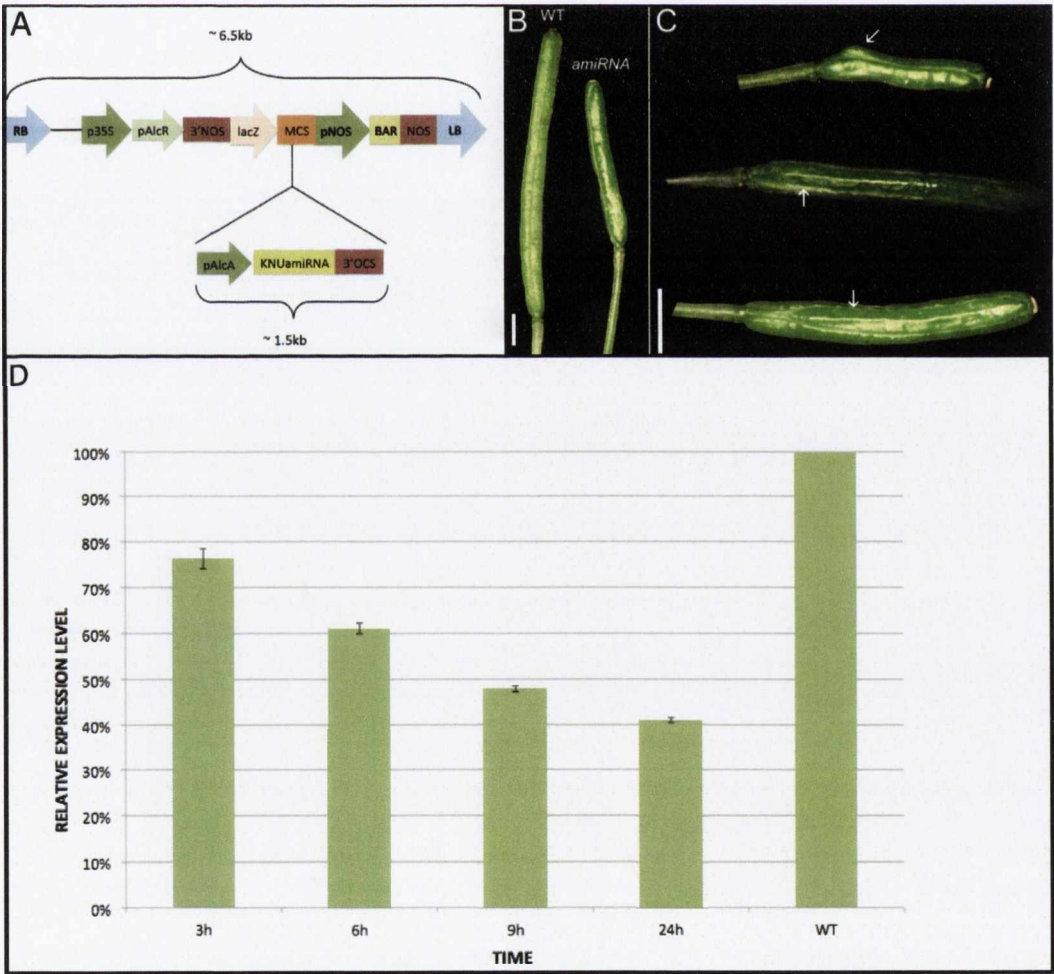


Figure 4.1 Effects of a specific induction of *KNU*-amiRNA1 expression. (A) A schematic representation of the T-DNA insertion mediating expression of the *KNU*-amiRNA1 from the

ethanol-inducible 35S_{pro}:AlcR/AlcA_{pro} promoter system. For a full description, see Section 2.15.1. **(B, C)** Phenotypes observed in siliques of 35S_{pro}:AlcR/AlcA_{pro}:KNU-amiRNA1 plants treated with ethanol vapor. **(B)** Siliques of wild-type and KNU-amiRNA1-expressing plants. **(C)** Bulged and tricarpeoloid siliques were observed in the transgenic plants after ethanol treatment. **(D)** *KNU* transcript levels 3, 6, 9 and 24 h after ethanol treatment of 35S_{pro}:AlcR/AlcA_{pro}:KNU-amiRNA1 plants (line #1.16). Error bars represent s.e.m. of the 3 biological replicates. To determine *KNU* mRNA levels, primer pairs KK-27/KK-28 and KK-29/KK-30 were used for *KNU* and a reference gene, respectively. Panels B and C adopted from (O'Maoileidigh, 2012)). 3'OCS, octopine synthase terminator sequence; GR, glucocorticoid receptor sequence; LhG4, chimeric transcription factor sequence; RB, right border of T-DNA; LB, left border of T-DNA, lacZ, a modified version of the β -galactosidase coding gene from *E. coli*; p35S, Cauliflower Mosaic Virus (CaMV) 35S promoter sequence; MCS, multi cloning site; 35S, Cauliflower Mosaic Virus (CaMV) 35S terminator sequence; pAlcA, ethanol responsive promoter; AlcR, ethanol responsive transcription factor from *Aspergillus nidulans*. Size bars in B and C: 1 mm.

Three hours after ethanol treatment the level of *KNU* mRNA decreased to ~75% of wild-type levels, and after 6 h, *KNU* expression levels had decreased further to about 60%. An additional reduction was detected after 9 h and 24 h, when *KNU* mRNA levels were at ~40% of wild-type levels.

Because the amiRNA-mediated knockdown of *KNU* from the AlcR/AlcA_{pro} system was not complete, an additional inducible promoter system (35S_{pro}:GR-LhG4/6xOP_{pro}) was tested (see below). However, because the 35S_{pro}:AlcR/AlcA_{pro}:KNU-amiRNA1 approach was in principle successful, the transgene was crossed into the FIS (see Section 2.9.1) and then used in the microarray experiment described in Section 4.3.8 below.

4.3.1.2 Characterization of a dexamethasone-inducible KNU-amiRNA1 line

In the first section of Chapter 3, it was demonstrated that the constitutive expression of the amiRNAs KNU-amiRNA1 and KNU-amiRNA2 led to a knockdown of *KNU* that was sufficient to disrupted its activity. Furthermore, in the previous section, I outlined the results experiments with the 35S_{pro}:AlcR/AlcA_{pro} ethanol-inducible promoter system, which showed that the amiRNA-mediated *KNU* perturbation can be made

conditional, which was a prerequisite for the gene expression profiling experiments that were to be conducted as part of this thesis. However, only a moderate *KNU* knockdown was observed with the 35S_{pro}:AlcR/AlcA_{pro} promoter system. I therefore investigated whether use of the 35S_{pro}:GR-LhG4/6xOp_{pro} dexamethasone-inducible promoter system would result in a higher knockdown efficiency. To this end, wild-type plants were transformed with a 35S_{pro}:GR-LhG4/6xOp_{pro}:KNU-amiRNA1 construct (Fig. 4.2 A) as described in Section 2.3.1. In total, 72 transformants were isolated and genotyped to verify the presence of the transgene. The progeny of 10 selected lines was then treated once with a solution containing 10 µM dexamethasone and screened for the presence of *knu*-like phenotypes. Seven lines displayed a strong mutant phenotype (Fig. 4.2 B-F) while the three others showed mild or no abnormal phenotypes. For the lines with the strongest mutant phenotypes the mean percentage of bulged siliques was determined (Fig. 4.2 G). In all but one of the lines the mean percentage of bulged siliques reached 10-21%. Moreover, as in *knu* mutants, the bulges contained ectopic carpels and stamens (Fig. 4.2 D-F).

One line (#42) was selected for a more detailed analysis. The inflorescences from axillary shoots of six plants of this line were collected and after total RNA extraction and DNaseI treatment, cDNA was synthesized. qRT-PCR was performed to determine the extent of the amiRNA-mediated knockdown of *KNU* (Fig. 4.2 H; dark green). This analysis showed that *KNU* mRNA levels were reduced to ~20% when compared to the wild type. In addition, the average percentage of bulged siliques was determined for different plants (Fig. 4.2 H; light green). Overall, the severity of the phenotypes correlated well with the degree of *KNU* knockdown. The line was then used for a pulsed perturbation analysis to determine *KNU* functions at different floral stages.

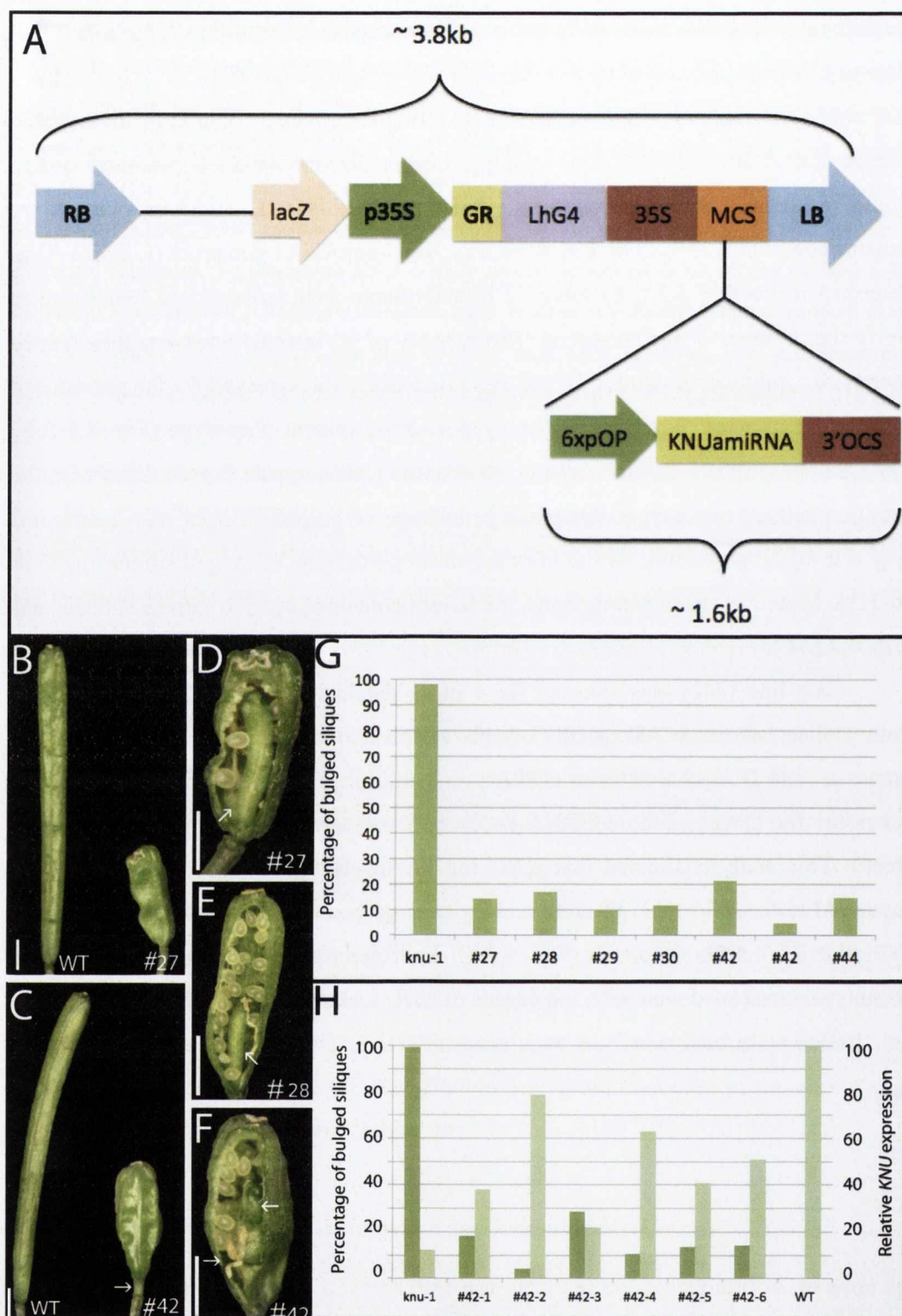


Figure 4.2 Phenotypes observed after a *KNU* knockdown in $35S_{pro}:GR-LhG4/6xOP_{pro}:KNU\text{-}amiRNA1$ plants. (A) A schematic representation of the T-DNA insertion mediating expression of the amiRNA under the 6xOP promoter. For full description,

see Section 2.15.1 **(B-F)** Phenotypes observed in plants of the T2 generation. After induction of amiRNA expression, siliques were shorter than in the wild type and contained ectopic carpels and stamens. Arrows indicate elongated gynophores. Numbers in panels refer to line numbers. **(G)** The average percentage of bulged siliques observed in selected lines. **(H)** Levels of *KNU* transcript were measured in T3 plants (line #42) by qRT-PCR (light green) and the percentage of bulged siliques relative to non-bulged siliques (dark green; at least 15 siliques were counted per plant) was determined. The line with the strongest phenotype is marked by an asterisk. Sets of oligonucleotides used for qRT-PCR: KK-27/KK-28 to amplify *KNU* and KK-29/KK-30 for the reference gene *REF2* (At4g34270). 3'OCS, octopine synthase terminator sequence; GR, glucocorticoid receptor sequence; LhG4, chimeric transcription factor sequence; RB, right border of T-DNA; LB, left border of T-DNA, lacZ, a modified version of the β -galactosidase coding gene from *E. coli*; p35S, Cauliflower Mosaic Virus (CaMV) 35S promoter sequence; MCS, multi cloning site; 35S, Cauliflower Mosaic Virus (CaMV) 35S terminator sequence; 6xpOP, 6 repeats of the operator promoter sequence. Size bars: 1 mm.

To this end, plants were treated every third day for three times with 10 μ M dexamethasone. Subsequently, floral phenotypes were assessed at the time of anthesis (stage 13) for a period of 24 d. During this time, a total of 32 flowers were observed. The first flowers that reached maturity after dexamethasone treatment (i.e. flowers that had been at advanced stages of development when the *KNU* knockdown occurred) exhibited stamen elongation defects. In addition, anthers failed to dehisce and release pollen grains (Fig. 4.3, day 5). These stamen elongation defects were observed up to 13 d after the first dexamethasone treatment. Flowers that were at an earlier developmental stage at the time of the *KNU* knockdown formed normal stamens that produced and dehiscence pollen grains.

Carpel defects were first observed in flowers 5 d after the first dexamethasone treatment. The gynoecia of flowers that had matured between 7 and 13 d were abnormally shaped and the carpels were enlarged compared to the wild type. At day 16, gynoecia became unfused and contained ectopic reproductive organs (carpels and stamens). An increased amount of carpel tissue was also observed (Fig. 4.3, 16 day). After day 16-17, plants started to produce siliques that resembled those of *knu-1* mutant plants. In summary, strong *knu*-like phenotypes were generated when the plants containing the 35S_{pro}:GR-LhG4/6xOP_{pro}:KNU-amiRNA1 transgene were treated with

dexamethasone. This showed that *KNU* activity could be inducibly perturbed in a temporary manner.

The line described above was isolated in a homozygous state and seeds were propagated. In addition, the transgene was crossed into an AP1_{pro}:AP1-AR *ap1-1 cal-1* background and plants homozygous for all transgenes and mutant alleles were isolated. These plants, which would allow the induction of flower development through the DHT-dependent activation of the AP1-AR fusion protein and the subsequent dexamethasone-dependent perturbation of *KNU* function, await further characterization.

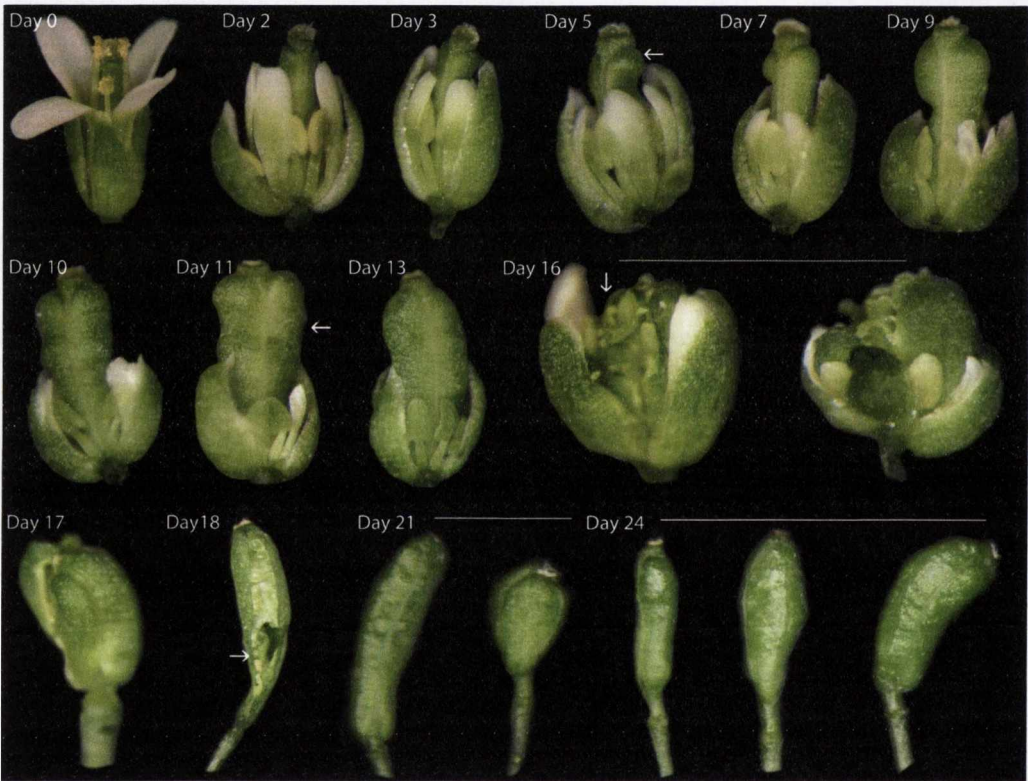


Figure 4.3 Stage-specific effects of an amiRNA-mediated *KNU* knockdown. Plants homozygous for the 35S_{pro}:GR-LhG4/6xOP_{pro}:KNU-amiRNA1 transgene were treated three times (every third day) with 10 μ M dexamethasone. Flowers produced after the treatment exhibited various defects depending on their developmental stage at the time of *KNU* perturbation. For each flower, the number of days between the first dexamethasone treatment and the time the photograph was taken is indicated. Flowers observed between day 2 and day 13 contained stamens that failed to elongate and dehiscence pollen. Flowers observed between day 5 and 16 contained abnormal gynoecia. At day 16, flowers contained additional stamens and carpels within gynoecia. These gynoecia were not fused and ectopic organs were visible. From day 17 onwards, plant produced siliques with ectopic organs, resembling *knu* mutant siliques.

4.3.2 Generation of an inducible *KNU* over-expressing line

Over-expression of a transcriptional regulator is a common method of perturbing a gene regulatory network and can be used to identify the functions of that regulator a specific biological process. Also, this approach is frequently being employed to identify genes that act downstream of the regulator under study. A line (35S_{pro}:*KNU*-AR) that mediates over-expression of *KNU*-AR was described before (Sun et al., 2009) but was unavailable for this study. I therefore generated transgenic lines that expressed *KNU* under control of the 35S_{pro}:GR-LhG4/6xOP_{pro} dexamethasone-inducible promoter system and the constitutive 35S promoter, respectively. The results of the characterization of these experiments are described below.

4.3.2.1 Characterization of a dexamethasone-inducible *KNU* over-expression line

For inducible over-expression of *KNU*, the coding region of the gene together with its 5'UTR was inserted into the 35S_{pro}:GR-LhG4/6xOP_{pro} promoter system (Fig. 4.4 A). After transformation of wild-type plants, 32 independent transformants were identified that contained the 35S_{pro}:GR-LhG4/6xOP_{pro}:*KNU* transgene. The progeny of selected lines was treated with a dexamethasone-containing solution and axillary inflorescence tissue from stage 0-10 (Smyth et al., 1990) was collected 24 h after the treatment. After RNA extraction and cDNA synthesis, qRT-PCR was conducted. This analysis revealed that, as expected, *KNU* mRNA levels were higher than in the wild type (Fig. 4.4 B), thus confirming that the over-expression construct was functional. Specifically, of 12 lines analyzed, 5 showed an at least 10-fold increase in expression when compared non-transgenic control plants. In the other lines, *KNU* mRNA levels were slightly elevated or similar to those in wild-type plants. The strongest line identified (#12), where *KNU* levels were about 20-fold higher than in the wild type, was used for further characterization.

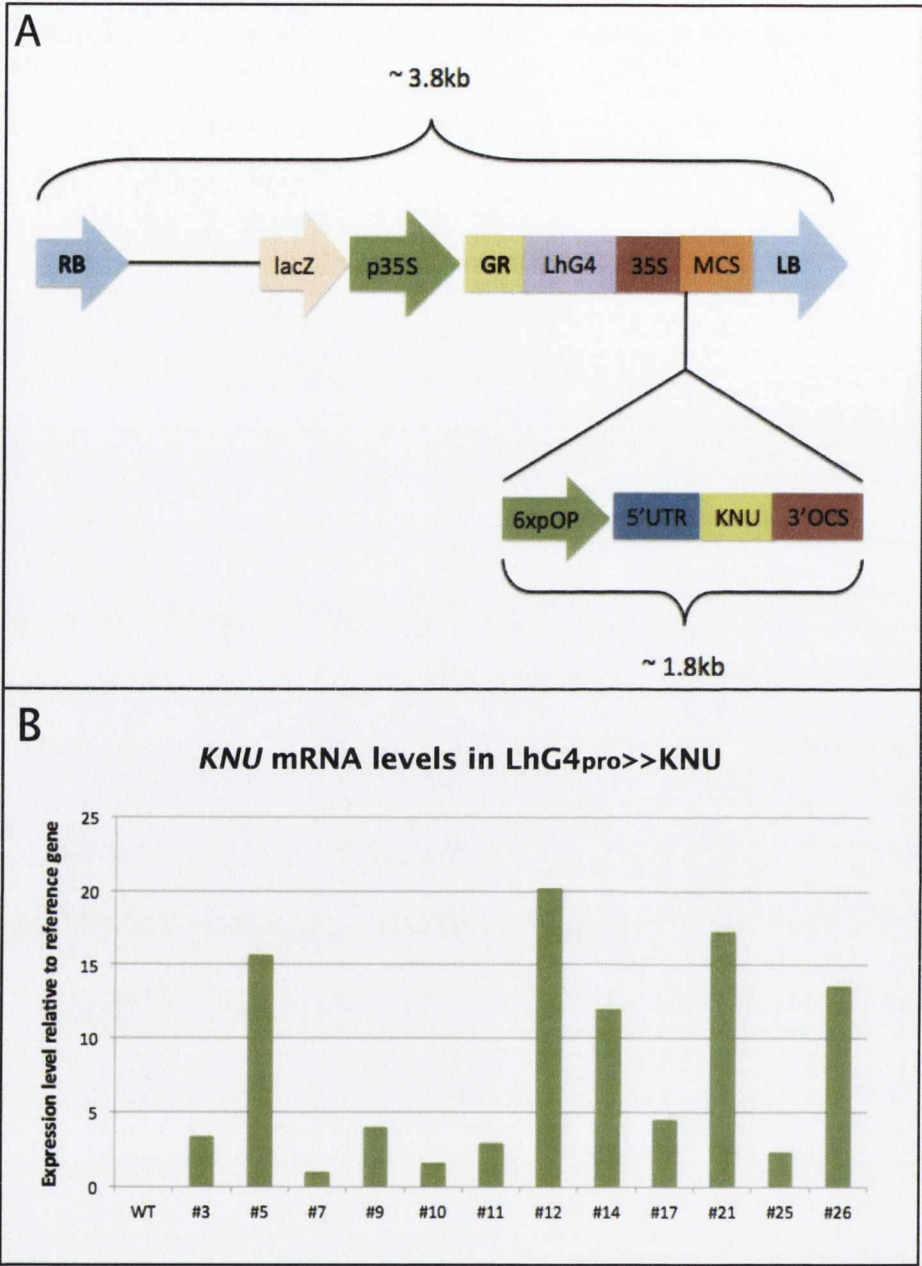


Figure 4.4 Induction of *KNU* over-expression from the 35S_{pro}:GR-LhG4/6xOP_{pro} promoter system. (A) A schematic representation of the T-DNA insertion mediating expression of *KNU* from the 35S_{pro}:GR-LhG4/6xOP_{pro} dexamethasone-inducible promoter system. For a full description, see Section 2.15.6. (B) *KNU* mRNA levels in selected T2 lines of axillary inflorescence tissue after a single treatment with 10 μ M dexamethasone. Expression levels were determined relative to those of a reference gene. Sets of oligonucleotides used for qRT-PCR: KK-27/KK-28 to amplify *KNU* and KK-29/KK-30 for the reference gene *REF2* (At4g34270). 3'OCS, octopine synthase terminator sequence; GR, glucocorticoid receptor sequence; LhG4,

chimeric transcription factor sequence; RB, right border of T-DNA; LB, left border of T-DNA, lacZ, a modified version of the β -galactosidase coding gene from *E. coli*; p35S, Cauliflower Mosaic Virus (CaMV) 35S promoter sequence; MCS, multi cloning site; 35S, Cauliflower Mosaic Virus (CaMV) 35S terminator sequence; 6xOP, 6 repeats of the OP promoter sequence; 5'UTR, 5' untranslated region.

To this end, T2 plants of line #12 were again submitted to dexamethasone treatments once, twice or thrice and a phenotypic characterization was performed (Fig. 4.5 A-G).



Figure 4.5 Phenotypes observed after *KNU* over-expression from the 35S_{pro}:GR-LhG4/6xOP_{pro} promoter system. (A-B) Flowers of plants treated once with 10 μ M dexamethasone exhibited organ elongation defects and did not produce pollen. In addition, siliques were abnormally shaped and shorter than in the wild type. (C-D) Phenotypes of flowers treated twice with dexamethasone. (C) Some flowers formed abnormal petals but stamens were largely unaffected and produced some pollen. (D) Shorter petals started to turn green. (E-G) Phenotypes observed in plants treated three times with 10 μ M dexamethasone. (E) The size of

flowers was drastically reduced when compared to wild-type flowers. Green petals and anthocyanin accumulation in the carpel was observed in flower treated with DEX. (F) Siliques of a wild-type plant and of plants after *KNU* over-expression. (G) The latter siliques were much shorter and formed a reduced number of seeds (arrows). Size bars: 1 mm.

Plants that were treated only once contained flowers that had developmental defects such as shorter sepals, petals and stamens than the organs of wild-type flowers (Fig. 4.5 A). Additionally, carpels were slightly kinked while stamens did not produce pollen (Fig. 4.5 B, indicated by an arrow). There was no significant phenotypic response in the siliques of these plants. Plants treated twice with 10 μ M dexamethasone showed abnormalities in floral organ development (Fig. 4.5 D) while some flowers continued to generate almost regular stamens that produce pollen grains (Fig. 4.5 C). Plants that were treated three times with the dexamethasone-containing solution exhibited the strongest mutant phenotype. The size of the flower was drastically reduced and the proportion of the length between the carpel and other organs was disturbed. Petals became green and carpels accumulated anthocyanin (Fig. 4.5 E, indicated with an arrow). Siliques of these plants were much shorter than those of wild-type plants and in addition, they exhibited abnormal seed development.

The phenotypes observed in these transgenic plants did not match the phenotypes published by (Sun et al., 2009). In order to gain more information on the effects of expressing *KNU* ectopically, I aimed to generate plants over-expressing *KNU* constitutively. The results of this experiment are described below.

4.3.3 Characterization of a line ectopically expressing *KNU*-GFP

As the *knu-1* mutant was first described in accession (Wassilewskija [Ws]), a 35S_{pro}:*KNU*-GFP transgene was introduced into Ws-0 plants. Twenty-three primary transformants were isolated and the presence of the transgene was confirmed. Among these T1 lines, 6 formed abnormal flowers and siliques while the other lines did not exhibit any developmental defects. Four of the 6 lines with mutant phenotypes were only mildly impaired, forming shorter petals and sepals (Fig. 4.6 B, C), as well as short siliques and flowers that were smaller than those of the wild type. Sepals often bore clusters of trichomes (Fig. 4.6 B) and some gynoecia formed branched trichomes on

carpel valves (Fig. 4.6 C). The other two lines with mutant phenotypes produced flowers with short sepals, exaggerated stigma (Fig. 4.6 D, indicated by an arrow) and carpel bore trichomes on their abaxial (outer) side (Fig. 4.6 E). Siliques of those plants were also much shorter than those of the wild type and produced fewer seeds (Fig. 4.6 F).



Figure 4.6 Floral phenotypes observed in 35S_{pro}:KNU plants. (A) Wild-type flower (accession: Ws). (B) Flower of a 35S_{pro}:KNU plant with shorter organs and clusters of trichomes forming on sepals (indicated by an arrow). (C) Branched trichomes forming on sepals. (D) Flower with smaller than normal sepals and enlarged stigmatic tissue. (E) The same line as (D) forming trichomes on the gynoecium. (F) Siliques contained fewer seeds and were shorter than wild-type siliques. Size bars: 1 mm.

I also generated a separate line that contained the KNU-GFP coding region in addition to the 5'UTR of *KNU*, which was expressed from the 35S promoter (for details on

generation of the construct see Section 2.15.5) (Fig. 4.7 A). A large number of *L-er* wild-type plants were transformed with the vector carrying the 35S_{pro}:*KNU*-GFP and 42 primary transformants were isolated that contained the transgene. Among them only 4 lines (#6, #7, #37, and #39) exhibited changes in the phenotype whereas the rest of the lines did not show any phenotypic abnormalities. The lines with the strongest phenotype were brought to the next generation and axillary inflorescences were collected in order to examine the level of *KNU* and *KNU*-GFP transcripts. After RNA extraction and cDNA synthesis, qRT-PCR was carried out. The lines that showed phenotypic differences were found to have increased levels of *KNU* mRNA. In case of line #7, generation of T2 plants consisted of plants of weak, medium and strong phenotype (Fig. 4.7 B), which suggested that those transgenes were segregating, explaining the different phenotypes in this line.

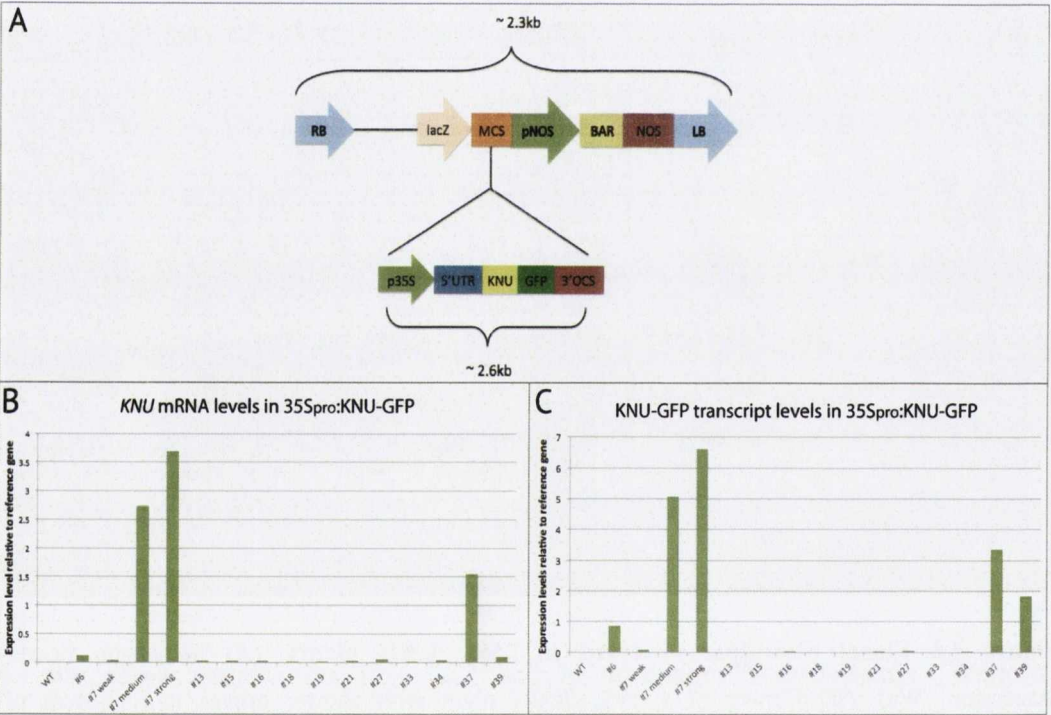


Figure 4.7 Levels of *KNU* mRNA in 35S_{pro}:*KNU*-GFP plants. (A) A schematic representation of the T-DNA insertion mediating expression of *KNU* from the 35S promoter. A full description is available in Section 2.15.5. **(B)** *KNU* mRNA levels in flowers of plants containing the transgene. Only 4 (#6, #7, #37 and #39) out of 13 analyzed lines showed mutant phenotype and in those *KNU* was expressed at higher levels than in the wild type. Cp values of duplicate measurements were within 0.5 units of each other. **(C)** Levels of the *KNU*-GFP transcript. The primers pairs KK-27/KK-28, KK-29/KK-30, and KK-247/KK-248 were used to amplify *KNU*,

the *REF2* reference gene and *KNU-GFP*, respectively. 3'OCS, octopine synthase terminator sequence; RB, right border of T-DNA; LB, left border of T-DNA, lacZ, a modified version of the β -galactosidase coding gene from *E. coli*; pNOS, nopaline synthase promoter sequence; BAR, BASTA® resistance gene; MCS, multi cloning site; NOS, nopaline synthase terminator sequence; KNU, *KNU* coding sequence; GFP, green fluorescence protein, 5'UTR, 5' untranslated region.

In general, the expression levels of the *KNU* and *KNU-GFP* transcripts were very low for the constitutively over-expressed line. In line #39, expression level reached 1.5 of wild-type levels whereas in line #7 'medium' and 'strong' the level of KNU expression were increased 2.5 and 3.5-fold, respectively. However, this level of *KNU* transcript appeared to be sufficient to induce developmental defects in the plants. In case of the *KNU-GFP* transcript, levels of enrichment were higher and span between ~1 in line #6 up to 5-6.5-fold in line #7 'medium' and 'strong', respectively. (Fig. 4.7 C). The weakest phenotype was observed in line #6. These plants had problems with pollen production but no other abnormal floral phenotypes were observed (Fig. 4.8 B), with the exception of the formation of tri-carpelloid siliques (Fig. 4.8 A, indicated by an arrow). Another line that showed slightly different phenotype was line #39. Here, flowers produced pollen, but carpel development was abnormal (Fig. 4.8 C). Similar phenotypes were observed in line #7 marked as 'weak' (not shown). Line #37 exhibited a medium strength phenotype. For instance, flowers showed problems in stamen elongation and siliques produced additional carpels (Fig. 4.8 D, E). Some flowers formed organs that consisted of fused petal and stamen and in addition displayed problems in stigma formation (Fig. 4.8 F, indicated by an arrows). A line that showed much stronger phenotypes was line #7 (named 'medium'). These flowers displayed a number of defects, e.g. elongation problems (Fig. 4.8 G) (flowers were smaller than those of the wild type), the formation of trichomes on carpel valves (Fig. 4.8 H), and carpel fusion defects (Fig. 4.8 I). The same line (#7), named 'strong' produced flowers with similar defects, including underdeveloped and unfused carpels (Fig. 4.8 J). In addition, some gynoecia were tri- and tetra-carpelloid. Moreover, flowers produced more trichomes on sepals when compared to the wild type (Fig. 4.8 K), and gynoecia exhibited trichome formation on their abaxial surfaces. Abnormal stamen and petal formation was also observed in these plants (Fig. 4.8 L).

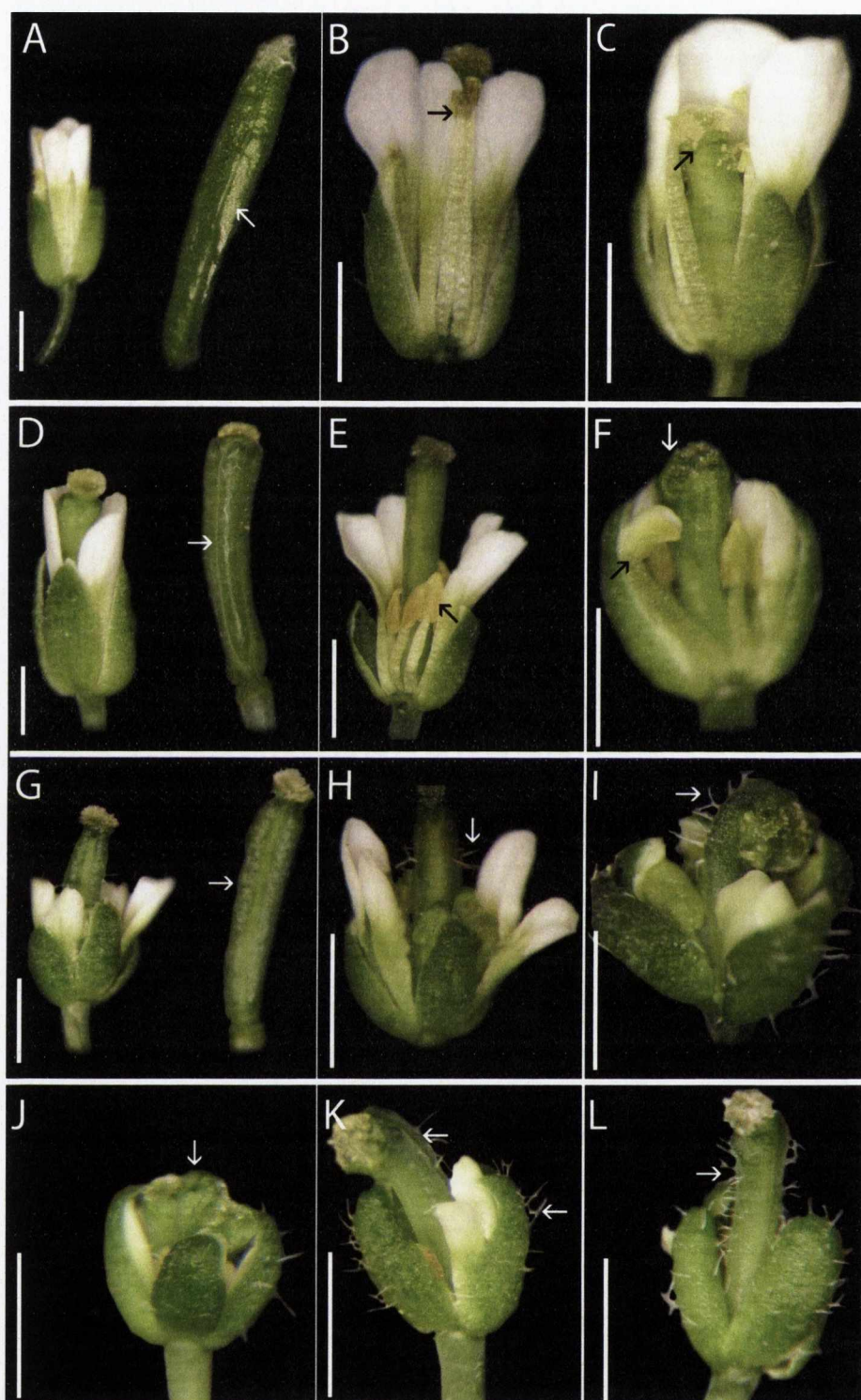


Figure 4.8 Phenotypes observed in flowers of 35S_{pro}:KNU-GFP plants. (A-C) Phenotypes observed in plants with *KNU* transcript levels similar to those in wild-type plants. (A) A silique

with a supernumerary carpel. **(B)** Some plants did not produce pollen, however, others made pollen but formed less stigmatic tissue than the wild type **(C)**. **(D-F)** Line expressing *KNU* mRNA at a higher level than the wild type. **(D)** A flower with a reduced size compared to the wild type and a tetracarpelloid silique. **(E)** Flowers with stamen elongation defects. **(F)** Flower showing mild homeotic transformations, producing petaloid stamens. **(G-I)** Phenotypes of flowers observed in plants with high expression of *KNU*. **(G)** Flower with reduced size and a short silique with bulged valves. **(H)** Flower with trichomes on silique. **(I)** Flower with unfused carpel carrying branched trichomes. **(J-L)** Phenotypes observed in the strongest lines. **(J)** Flower with unfused carpels. **(K)** Flower with a tetracarpelloid gynoecium carrying trichomes. Sepals formed branched trichomes. **(L)** Flower with multiple branched and unbranched trichomes on the gynoecium. Abnormal stamen and petal development were also observed. Size bars: 1 mm.

4.3.4 Testing the activity of the EAR-like domain in *KNU*

As mentioned earlier, *KNU* is a transcription factor that contains an EAR-like motif, suggesting that it predominately acts as a repressor of gene expression. To determine the validity of this assumption, a translational fusion of the *KNU* coding sequence with the strong VP16 transcriptional activation domain (Triezenberg et al., 1988) was expressed from the 35S promoter (Fig. 4.9 A). This fusion protein should act as a transcriptional activator, however, the EAR-domain may interfere with the activity of the VP16 domain. Therefore, the EAR-like motif was mutated to test its function in the context of *KNU*-VP16 activity (Fig. 4.9 B).

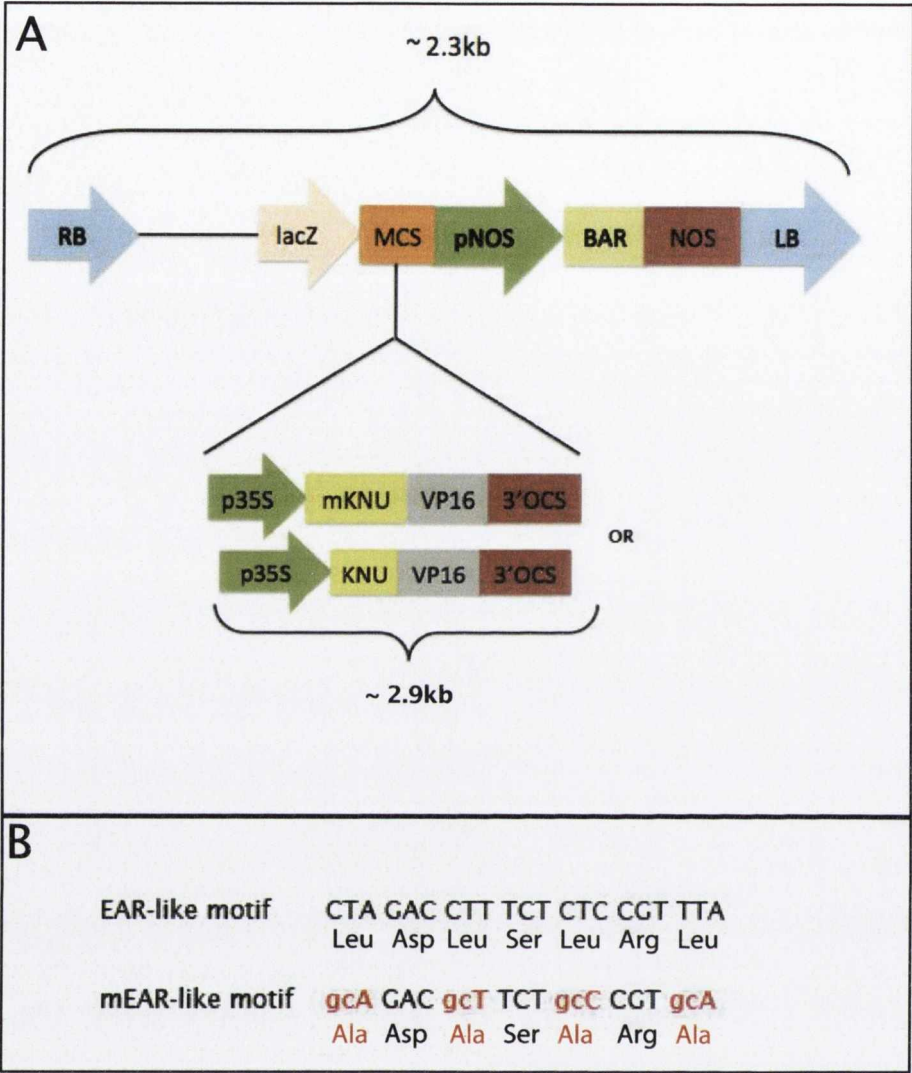


Figure 4.9 Construction of plasmids for KNU-VP16 fusions. (A) A schematic representation of the T-DNA insertion mediating expression of fusion between the VP16 activation domain and KNU or mutated KNU, respectively, from the 35S promoter. A full description of these plasmids is available in Section 2.15.10. (B) Sequences of wild-type and mutated EAR-motifs used for this study. Nucleotide sequences and corresponding amino acid sequences are shown. Nucleotides mutated to obtain the mEAR-like motif are indicated by lowercase letters. Red indicates the resulting amino acid changes. 3'OCS, octopine synthase terminator sequence; RB, right border of T-DNA; LB, left border of T-DNA, lacZ, a modified version of the β -galactosidase coding gene from *E. coli*; pNOS, nopaline synthase promoter sequence; BAR, BASTA® resistance gene; MCS, multi cloning site; NOS, nopaline synthase terminator sequence; KNU, *KNU* coding sequence; mKNU, *KNU* coding sequence with mutated EAR-like motif; VP16, activation domain from *Herpes simplex*.

4.3.4.1 Characterization of plants expressing a KNU-VP16 fusion protein

The *KNU* coding sequence was fused to the coding sequence of the VP16 activation domain from *Herpes simplex* and expressed in wild-type plants from the 35S promoter. Thirty-five transformants were isolated that contained the transgene. Twenty-eight of those were phenotypically different from wild-type plants. In general, plants were smaller and produced smaller floral organs. Flowers bore more trichomes on the abaxial side of sepals (Fig. 4.10 A) and produced petite and misshapen petals (Fig. 4.10 B). Leaves of plants containing the transgene were misshapen and curved. Moreover, their shape was more pointed and the leaves were chlorotic compared to wild-type plants (Fig. 4.10 C). Also, siliques were much shorter (Fig. 4.10 D) and produced fewer seeds.

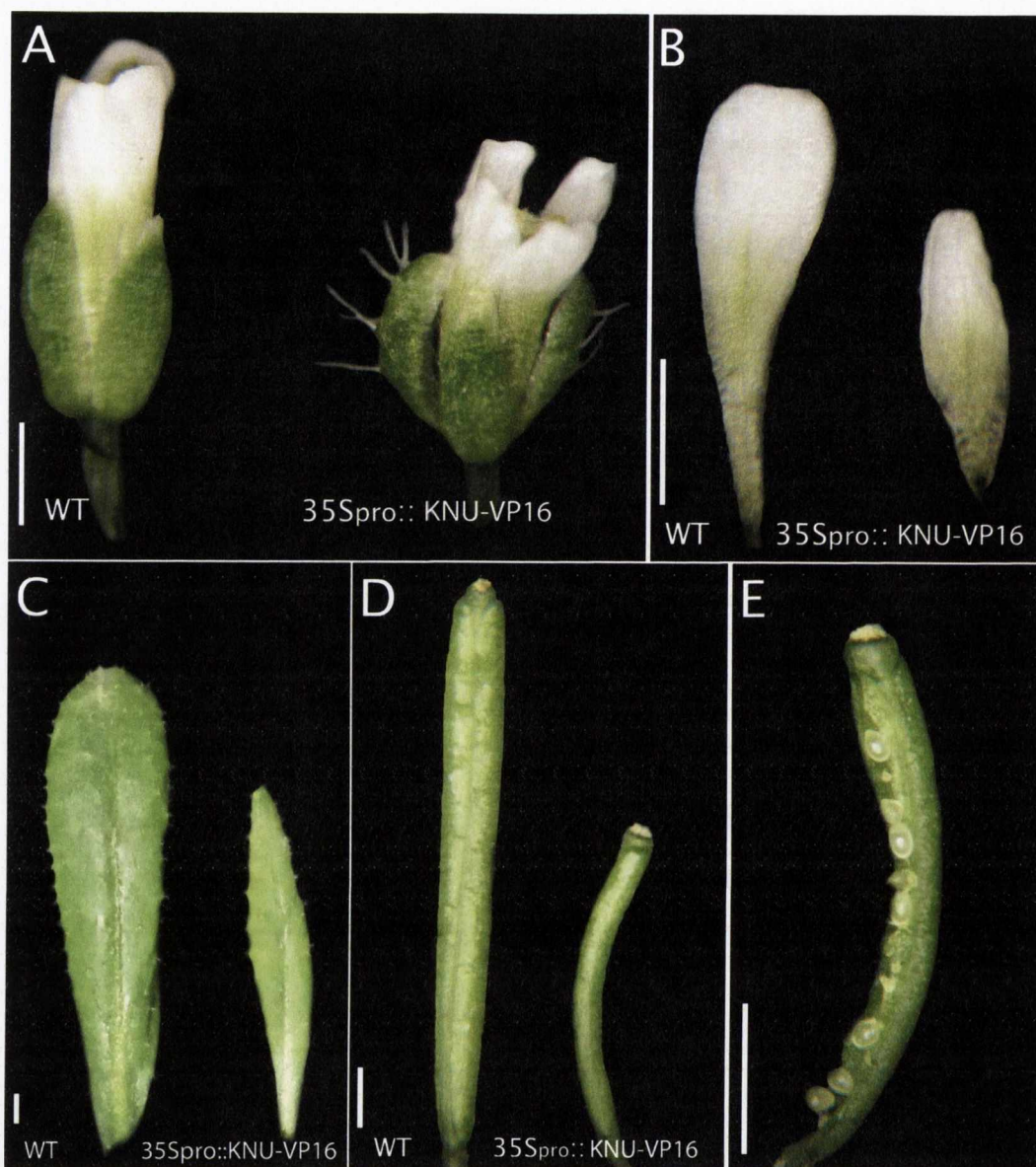


Figure 4.10 Floral phenotypes of 35Spro::KNU-VP16 plants. (A) Flower of a transgenic plant with reduced size compared to the wild type, and numerous trichomes on sepals. (B) Comparison of wild-type petals and petals of a transgenic plant. (C) Plants carrying the transgene produced small, curled leaves. (D) Siliques of wild-type and 35S_{pro}::KNU-VP16 plants. (E) Siliques of transgenic plants were shorter and produced fewer seeds. Size bars: 1 mm.

4.3.4.2 Characterization of plants expressing a KNUmEAR-VP16 fusion protein

In order to disrupt the repressive effect of the EAR-like motif that is included at the N-terminal region of KNU, four leucine residues, which are known to be important for the

function of the EAR domain, were replaced with alanines (Fig. 4.9 B). Wild-type plants were then transformed with a plasmid containing the VP16 activation domain fused to the mutated *KNU* coding sequence and expressed from the 35S promoter. Twenty-nine transformants were isolated and genotyped for the presence of the transgene. The primary transformants fell into three phenotypic classes: seven out of 26 plants showed weak mutant phenotypes. These plants were smaller than wild-type plants (Fig. 4.11 A), displayed abnormal phyllotaxis and flowered considerably longer than the wild type. They also showed defects in silique development with most siliques being tricarpelloid (Fig. 4.11 B, indicated by an arrow). (Fig. 4.12 A-C). Some flowers produced curved petals (Fig. 4.12 A), while others displayed a phenotype with strongly bent carpels and stamens (Fig. 4.12 B). In addition, flowers often produced filamentous organs in place of stamens (Fig. 4.12 C).

In the second phenotypic class, plants displayed growth problems and were only half the size of the wild type grown under the same conditions. In addition to problems with phyllotaxis, which was strongly affected (Fig. 4.11 A, in the middle), siliques were tetra-carpelloid (Fig. 4.11 C, indicated by an arrow) and contained some seeds. Flower phenotypes were much stronger compared to the changes observed in the phenotypically weak group of plants (Fig. 4.12 D-F). In these flowers some mild homeotic transformations were observed.

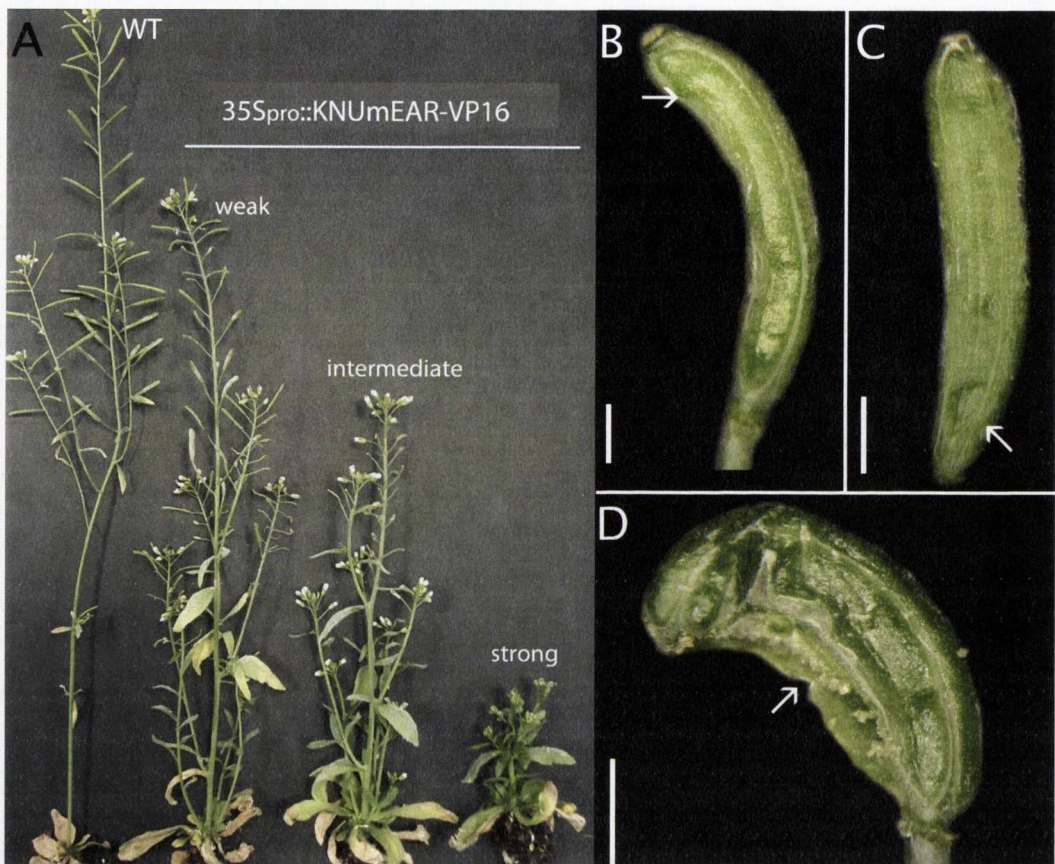


Figure 4.11 Phenotypes of plants carrying the 35S_{pro}:KNUmEAR-VP16 transgene. (A) A wild-type plant compared to transgenic lines with different phenotypic strengths (weak, intermediate or strong). (B) Tricarpelloid silique of a phenotypically weak plant. (C) Tertacarpelloid silique of a phenotypically intermediate plant. (D) Bent and unfused silique from a phenotypically strong plant. Size bars: 1 mm.

For instance, stamen transformation into a carpel-resembling tissue with visible ovules (Fig. 4.12 D, indicated by an arrow). Moreover, the flowers with stamens with the petal-like tissue on the top of anther were observed (Fig. 4.12 E). Additionally, some plants exhibited bifurcated stamens with two anthers (Fig. 4.12 F).

The last group of plants comprised of 6 (out of the 29) T1 lines exhibited very strong phenotypes. These plants were dwarfed (Fig. 4.11 A, far right) and siliques were multi-carpelled, curved, and unfused (Fig. 4.11 D). They did not produce any seeds. In addition, floral organs were strongly affected (Fig. 4.12 G-I). Carpels were often curled and appeared to accumulate anthocyanins, while petals and stamens turned green (Fig. 4.12 G, indicated by arrows). Other floral phenotypes in this group of plants resembled strong homeotic transformation, where unfused carpels did not contain seed and petals

turned green (Fig. 4.12 H). The strongest phenotype observed included flowers containing petals and presumably stamens transformed into sepal-like tissue. These flowers failed to produce seeds as the carpel was unfused and strongly misshapen (Fig. 4.12 I).



Figure 4.12 Floral phenotypes observed in 35S_{pro}:KNumEAR-VP16 plants. (A-C) Developmental abnormalities of phenotypically weak lines. Flowers with (A) curved petals, and (B) carpels and stamens were observed. (C) Some flowers produced filamentous organs. (D-F) Mild homeotic transformations were observed in flowers of lines with intermediate phenotypes. (D) Transformation of a stamen into a carpel-like organ. (E) Stamen with petaloid tissue growing out of the anther. (F) A branched stamen. (G-I) Homeotic transformations observed in lines with strong phenotypes. (G) Stamens and petals with green tissue. A bent gynoceium

accumulating anthocyanin. (H) Flower with curved, misshaped and green petals. The tricarpeloid gynoecium exhibited reduced seed set. (I) Stamens and petals transformed into sepal-like organs. Unfused carpel-like organs with visible unfertilized ovules were produced in these plants. One sepal was removed from flowers in panel A, B, C, D, E and G for better visibility of the inner whorl organs. Size bars: 1 mm.

4.3.5 Translational fusion of an mGFP5 reporter to KNU

The identification of a functional KNU-amiRNA (see Chapter 3) prompted the generation of an amiRNA-resistant, epitope-tagged version of KNU that could rescue the mutant phenotype observed in lines expressing KNU-amiRNA. This line could be used in ChIP experiments in order to identify the genomic regions bound by KNU. To this end, Dr Diarmuid O'Maoileidigh conducted an experiment where, an mGFP5 reporter gene was translationally fused to the 3' end of the KNU coding region (see Section 2.15.7) and was placed under the control of the endogenous KNU promoter (Fig. 4.13 A). A T-DNA construct carrying this fusion as well as a kanamycin resistance gene were transformed into homozygous 35S_{pro}:KNU-amiRNA1 plants. Transformants were identified based on their resistance to kanamycin and by PCR-based genotyping (see Section 2.15.7). Of 25 independent transformants that contained the KNU_{pro}:mKNU-mGFP5 transgene (these plants are referred to as KNU-GFP plants hereafter), 10 did not produce any bulged siliques, suggesting that in these lines, the KNU-mGFP5 fusion protein could compensate for the amiRNA-mediated knockdown of endogenous *KNU* activity. These 10 plants were then crossed with AP1_{pro}:AP1-GR *ap1-1 cal-1* plants (for detailed description see Section 2.15.8) to generate KNU-GFP,FIS plants. In order to carry out a preliminary characterization of these KNU-GFP,FIS lines, inflorescence tissue from F1 plants (none produced *knu*-like siliques), as well as from wild-type plants, was collected. After RNA extraction and cDNA synthesis, qRT-PCRs were performed to determine *KNU* transcript levels. *KNU* mRNA levels in these lines varied from 35% of wild-type levels to a 10-fold increase in *KNU* mRNA when compared to wild-type plants (Fig. 4.13 B). Although *KNU* mRNA levels in certain lines (#2, #4, #5 and #17) were lower than in the wild type, no *knu*-like mutant phenotypes were observed, suggesting that the remaining KNU activity was sufficient to carry out the functions of KNU. Lines #12, #16 and #23 were selected for further analysis because *KNU* mRNA levels appeared to be similar to those in wild-type

plants. Homozygous plants for line #16 have been isolated in the floral induction system and levels of *KNU-GFP* transcripts in F2 plants (#16.6) were examined in the plants that responded to dexamethasone treatment. Tissue was collected at 0 d and 7 d after the treatment. In addition, tissue of treated inflorescence at 7 d of AP1-FIS plants and buds from stage 0-8 of 35S_{pro}:*KNU*-amiRNA plants were included. *KNU* transcript levels in *KNU*-GFP, FIS were ~140 fold higher than in control lines, confirming strong expression of the translational fusion of mGFP to *KNU* (Fig. 4.13 D). The presence of *KNU-GFP* transcript was confirmed by treating four plants of a F2 segregation population with dexamethasone, followed by tissue collection, RNA extraction, cDNA synthesis, and qRT-PCR analysis. Strong expression of the transcript was observed in dexamethasone-treated plants (lines 1-4) in contrast to plants not treated with the hormone (line 5) or plants containing only the 35S_{pro}:*KNU*-amiRNA transgene (line 6) or AP1-FIS plants (line 7) (Fig. 4.13 C). This line was later used for a detailed characterization before chromatin immunoprecipitation experiments commenced.

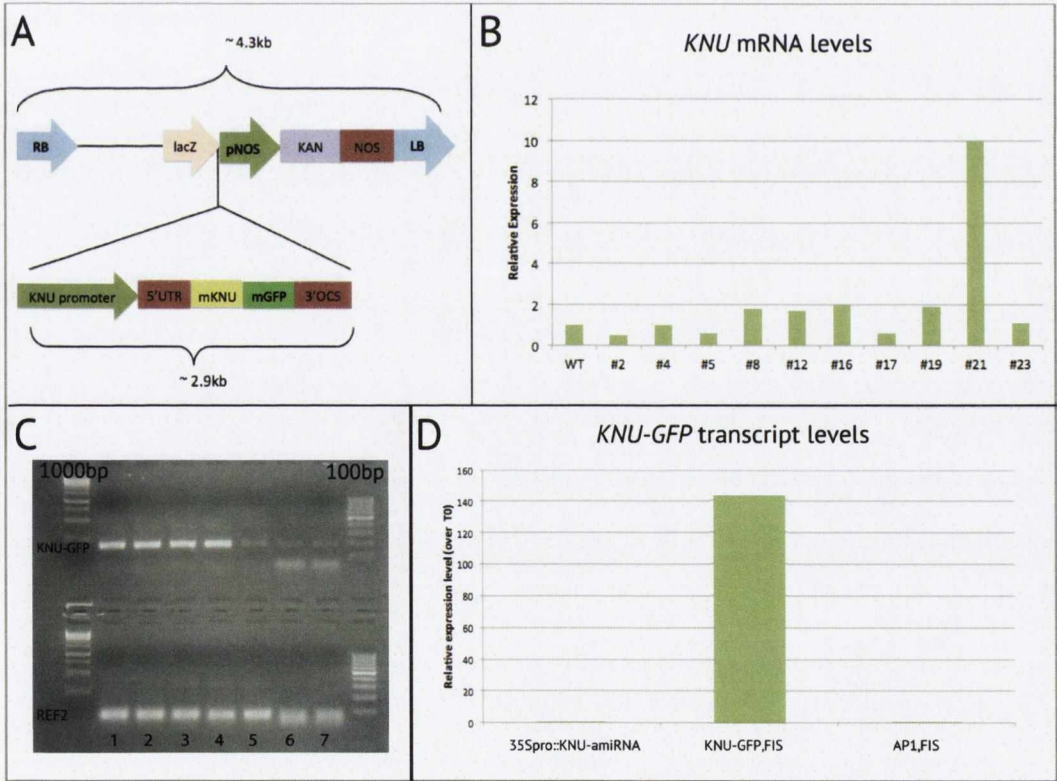


Figure 4.13 Accumulation of *KNU* and *KNU-GFP* transcripts in *KNU*-GFP expressing lines. (A) A schematic representation of the T-DNA insertion mediating expression of the *KNU* coding sequence fused to GFP under control of the endogenous *KNU* promoter. (B) Levels of

KNU transcripts in plants that contain both the 35S_{pro}:*KNU*-amiRNA and the *KNU*_{pro}:m*KNU*-GFP transgene. No error bars are presented for expression assays because these values represent technical duplicates (adapted from (O'Maoileidigh, 2012)). (C) RT-PCR on plants carrying *KNU*_{pro}:m*KNU*-GFP in a FIS background. Expression of *KNU*-GFP transgene in F2 plants of line #16.6 (those which responded to dexamethasone treatment). Lines 1-4 plants treated with dexamethasone. Line 5, untreated plant. Line 6, 35S_{pro}:*KNU*-amiRNA. Line 7, FIS plants. Thirty-four cycles were used at 58°C to amplify the transcripts. (D) *KNU*-GFP transcript levels in *KNU*-GFP, FIS plants. The level of *KNU*-GFP was measured 7 d after dexamethasone treatment and was compared to untreated plants (T0). Plants containing the 35S_{pro}:*KNU*-amiRNA1 transgene and FIS plants served as controls. Cp values of duplicate measurements were within 0.5 units of each other. Primer pairs KK-27/KK-28 and KK-247/KK-248 were used to amplify *KNU* and *KNU*-GFP transcripts, respectively. Expression levels are given relative to those of a reference gene (*REF2*) amplified using primers KK-27/KK-28. 3'OCS, octopine synthase terminator sequence; RB, right border of T-DNA; LB, left border of T-DNA, lacZ, a modified version of the β-galactosidase coding gene from *E. coli*; pNOS, nopaline synthase promoter sequence; KAN, kanamycin resistance gene; NOS, nopaline synthase terminator sequence; m*KNU*, amiRNA1-resistant sequence of *KNU*; GFP, green fluorescence protein, 5'UTR, 5' untranslated region.

4.3.6 Characterization of plants containing 35S_{pro}:*KNU*-amiRNA and *KNU*_{pro}:m*KNU*-mGFP in an AP1_{pro}:AP1-GR *ap1 cal* background

The line described in the previous section was brought to the next generation and genotyped for two transgenes as described in Section 2.15.8, and a segregation analysis was carried out due to a lack of a genotyping assay for one of the transgenes (see Section 2.15.8). Inflorescences of the homozygous plants were then treated with a dexamethasone-containing solution and examined for synchronous flowering. Lines #16.6.2-3 and 16.6.6-7 responded best to the dexamethasone treatment and exhibited synchronous flower development (Fig. 4.14 A, B; line #16.6.6). In order to verify that the *KNU*-GFP protein was present in its appropriate domain, fluorescence microscopy was used to image dexamethasone-treated inflorescence-like meristems.

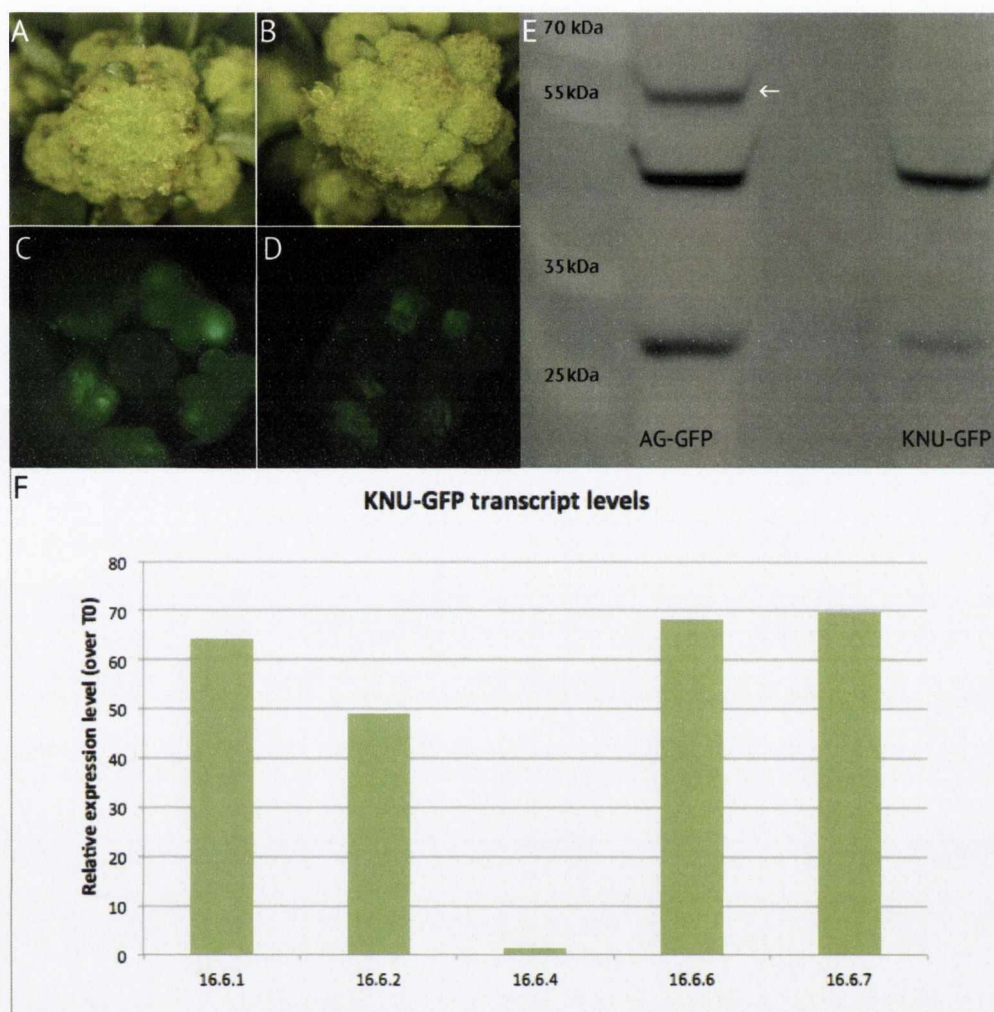


Figure 4.14 Characterization of $35S_{pro}:KNU\text{-}amiRNA$, $KNU_{pro}:mKNU\text{-}mGFP$, $API_{pro}:API\text{-}GR$ *ap1-1 cal-1* plants. (A) Inflorescence of an untreated plant. (B) An inflorescence 4 days after being treated with a dexamethasone-containing solution. Synchronous flowering was observed up to 8 d after dexamethasone treatment (not shown). (C-D) Fluorescent microscope images of a flower 8 days after dexamethasone treatment (C) and anthers of a plant at the same stage of development (D). Negative controls are not shown. (E) Results of Western blot analysis of nuclear extracts from inflorescence tissue of plants expressing either KNU-GFP or AG-GFP using GFP antibody. No band was observed at the size expected for KNU-GFP; a band for AG-GFP was detected at ~56 kDa (indicated by an arrow). (F) *KNU-GFP* transcript levels in KNU-GFP, FIS plants (F3 generation). Strong *KNU-GFP* expression was detected in four out of five KNU-GFP, FIS plants. The levels of *KNU-GFP* were measured 8 d after dexamethasone treatment and were compared to those of a reference gene. Cp values of duplicate measurements were with 0.5 units of each other. Primer pairs KK-

247/KK-248 and KK-27/KK-28 were used to amplify *KNU-GFP* and a reference gene (*REF2*), respectively.

In the *KNU-GFP*, FIS plants that were treated with dexamethasone clear fluorescent signals were observed in anthers 8 days after the treatment (Fig. 4.14 C-D). In addition, Western blotting (Section 2.11) was conducted on nuclear protein extracts that were generated using plants expressing *KNU-GFP* and *AG-GFP* (as a positive control) (O'Maoileidigh, 2012) using an α -GFP antiserum. The method identified a band at ~56 kDa, which corresponded to the size of the *AG-mGFP* fusion protein (Fig. 4.14 E, indicated by an arrow) and indicated that the detection method had worked in principle. However, the *KNU-GFP* fusion protein (expected size: ~45 kDa) could not be identified perhaps due to low expression levels or the small expression domain of *KNU* in flowers. To test the levels of *KNU-GFP* transcript in the plants of the F3 generation, the inflorescences of different lines were treated with dexamethasone and after tissue collection, RNA extraction and cDNA synthesis, qRT-PCR was conducted. The expression levels of *KNU-GFP* transcripts in plants 8 d after dexamethasone treatment were measured and compared to those in plants that were not treated with dexamethasone (Fig. 4.14 F). Five lines were examined and four of them showed a high level of *KNU-GFP* transcript accumulation, ranging from a ~50 to ~70 fold increase relative to the mock-treated control plants.

Even though the *KNU-mGFP* protein fusion could not be detected by Western blotting, the transgenic line was propagated and used in chromatin immunoprecipitation experiments as described in the Section 4.3.6.

4.3.7 Global identification of genomic regions bound by KNU

4.3.7.1 Generation of ChIP-seq libraries

In order to identify genomic regions bound by *KNU* on a global scale, two independent large-scale ChIP experiments were performed (referred to as *KNU-1* and *KNU-2* hereafter) using *KNU-GFP*, FIS transgenic plants and the α -GFP antiserum Ab290 from Abcam (see Section 2.12.3). To this end, inflorescence-like meristems were harvested 8 days after treatment with a dexamethasone-containing solution and then processed using the ChIP protocol (Diagenode ChIP kit) that was optimized for inflorescence tissue

(Section 2.12).

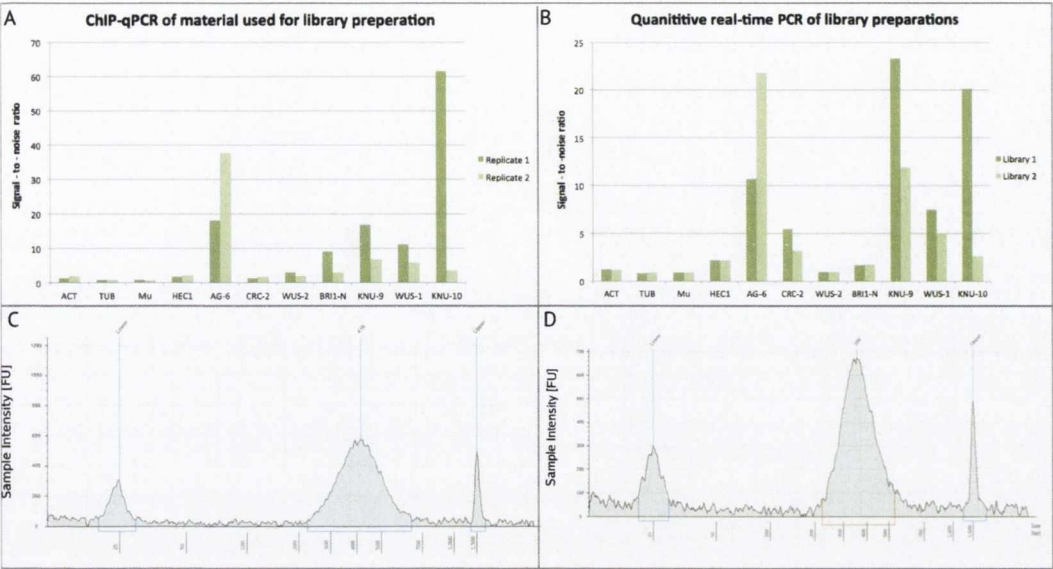


Figure 4.15 Preparation of ChIP-seq libraries for the genome-wide identification of KNU binding sites. (A) Quantitative real-time PCR for selected predicted targets of KNU (as indicated) using immunoprecipitated genomic DNA from dexamethasone-treated inflorescences of KNU-GFP, FIS plants. Two independent biological samples were generated (Replicate 1 and 2). The signal-to-noise ratios were calculated as outlined in Section 2.10. (B) Quantitative real-time PCR of ChIP-seq libraries prepared from the material used in (A). The signal-to-noise ratios were calculated as outlined in Section 2.10. Input DNA from both ChIP preparations was used to calculate the relative enrichments observed in the library preparations. (C-D) DNA size distribution (analyzed with an Agilent Tape Station) the ChIP-seq libraries used for ultra high-throughput sequencing. Lower and upper markers are at 25 bp and 1500 bp, respectively. FU, fluorescence units; bp, base pairs. The primer pairs DM-323/DM-324, DM-325/DM-326, SW-369/SW-370, SW-633/SW-634, DM-523/DM-524, DM-319/DM-320, DM-565/DM-566, SW-627/SW-628, DM-339/DM-340, DM-563/DM-564, and DM-347/DM-348 were used to amplify regions of: *ACT* (*ACTIN*), *TUB* (*TUBULIN*), *Mu*, *HEC1* (*HECETE1*), *AG-6*, *CRC-2* (*CRABS CLAW*), *WUS-2*, *BRI1-N* (*BRASSINOSTEROID INSENSITIVE 1*), *KNU-9*, *WUS-1*, and *KNU-10*.

For both experiments, ~1 mL of inflorescence tissue were used as starting material. After the ChIP reactions, the precipitated genomic DNA was purified and quantified using a fluorescence-based assay. Experiment 1 resulted in a total DNA yield of 35 ng, while experiment 2 yielded 24 ng of genomic DNA. To test the specificity of the ChIP

assays, aliquots of the DNA preparations were subjected to qPCR analysis using primers specific to regions that were presumed to be bound by KNU (*WUS-1*, *-2*, *AG-6*, *HEC2*, *CRC* and *KNU-9*, *10*) and negative (*ACT*, *TUB* and *Mu*) control regions. The positive regions were selected based on the function that KNU plays in meristem maintenance and possibly in carpel development. Five out of eight positive controls were enriched above background (Figure 4.15 A). These results indicated that the ChIP assays were successful. Using the precipitated chromatin from the two experiments, two Diagenode ChIP-seq libraries were prepared (see Section 2.12.6). Briefly, the ends of the ChIP-enriched DNA fragments of Diagenode-specific oligonucleotide adapter sequences (that were based on the pattern of Illumina adapter sequences) were ligated to the modified ChIP DNA and amplified. Next, the adapter-DNA mixture was purified using magnetic beads and the size distribution of the library was examined by electrophoresis (Fig. 4.15 C, D) using TapeStation technology. The average size was 416 and 356 bp for KNU-1 and KNU-2 libraries, respectively. An aliquot of these libraries was taken and qPCR analysis was performed. Input DNA from each ChIP was used to normalize the data, as described above. For both libraries the same positive regions were tested and all of them except one (*WUS-2*) were enriched (Fig. 4.15 B). Although the signal-to-noise ratios in general were lower when compared to the initial ChIP-enriched DNA, in some cases the enrichment was in fact higher. For example, the *CRC* or *KNU-9* positive control region was higher when compared to the initial ChIP-enriched DNA (Figure 4.15 B). Although it remained unclear why the signal-to noise ratios had been compressed during library preparation, these two libraries were sent for ultra high-throughput sequencing, which was carried out using an Illumina Genome Analyzer.

4.3.7.2 Quality assessment of ChIP-seq data

The libraries generated by ChIP from KNU-GFP, FIS plants (denoted KNU-1 and KNU-2 hereafter) and the control libraries derived from the ChIP material that was not submitted to immunoprecipitation (denoted KNU-INPUT-1 and KNU-INPUT-2 hereafter) were subjected to Illumina next-generation sequencing. Dr. Beibei Zheng assisted with the analysis of the data obtained from Illumina sequencing. In brief, the data produced by the Illumina sequencing pipeline included the sequence string output and a quality score. The quality score reflects the probability that a given base call at a

given position is correct. These quality scores and sequence strings were uploaded to the online quality control tool, *FastQC* (www.bioinformatics.bbsrc.ac.uk).

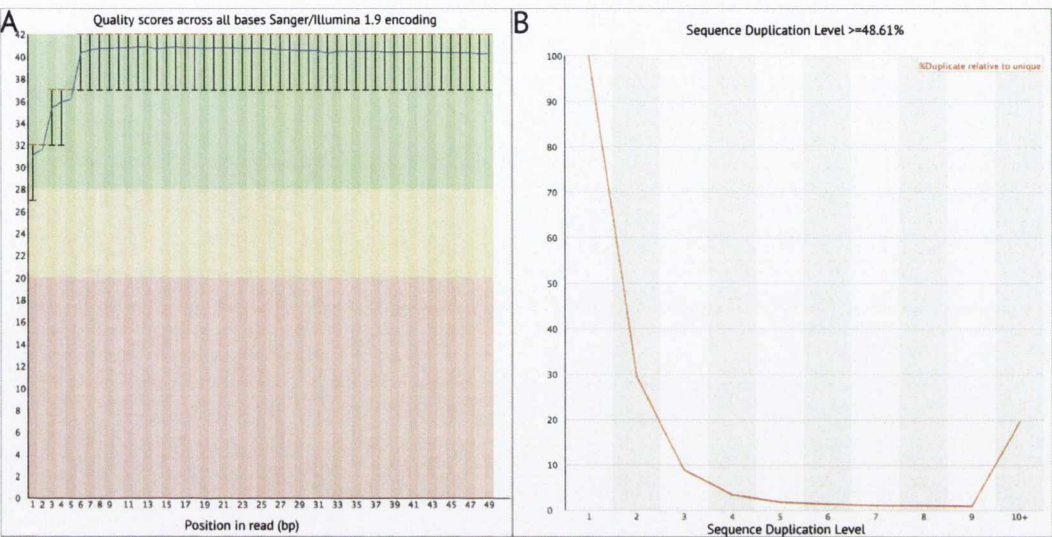


Figure 4.16 Results of ChIP-seq quality controls. (A) Quality scores at a given position within the sequence of all reads from the KNU-1 ChIP-seq experiment, as determined by the *FastQC* software. The box-plots show the distribution of per-base qualities for all reads in the KNU-1 library. The average quality score is high, indicating a good quality dataset. **(B)** Duplication levels of the reads from the KNU-1 library. The number of single reads is represented as 100% and duplicated sequences are represented as a fraction of this. A duplication level of 10 is equivalent to the percentage of sequences that were present 10 or more times. The sequence duplication level reached ~49%.

The *FastQC* software generated a box plot that illustrated the distribution of per-base qualities of all reads (Fig. 4.16 A, the results for the KNU-1 library are shown as an example). The colored blocks indicate high quality calls (green), calls of reasonable quality (orange), and calls of poor quality (red). The box-plots produced for all sequenced samples indicated that the sequencing data were generally of very high quality. This trend was similar for the data from all four sequenced libraries. The *FastQC* software analyses various other parameters of the sequencing run including the number of duplicated sequences. A high level of duplication indicates that there is some enrichment bias present. A high level of sequence duplication was observed in all the libraries sequenced (not shown). The KNU-1 library contained 49% duplication level, which is a substantial amount of multiple reads (Fig. 4.16 B). Given the large data

volume resulting from ChIP-seq experiments, these sequences can be readily removed without affecting the overall sequencing coverage.

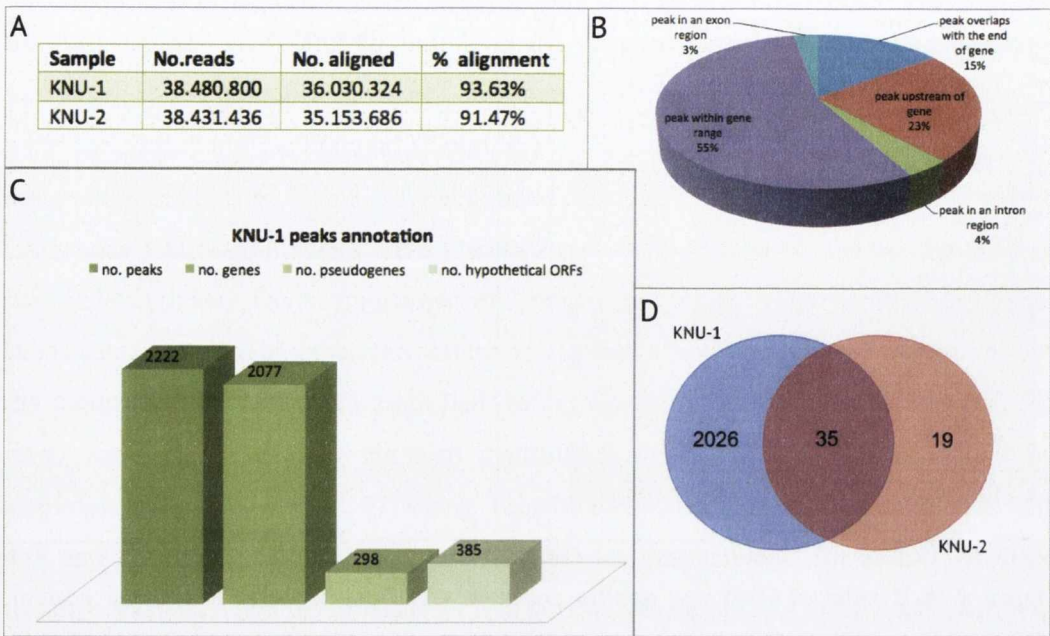


Figure 4.17 Analysis of data from the KNU ChIP-seq experiment. (A) Number of unique reads obtained from two ChIP-seq experiments and their alignment to the *Arabidopsis* genome. (B) The distribution of peaks in KNU-1. Peaks were assigned based on the region closest to a given peak. (C) Number of peaks identified in the *Arabidopsis* genome for the KNU-1 ChIP-seq library. The number of (protein-coding) genes, pseudogenes and hypothetical ORFs associated with the peaks are shown. (D) A Venn diagram depicting the overlap in genes associated with binding peaks in the KNU-1 and KNU-2 libraries.

Next, the *HOMER* (<http://homer.ucsd.edu/>) software was used to align the unique short read sequences obtained to the *Arabidopsis* genome (TAIR release 10, www.arabidopsis.org). For both the KNU-1 and KNU-2 libraries, ~38.5 million reads were obtained and over 90% of these reads could be aligned to the *Arabidopsis* genome. In the KNU-1 dataset, ~23% of peaks were located upstream of a transcriptional initiation site, ~55% of peaks overlapped with the intragenic regions and ~15% of peaks were downstream of the translational termination site (Fig. 4.17 B). Because it was shown that *Arabidopsis* transcription factors often bind to sequences in close proximity to the transcription initiation sites of genes (e.g., (Kaufmann et al., 2010), it was of an interest to investigate why so many peaks were represent in the intragenic regions. The

use of the *HOMER* method provided a simple and fast identification of peaks in the genome. However, the list of genes generated by this software includes genes which are far away from the regulatory regions of their associated gene, and also contains peaks associated with pseudogenes, transposable elements and small ORF's, which could be in the regulatory regions of other genes. Indeed, the number of pseudogenes associated with peaks was around 13% and the number of hypothetical ORFs was around 17% in the KNU-1 dataset (Fig. 4.17 C). Using the *MACS* peak calling method outlined in Section 2.14.5, 2,222 binding peaks were defined as being enriched above background in the KNU-1 library, but only 55 binding peaks were identified for KNU-2 (Fig. 4.17 D). Because based on prior experience in the laboratory, plant transcription factor ChIP experiments result in at least several hundred binding peaks in the *Arabidopsis* genome, the KNU-2 dataset was deemed to be of insufficient quality and therefore excluded from further analysis.

4.3.7.3 Detection of KNU binding sites in the *Arabidopsis* genome

In order to identify genomic fragments bound by the KNU transcription factor, the enrichments of reads that were aligned to KNU binding sites were investigated first. The *IGV* viewer (www.broadinstitute.org) was used to display genomic regions bound by KNU generated by *MACS* peak calling method. Predicted earlier genomic regions bound by KNU were enriched at different level to what was indicated by the peak score. Strong enrichments were observed in the promoter region (peak score 119) and second intron (peak score 50) of the *AG* locus in the KNU-1 dataset (Figure 4.18 A, green track). Enrichment was also observed at the *BRI1* locus (peak score 97.7), in the promoter region (Fig. 4.18 B, green track). The whole promoter region of *CRC* was bound by KNU and a high peak (peak score: 57) was observed (Fig. 4.18 C). A large enrichment was observed in the promoter region and gene body of *KNU* (Fig. 4.18 D) in both, the control (in grey) and the KNU-1 library (in green), which is probably reflective of the extra copies of this genomic sequence in the KNU-GFP, FIS line. This region was not recognized as significantly enriched in the dataset and the peak was not called using *MACS* method.

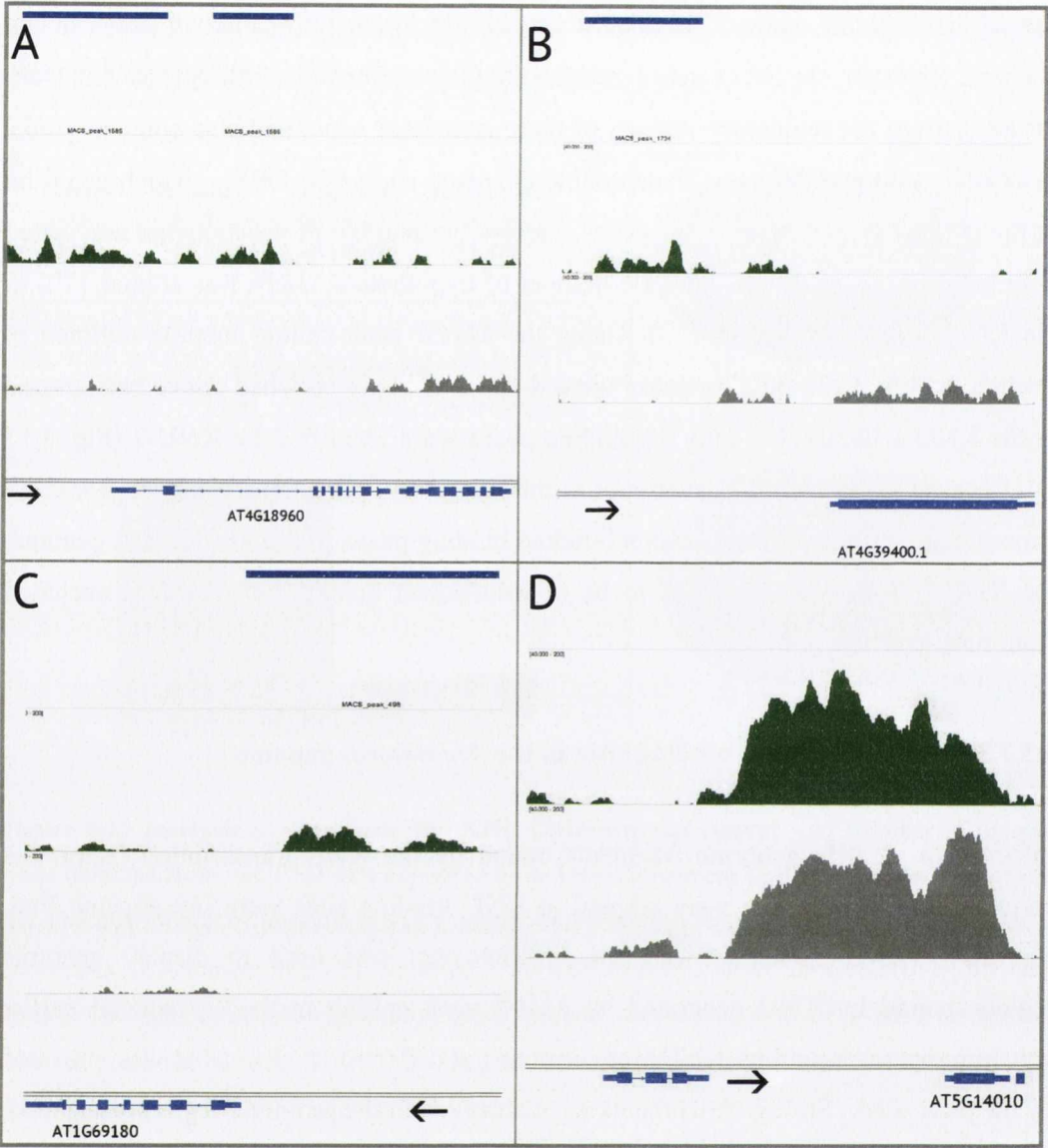


Figure 4.18 Genomic regions with predicted KNU binding sites. (A-D) KNU binding sites at the *AG* locus (A); the *BRI1* locus (B); the *CRC* locus (C), and the *KNU* locus (D). Peaks identified in the KNU-1 library are marked in green. Gene models are shown at the bottom of each graph (in blue). The rectangular blue boxes at the top track represent peaks called by the *MACS* software. The grey tracks represent the results for the control library (KNU-INPUT-1). The peaks were visualized using the *IGV* genome viewer.

Next, a Gene Ontology (GO) term analysis was performed using genes that were associated with binding peaks. This analysis was performed using the software *PANTHER* (<http://pantherdb.org/tools/compareToRefList.jsp>) (Thomas et al., 2006). Using the Bonferroni correction for multiple hypothesis testing, an adjusted *p*-value cut-

off of <0.05 was employed to identify over-represented GO terms. This analysis yielded 88 enriched GO terms in total (Figure 4.19). These terms were further processed in that related GO terms were placed into one of the following parent categories: Biosynthesis; Development; Patterning; Transcription; Metabolic Processes; Hormone Regulation; Patterning and Meristem Regulation. The GO terms *Regulation of Transcription* and *Regulation of RNA Biosynthetic Processes* and *Metabolic Processes* were the highest ranked terms amongst the GO terms. The *Shoot System Development* and *Leaf Development* were enriched in the Development category. The Hormone Regulation section contained terms like *Response to Auxin* and *Regulation of Hormone Levels*. *Regionalization* and *Axis Specification* were significantly enriched in the Patterning category. Finally, *Regulation of Meristem Development* and *Meristem Maintenance* were included in the Meristem Regulation category. The enrichment of all of some of the terms (Meristem Maintenance, Regulation of Transcription) in the ChIP-seq dataset is consistent with the known role of KNU as a regulator of floral meristem determinacy. These enriched GO terms indicated that KNU binds to genes involved in the corresponding processes, which suggests new roles for this transcription factor.

Significant binding peaks were present in the KNU-1 library for many known regulatory genes including *API*, *CLV3*, *GLABRA3 (GL3)*, *SEALLATAPI (SEPI)*, *SUP* and *SEUSS (SEU)* (Fig. 4.19 B-G). A large enrichment was observed in the promoter region and gene body of *API* (Fig. 4.19 B) in both, the control (in grey) and the KNU-1 library (in green), which is probably reflective of the extra copies of this genomic sequence of the AP1-GR originating from the FIS line. Other regions of genes that are specific for different roles in the flower formation were also identified as putative direct targets of KNU. Some of the most interesting genes, in addition to those indicated above, play a role in trichome formation. *TRIPTYCHON (TRY)* together with other genes that encode for zinc finger domain proteins *ZINC FINGER PROTEIN2, 5 and 10 (ZFP2, 5 and 10)* act in parallel and downstream of AG (O'Maoileidigh et al., 2013; Stewart, 2016).

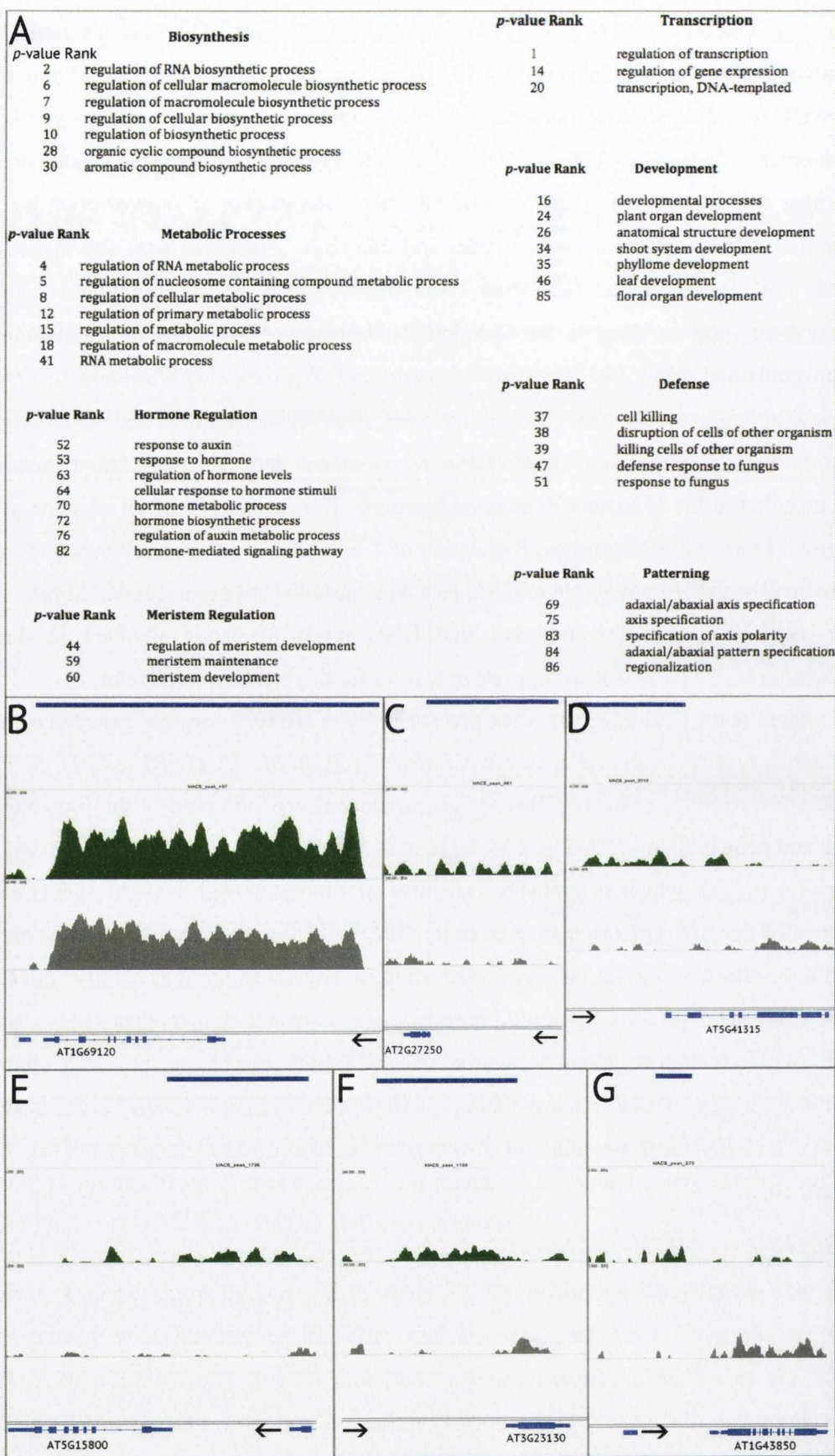


Figure 4.19. ChIP-seq results for selected KNU targets. (A) Selected gene ontology (GO) terms enriched among genes bound by KNU. An adjusted (Bonferroni correction for multiple testing), p -value < 0.05 was employed to identify over-represented GO terms. The column “ p -value Rank” separates the most significant p -value (Rank 1) from the least significant (Rank 88). The GO terms were categorized into four groups that broadly represent the biological processes involved. (B-G) Genomic regions bound by KNU surrounding the *API* locus (B); the *CLV3* locus (C); the *GL3* locus (D); the *SEP1* locus (E); the *SUP* locus (F), and the *SEU* locus (G). Peaks identified in the KNU-1 library are marked in green. Gene models are shown at the bottom of each graph (in blue). The rectangular blue boxes at the top track represent peaks called by the *MACS* software. The grey tracks represent the results for the control library (KNU-INPUT-1). The peaks were visualized using the *IGV* genome viewer.

The basic Helix-Loop-Helix family of transcription factors is one of the gene groups that are significantly enriched in the dataset. Among them, *GL3* plays a key role in trichome formation (Bernhardt et al., 2005). In addition, a large group of genes that encode MYB transcription factors, which are responsible for the regulation of secondary metabolism, cellular morphogenesis as well as meristem formation and cell cycle control (Jin and Martin, 1999), was present in the ChIP-seq dataset. Among this group were genes such as *MYB58* and *MYB85*, which activate lignin biosynthesis in fibers and vessels (reviewed in: (Dubos et al., 2010)). Moreover, genes that are responsible for meristem maintenance or development such as *CLV3* (Fletcher et al., 1999) and *STM* (*SHOOT MERISTEMLESS*) (Long et al., 1996) were identified as direct targets of KNU. Furthermore, genes that are involved in hormone pathways or signaling were identified. Among those genes are *PIN-FORMED5* (*PIN5*) (Mravec et al., 2009) and *CYTOKININ OXIDASE3* (*CKX3*) (Bartrina et al., 2011), which are known regulators of reproductive meristem size.

Because no DNA binding studies have been conducted with KNU prior to this study, the DNA specificities of the transcription factor were unknown. To define putative KNU binding sites in the *Arabidopsis* genome, the DNA motif discovery software *HOMER* (for: Hypergeometric Optimization of Motif EnRichment) (Heinz et al., 2010) was used to identify DNA sequence motifs that are enriched in the regions bound by KNU-GFP. The most representative motifs were selected for each of the five *Arabidopsis* chromosomes. The significance of a given motif was specified based on the number of hits. As some of the motifs obtained in this analysis had already been

identified for selected C2H2 transcription factors (Franco-Zorrilla et al., 2014), they were compared to those previously described (Fig. 4.20 B). The DNA motifs are represented in the form of position weight matrices (PMW), which specify, for each position in the DNA binding site, the contribution of each possible nucleotide. One of the most prevalent motifs found was 5'-[C/G]ATGTGTATG-3' (Fig. 4.20 A1). This motif confers a E-box motif (consensus: 5'-CANNTG-3') that is often bound by basic Helix-Loop-Helix transcription factors (Heim et al., 2003). Another motif that was identified as enriched in the genomic fragments bound by KNU was the “A”-rich motif (Fig. 4.20 A4). This motif contained the sequence similar to the 5'-CA[C/G]T-3' motif bound by the ZAT2 C2H2-zinc finger transcription factor (Fig. 4.20 B, top) (Franco-Zorrilla et al., 2014).

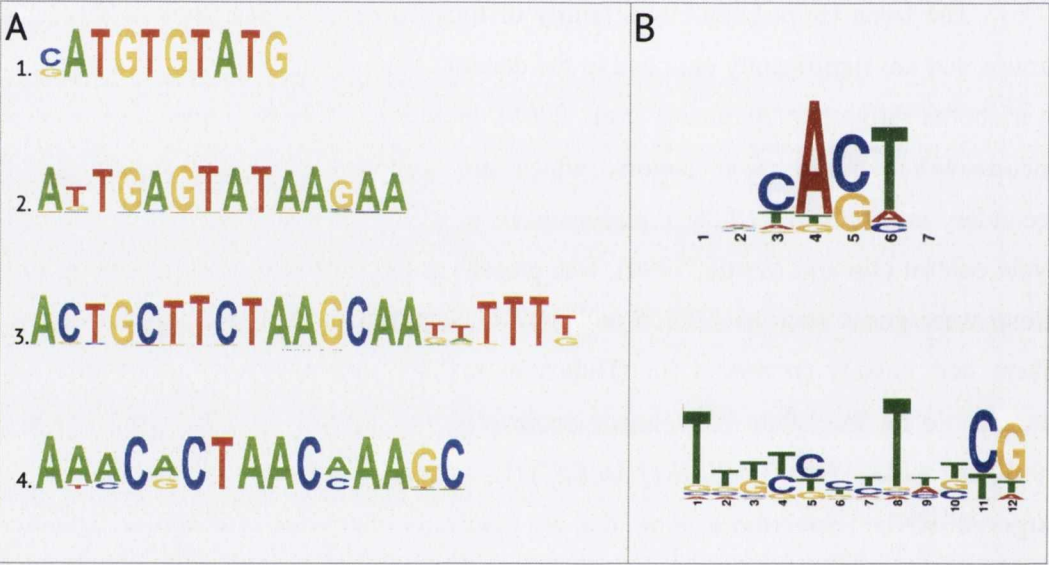


Figure 4.20 Sequence motifs enriched in regions bound by KNU. (A) The position weight matrixes of the binding motifs most frequently found within peak regions are represented in the form of sequence logos. The DNA motif discovery software *HOMER* was used to identify the most abundant DNA motifs in the regions bound by KNU-GFP. (B) Binding motifs identified for some members of C2H2-zinc finger transcription factor family (Franco-Zorrilla et al., 2014).

In summary, putative binding sites of KNU were identified in the KNU-1 library but not in the KNU-2 library. Several processes directly under the control of KNU have been highlighted by the ChIP-seq data including its role in regulating meristem maintenance (*CRC*, *SUP*), trichome formation (*GL3*, *TRY*, *ZFP5*), cytokinin signaling

pathway (*CKX3*, *CKX7*) and flower development (*AP1*, *AP2*, *SEP1*). This data can be further mined and combined with other datasets in order to identify processes and genes regulated by KNU.

4.3.8 Genes acting downstream of KNU

In order to confirm the molecular data from genome-wide studies on the interaction between *KNU* and genes that are bound or act downstream of KNU, genetic analyses were conducted for some genes that were identified to be putative direct targets.

4.3.8.1 Mutations in *CRABS CLAW* enhance the over-proliferation of ectopic tissue in *knu-1*

One of the genes that was enriched in the ChIP-seq data was *CRC*, which is implicated in stem cell maintenance (see Section 3.1.5). *knu-1* and *crc-1* mutants and a line homozygous for both alleles (for genotyping assays, see Section 2.4.1) was isolated and their floral phenotypes were characterized. As described in Section 1.3.1, *knu-1* mutant flowers do not show any specific changes in their architecture (Fig. 4.21 A), with the exception of defects in pollen production, when plants are grown at 16°C (Payne et al., 2004). However, at higher temperatures, it produces ectopic carpels and stamens in siliques. The *crc-1* allele mutant exhibits slight defects in gynoecium development, where the top part of the style is not fused (Fig. 4.21 B, indicated by an arrow). The double mutant *knu-1 crc-1* produced flowers with an unfused gynoecium and ectopic organs within the carpel. Moreover, some defects in stamen elongation were observed. The fruit produced over-proliferated carpel tissue (Fig. 4.21 D) and continued to elongate, forming rounds of carpels and stamens that appeared in a spiral pattern (Fig. 4.21 E). With the further growth of the plants, which had longer life span than the wild type or single mutant plants, the siliques continued to produce internodes with an extra mass of carpel-like tissue (Fig. 4.21 F). To validate these results, the *crc-1* allele was introgressed into the 35S_{pro}:KNU-amiRNA1 background (described in Section 3.2.2), which exhibits a weaker *knu*-like phenotype than the *knu-1* allele. In agreement, with the 35S_{pro}:KNU-amiRNA1 line having reduced KNU activity, the phenotypes of 35S_{pro}:KNU-amiRNA1 *crc-1* plants were overall weaker than those of the *knu-1*

crc-1 double mutants (Fig. 4.21 G). In contrast to the *knu-1 crc-1* double mutant, flowers of this line formed elongated stamens that produced pollen (Fig. 4.21 H).

Taken together, the results of this analysis confirms that both, *KNU* and *CRC*, play roles in the maintenance of floral meristems and may act at least in part synergistically.

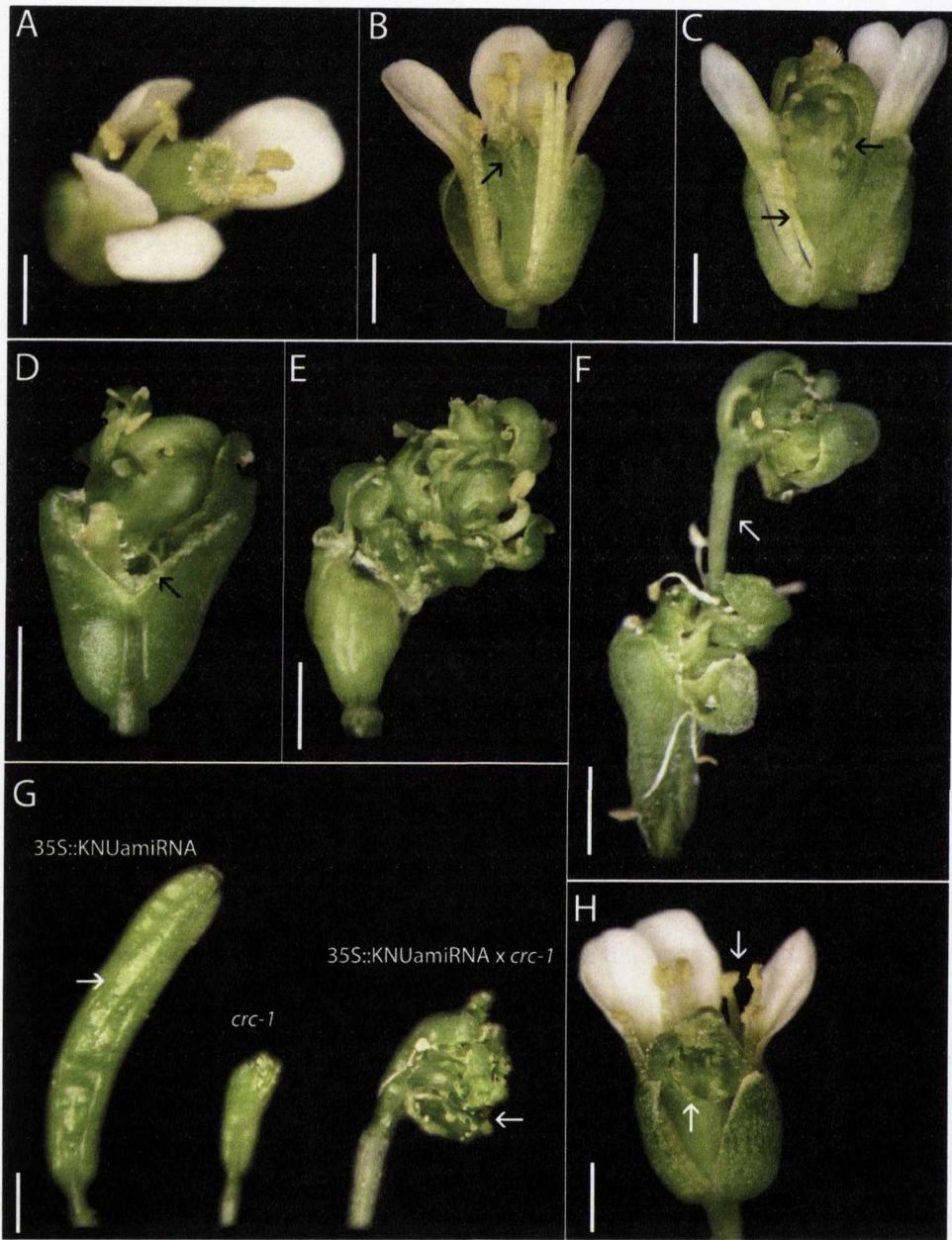


Figure 4.21 *CRC* and *KNU* function together in floral meristem maintenance. (A) *Knu-1* mutant flower with no apparent phenotypic abnormalities. (B) *Crc-1* mutant flower with unfused carpel at the top of the gynoecium. (C) *knu-1 crc-1* double-mutant flower with shorter

stamens, which do not produce pollen, and ectopic tissue inside the gynoecium. **(D)** Early-arising silique of the double mutant with an unfused gynoecium and ectopic carpel-like tissue. **(E)** Later arising silique of the *knu-1 crc-1* double mutant containing a mass of carpel- and stamen-like tissue. **(F)** Silique from a late arising flower producing a ‘silique within silique’. **(G)** Siliques of 35S_{pro}:KNU-amiRNA1 *crc-1* plants were similar to siliques of *knu-1 crc-1* plants. **(H)** Flower of a 35S_{pro}:KNU-amiRNA1 *crc-1* plant with unfused gynoecium but producing pollen. Size bars: 1 mm.

4.3.8.2 *knu-1* partially rescues the supernumerary stamen phenotype of *sup-5*

It has been reported that SUP regulate *KNU* expression by repressing its activity at stage 5 of flower development (Stewart, 2016). Moreover, a binding peak was identified in the *SUP* locus in the KNU ChIP-seq experiment discussed above, suggesting that *SUP* may be a direct target gene of KNU. To determine how *SUP* and *KNU* interact genetically, a *knu-1 sup-5* double mutant was generated and characterized. As described in the previous section, *knu-1* does not exhibit any specific changes in floral organ numbers (Fig. 4.22 A). In contrast, *sup-5* forms supernumerary stamens and exhibits defects in carpel formation and fusion (Fig. 4.22 B) (Gaiser et al., 1995). The double mutant *knu-1 sup-5* (Fig. 4.22 C-F) showed an intermediate phenotype in that flowers made fewer stamens than *sup-5*. The developmental problem in stamen elongation was detected in addition to failure in pollen production (Fig. 4.22 C). Ectopic organs are produced inside the double mutants’ gynoecium (Fig. 4.22 D), what was not observed in the single *sup-5* mutant. Moreover, a bent petal phenotype was noticed (Fig. 4.22 E) as well as tricarpelloid carpels that are fused and contained ectopic organs (Fig. 4.22 F). Additional examination of the double mutant plants is needed to provide more detailed insights into the nature of this interaction.

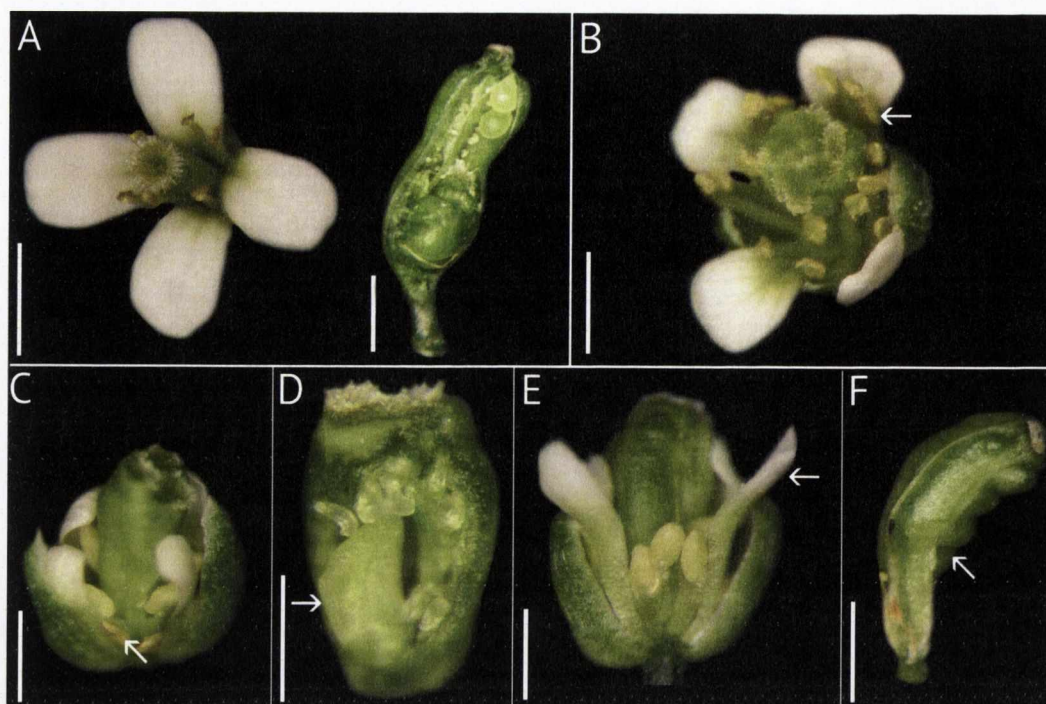


Figure 4.22 Genetics interaction between *KNU* and *SUP*. (A) Flower and silique of a *knu-1* mutant. (B) Flower phenotype of *sup-5* with supernumerary stamens and an abnormal gynoecium. (C-F) Phenotypes observed in *knu-1 sup-5* double mutants. (C) Flowers were smaller and contained fewer stamens than *sup-5*. (D) Gynoecium with anthers fused to the gynoecium. (E) Flower with bent petals (arrow). (F) Bulged silique of the double mutant. Size bars: 1 mm.

4.3.8.3 Gynoecia of *knu-1* flowers are sensitive to exogenous cytokinin treatment

As described in Section 4.3.7.3, two of the genes that regulate cytokinin-dependent pathways in *Arabidopsis*, *CKX3* and *CKX7*, were identified as a putative direct targets of *KNU*. In order to test if *KNU* is involved in the regulation of cytokinin response, as indicated by the data described above, *knu-1* mutant plants were treated with 100 μ M 6-benzylaminopurine (BAP), a synthetic cytokinin. Fourteen days after the initial treatment, *knu-1* plants began to show changes in the silique architecture, whereas there was no significant change in the flower development observed. The most characteristic changes were observed two weeks after the treatment and included overproliferation of carpel-like tissue (Fig. 4.23 E). Although, the *knu-1* mutant contains ectopic carpels in the silique, the amount of the carpel-like tissue in plants treated with the BAP was significantly higher. Moreover, the increase in production of stamen-like tissue and

ovules was observed in the siliques of *knu-1* treated with BAP (Fig. 4.23 F). It has been recently reported that cytokinin plays a role in medial tissue proliferation at early stages of the developing gynoecium (Fig. 4.23 B-C) (Marsch-Martinez et al., 2012). This may explain the phenotypic response of the *knu-1* mutant to exogenous cytokinin treatment.

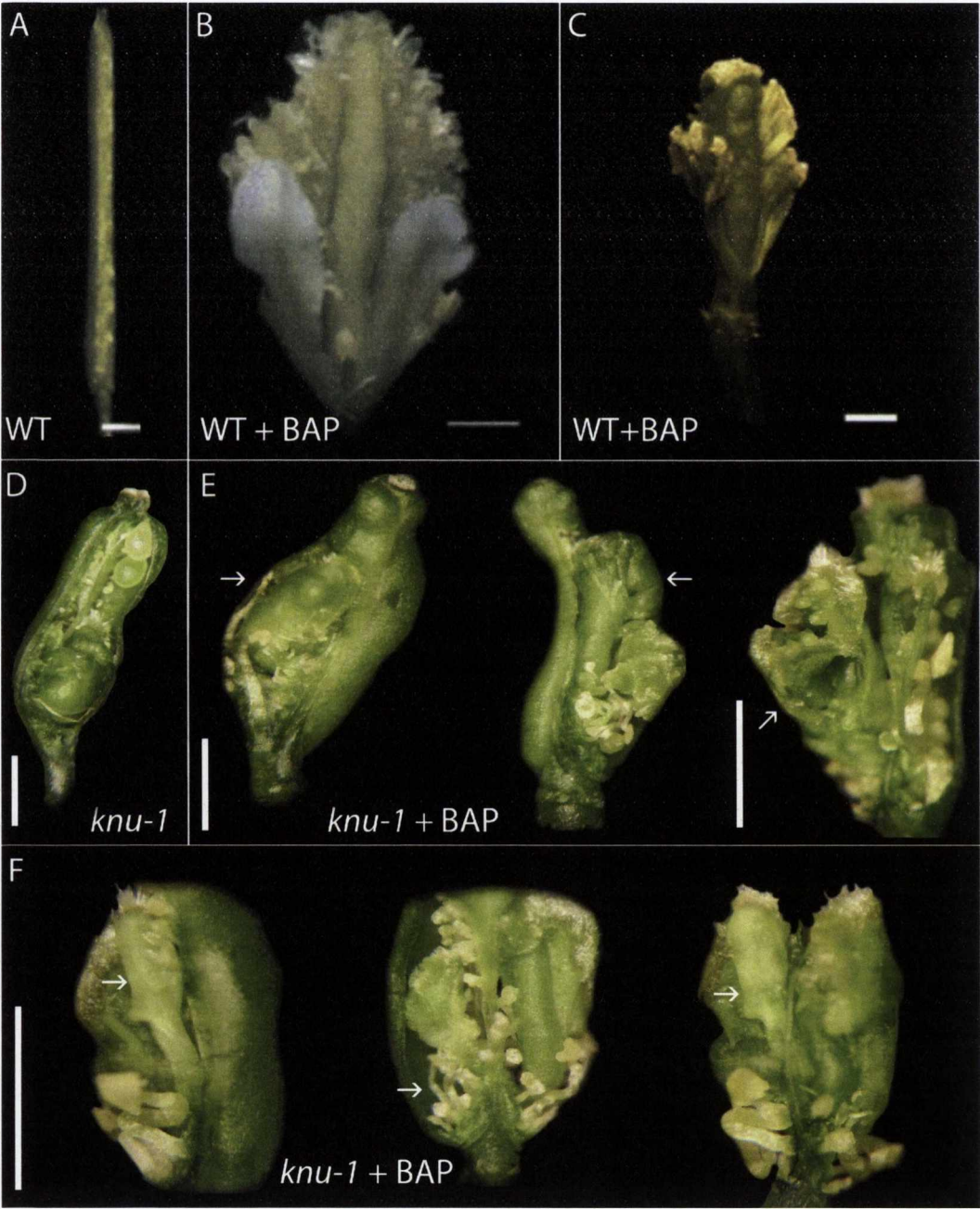


Figure 4.23 Exogenous cytokinin treatment causes overproliferation of carpel and stamen tissue in *knu-1* mutants. (A, B) Fruit of wild-type plants treated with mock solution (A) or 100 μ M BAP (B). (C) Gynoecium of wild-type plants treated with 100 μ M BAP. (D) An untreated

knu-1 mutant flower with ectopic carpels and stamens. (E) Siliques of *knu-1* mutant treated with 100 μ M BAP. Arrows indicate overproliferation of carpel tissue. (F) Siliques of a *knu-1* plant two weeks after treatment with 100 μ M BAP. Arrows indicate the overproduction of stamen-like tissue at the base of the silique. Size bars: 2 mm for A and B, 1 mm C-F. A-C modified from: (Marsch-Martinez et al., 2012).

4.3.9 Identification of genes acting downstream of KNU through inducible gene perturbation

In order to characterize processes that KNU might be involved in, a microarray experiment was carried out on the 35S_{pro}:KNU-amiRNA1 (see Section 3.2.2) that was crossed to AP1_{pro}:AP1-GR *ap1-1 cal-1* (referred as KNU-amiRNA, FIS) (for details see Section 2.15.2) in order to allow for synchronised flower development. Microarray expression analysis was then performed to contrast global expression patterns between KNU-amiRNA, FIS that was treated with dexamethasone and the plants with the same background that were not submitted to dexamethasone treatment. Four replicates of tissue for each of 4 time-points at 0, 5, 6 and 7 days after dexamethasone induction were collected. After the RNA extraction and cDNA preparation, *KNU* levels were examined across all the replicates. The *KNU* level at 0 d was the highest, but did not reached the level of *KNU* in wild-type plants (Fig. 4.24 A). Five days after the dexamethasone treatment transcript levels for *KNU* dropped to 30%, and further declined at the 6 and 7 d time-points to ~20% of wild-type levels, indicating an efficient knockdown of *KNU* across all the biological replicates (Fig. 4.24 A).

The RNA extracted from the tissue samples was subjected to labeling with fluorescence dyes, where material from treated and untreated samples was labeled with different dyes for two-color microarray hybridizations (for details see Section 2.13.1). Subsequently, samples were co-hybridized onto microarray slides (for description see Section 2.13.2-3). After signal quantification, quality plots were generated by the feature extraction software in order to test for any problems in the hybridization method. The MA-plots illustrate the mean processed signal intensity (*x*-axis) against the log₂-transformed ratios of the mean fold change values (*y*-axis). The generated plots for time-point 5 and 6 d after the dexamethasone treatment (Fig. 4.24 B-C) indicated reasonable (judged based on the results of similar experiments previously conducted in

the laboratory for other floral regulators) numbers of genes that were up-regulated (red dots) or down-regulated (green dots).

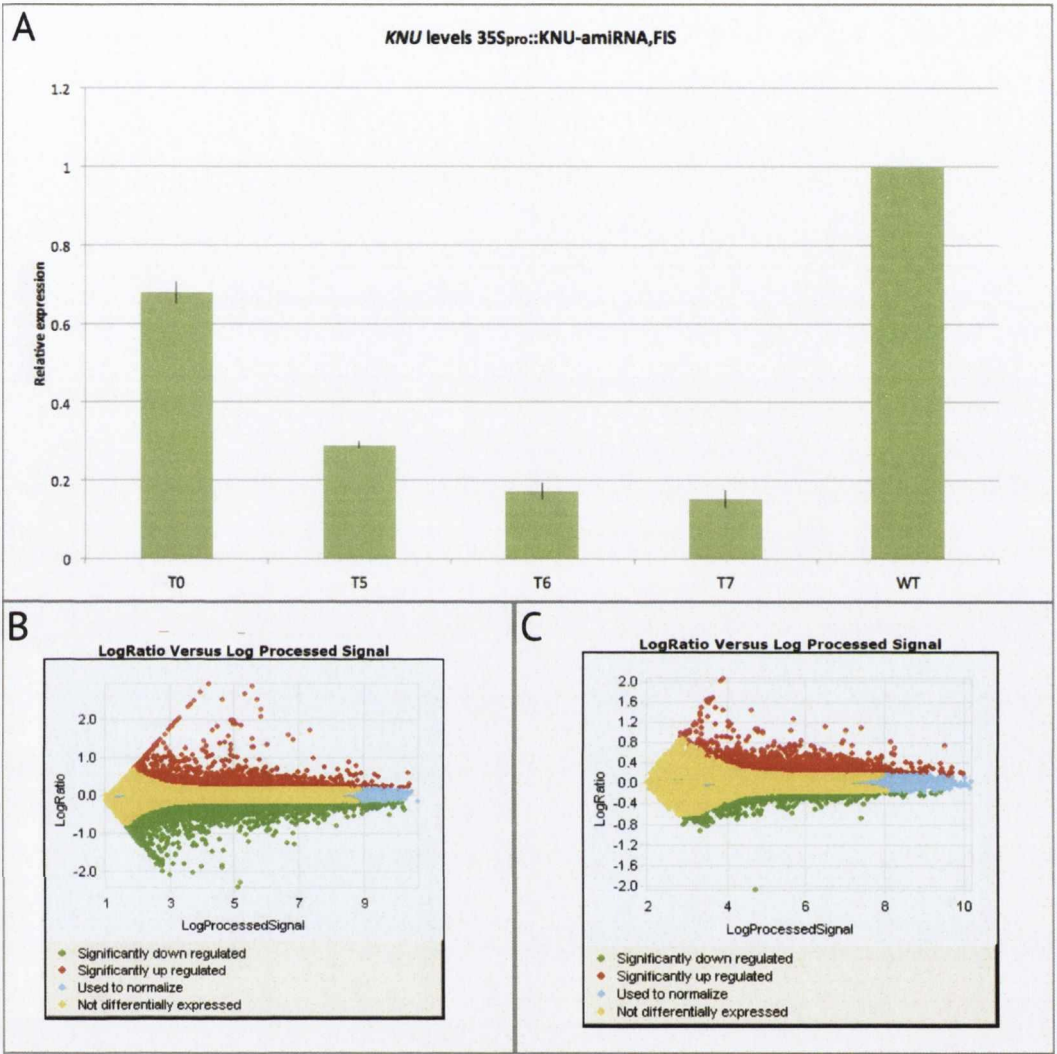


Figure 4.24 Quality assessment of samples used for microarray experiments. **(A)** *KNU* mRNA levels across the different time-points (T0, T5, T6, T7) relative to the wild type (WT). The error bars represent s.e.m. of four biological replicates. **(B, C)** MA-plots representing differentially expressed genes at time-points T5 (B) and T6 (C). Yellow dots indicate an absence of differential expression; blue dots indicate reference genes that were used to normalize the microarray data; red dots indicate genes that are significantly up-regulated; green dots indicate genes that are significantly down-regulated in the plants treated with dexamethasone relative to untreated control plants. Primer pairs KK-27/KK-28 and KK-29/KK-30 were used to amplify *KNU* and transcripts of a reference gene, respectively.

The analysis of the microarray data was done with the help of Dr Patrick Ryan, using the *limma* package, with microarray quality being assessed after normalization to remove variance related to the technology (see Section 2.14.1). Examination of correlation within and between replicates revealed that a number of microarrays exhibited low correlation compared to their replicates.

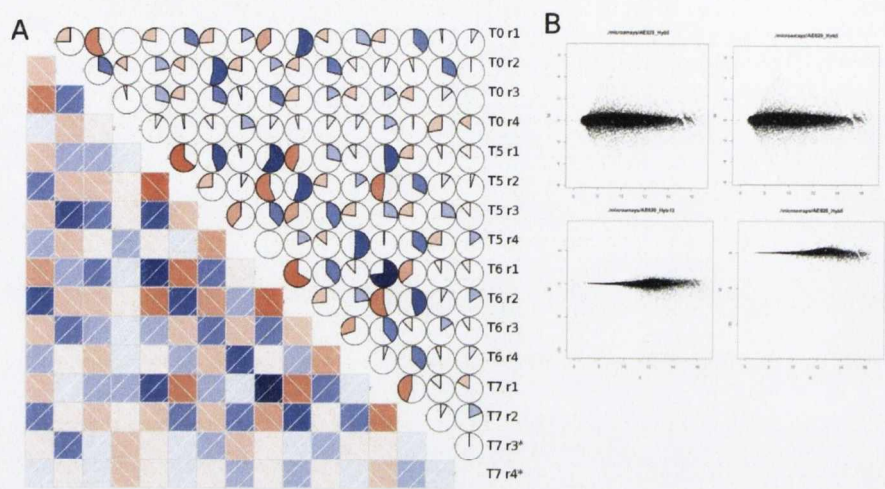


Figure 4.25 Quality assessment of microarray data contrasting expression in treated and untreated KNU-amiRNA, FIS plants. (A) Correlating the M values of replicates after normalization to minimize variance between microarrays identified two replicates with low correlation within their own group (indicated by an asterisk). **(B)** MA plots for these replicates were then contrasted against other hybridizations exhibiting normal correlations. The top two panels are representative of the expected shape of an MA plot following a successful hybridization. The bottom two panels are the MA plots for the abnormal replicates. The flattened pattern indicates that there is little difference between dye-channels, suggesting a technical issue with that experiment.

This prompted an examination of the MA plots for the affected replicates. This identified abnormally flattened variance patterns indicative of technical issues with the slides, prompting the removal of two of the 7 d replicates from further analysis (Fig. 4.25 A-B).

After the fitting of linear models to the data, genes which exhibited a $\log_2$ fold change in expression of ± 0.5 and a p -value of 0.01 (adjusted for multiple testing using

the Benjamini Hochberg method to control false discovery rates) were considered to be differentially expressed. This led to the identification of 397 genes which were differentially expressed after amiRNA knockdown of *KNU* at either T5, T6 or T7. The time-point containing the highest number of genes differentially expressed was observed to be T7, which corresponds to peak *KNU* expression. At the 5 d time-point, over 100 genes were up-regulated while 67 were down-regulated (Fig. 4.26 A). The number of genes up- or down-regulated at the 6 d time-point was almost the same, with 100 and 97 genes, respectively. At the 7 d time-point, most of the genes were down-regulated (170), while 75 were up-regulated. Next, the overlap between the three time-points was determined using a Venn diagram (www.bioinformatics.psb.ugent.be). This calculation resulted in comparison of genes that were differentially expressed at the different time-points. A large number of genes (74) were present across all time-points (Fig. 4.26 B). The number of genes that were in common for 5 and 6 d time-point was 45. In contrast, only 10 genes were judged as differentially expressed in both the 6 and 7 d time-points. This indicates that genes responding to *KNU* knockdown at 7 d after dexamethasone act differently than those at earlier stages of development. Further analysis of the genes that were differentially expressed indicated reduced levels of *KNU* transcript in plants treated with dexamethasone compared to untreated plants (Fig. 4.26 C). This was expected, as the experiment uses the loss-of-function approach, which indicates sufficient knockdown of *KNU* levels across the different time-points.

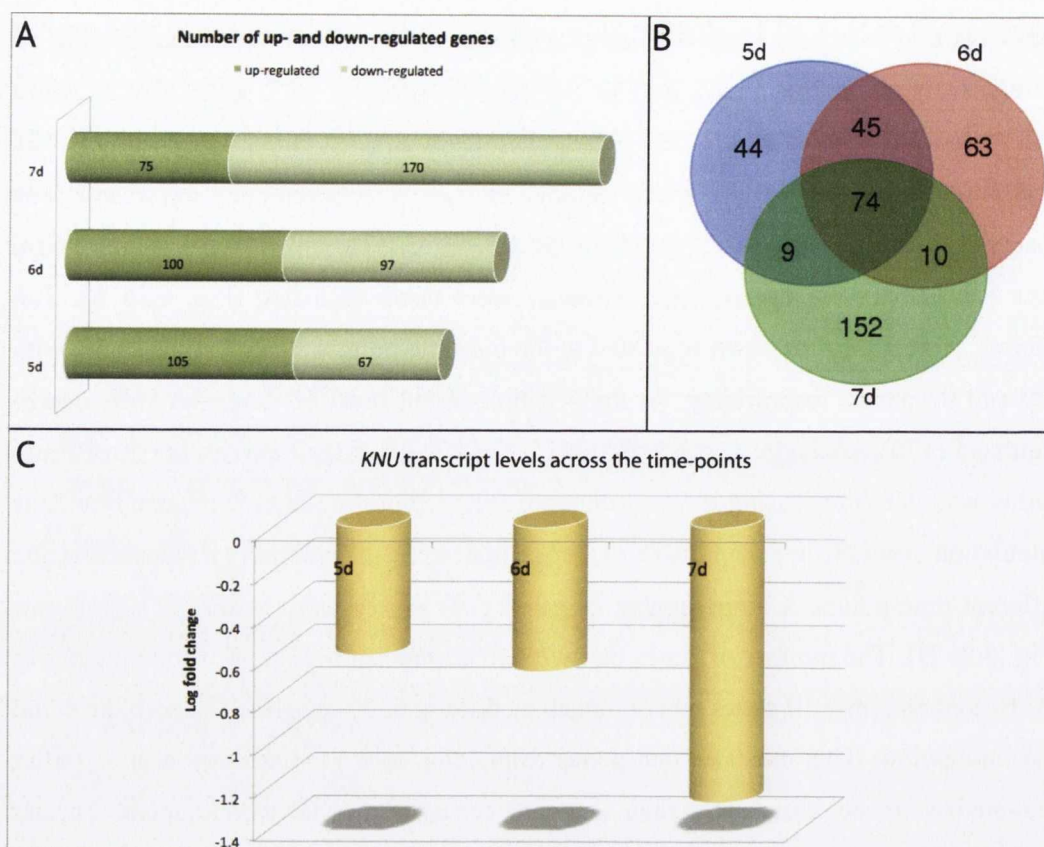


Figure 4.26 Genes differentially expressed at different time-points in response to *KNU* perturbation. (A) Number of genes identified as differentially expressed in the 5, 6 and 7 d time-points by microarray analysis. Genes, which exhibited a $\log_2$ -transformed fold-change in expression of 0.5 or greater and had a p -value of less than or equal to 0.01 after adjustment to control false discovery rates, were considered differentially expressed at a given time-point. (B) A Venn diagram depicting the overlap of genes differentially expressed in the *KNU*-amiRNA microarray experiments at the 5, 6 and 7 d time-points. (C) Levels of *KNU* transcript across the 5, 6 and 7 d time-points. Data shown are $\log_2$ -transformed fold-change expression ratios.

Next, a Gene Ontology (GO analysis) was performed after the fitting of linear models to identify GO terms, which were over-represented among potentially differentially expressed genes using a p -value cutoff of 0.005, adjusted to control (Fig. 4.27). This identified terms such as pollen wall assembly and tapetal layer development as being over-represented, which is in agreement with the ectopic formation and conditional sterility of male organs observed in *knu-1* mutant plants. Among genes that were significantly down-regulated at 7 d, *MS5* (Glover et al., 1998), which is essential for male fertility, was detected. It has been reported that mutants of this gene exhibit

problems in microsporocyte development (Chaudhury, 1993). Moreover, *KOMPEITO* (*KOM*), a gene that has a role in pollen wall assembly was identified as down-regulated in the dataset (Kanaoka et al., 2005). *GLUCAN SYNTHASE-LIKE2* (*GLS2*), was also found in the group of genes over-represented in the dataset. This gene is responsible for exine formation during microgametogenesis and for pollen viability (Dong et al., 2005). Taken together, the observations that differential expression in response to *KNU* knockdown corresponds to normal *KNU* activity, and the relationship of the genes involved to processes related to *knu-1* phenotypes established by gene ontology, indicate that the genes observed as differentially expressed are likely to be regulated by *KNU*.

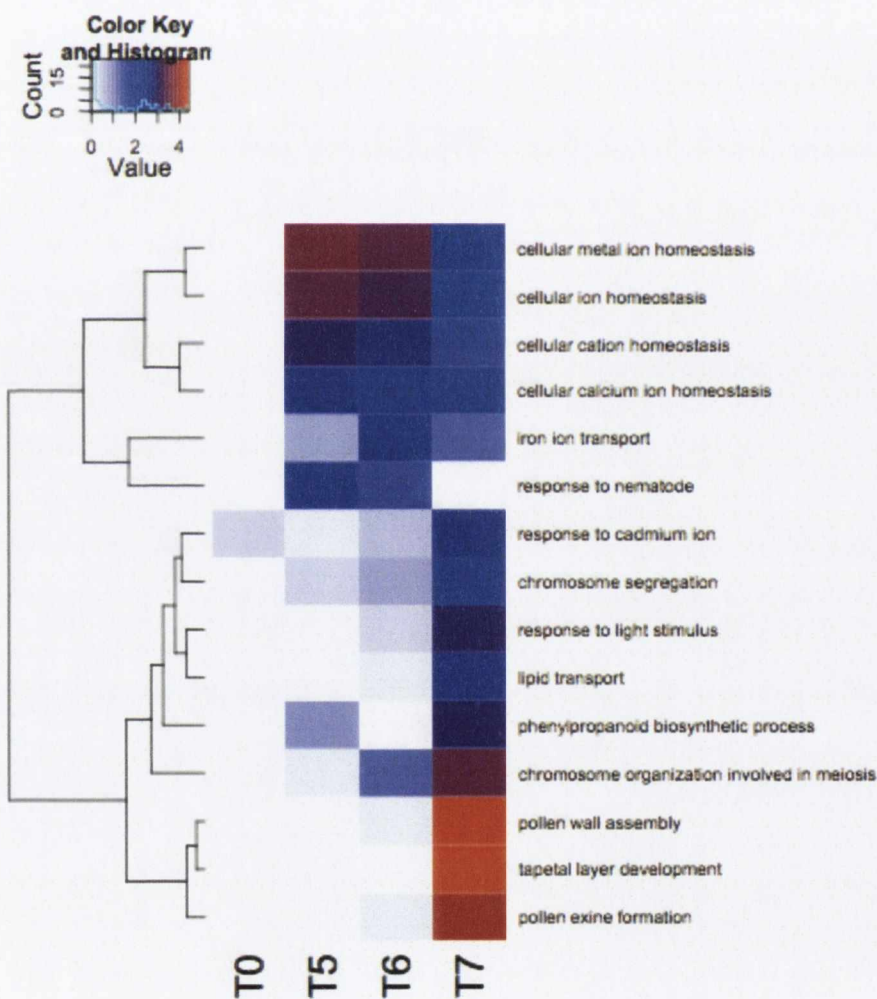


Figure 4.27 GO terms found to be enriched among differentially expressed genes identified in the *KNU*-amiRNA microarray experiment. The heatmap represents the

significance levels for an enrichment of selected GO terms represented among the differentially expressed genes identified in the microarray experiments.

The results from the ChIP-seq experiment (see above) prompted an additional microarray experiment, which was carried out using 8 d samples. The flower tissue was collected from the KNU-amiRNA, FIS plants 8 d after the dexamethasone treatment and also from untreated plants. In addition, two other lines were also included. One was the ethanol-inducible line that was described in Section 4.3.1.2. These plants were treated with dexamethasone and 7 d after the treatment, 24 h ethanol vapour was applied in order to induce a *KNU* knockdown. Tissue was collected from inflorescences of plants treated with ethanol and water-vapor, respectively. In addition, the *knu-1* allele was introgressed into the FIS and the analysis was carried out on *knu-1*, FIS homozygous plants (that were selected from F2 population by phenotyping and genotyping) that were compared with plants heterozygous for *knu-1*, together with wild-type FIS plants.

After RNA extraction and cDNA preparation, the levels of *KNU* were tested. The 35S_{pro}:KNU-amiRNA, FIS line expressed *KNU* at the lowest level (~18%). The level of *KNU* transcript at this stage was similar to the expression of *KNU* at 7 d after dexamethasone induction (see Fig. 4.24 A). Plants that contain AlcR_{pro}:KNU-amiRNA, FIS transcript (for details see Section 2.15.9) expressed *KNU* at higher level, reaching ~40% of the wild-type transcript. The *knu-1*, FIS plants expressed *KNU* at ~33% of wild-type levels (Fig. 4.28). All of the samples were subjected to labeling and hybridization (for details see Sections 2.13.1-3). The data for these microarray experiments have yet to be analysed and later can be compared against each other and the ChIP-seq data.

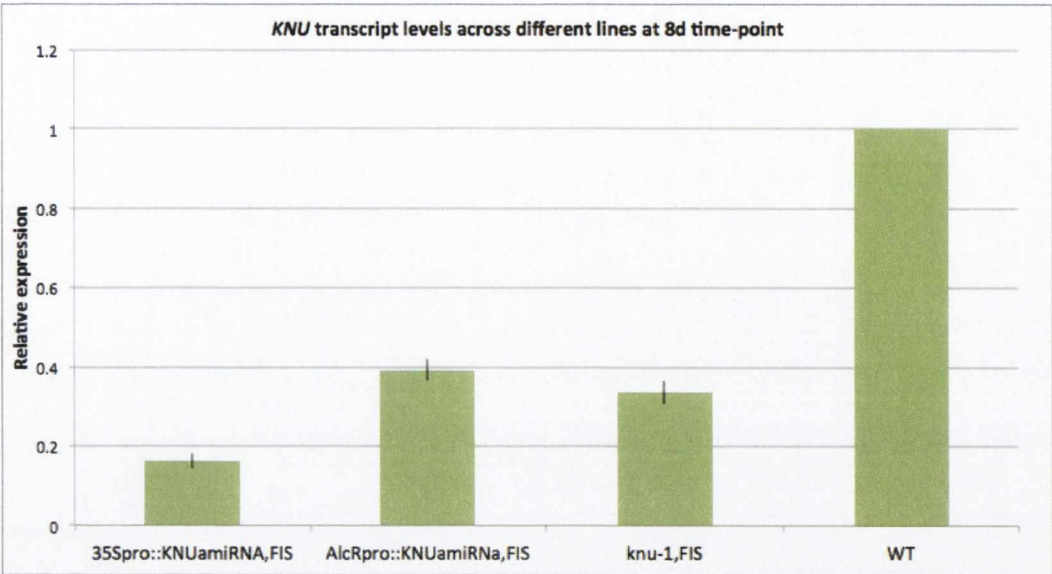


Figure 4.28 *KNU* transcript levels in different knock-down lines. The levels of *KNU* mRNA were determined by qRT-PCR in different lines (as indicated) and compared to those in the wild type (WT). Error bars represent s.e.m of four biological replicates. Primer pairs KK-27/KK-28 and KK-29/KK-30 were used to amplify *KNU* and *REF2* (reference gene), respectively.

In conclusion, the results from the microarray experiment confirms that *KNU* regulates the expression of various genes involved in the development of anthers and what comes with that, pollen formation. In order to identify processes that were clasified as overrepresented in the ChIP-seq data, more experiments are needed with the use of different knock-down lines, that already have been started.

4.3.10 Comparison of ChIP-seq and microarray data

As mentioned in Section 1.5, the aim of this study was the identification of genes regulated directly or indirectly by *KNU*. The combination of the datasets from ChIP-seq analysis and the data coming from microarray analysis gives the opportunity to recognize the genes that act downstream of *KNU*. This analysis also allows for identification of direct targets of *KNU* and recognizes the interaction between the candidate genes and *KNU*. Because ChIP-seq and microarray data were available only at different stages of flower development, just a small subset of genes have been identified to be over-represented in both the microarray data and the ChIP-seq dataset

(Fig. 4.29 A). For that, the number of genomic regions bound by KNU from the ChIP-seq data was reduced by eliminating pseudogenes and hypothetical ORFs (for details see Section 4.3.7.2). The largest overlap between the genome binding (8 d time-point) and transcriptomics datasets was at 7 d time-point. Only 17 genes were identified to be common in both time-points. The group of genes that were identified between them encode for Nodulin MtN3 family proteins. Among them, *SWEET7*, which encodes a sucrose membrane transporter and exhibits the highest expression in the anthers and during petal differentiation, was identified (Bock et al., 2006). The group of these transporters play a pivotal role in proper development of the male gametophyte and influence male fertility in *Arabidopsis* (Bock et al., 2006).

Although some of the genes were shared between both datasets, the number of genes directly under the control of KNU is still small. To address this, additional microarray datasets were generated (see above), which still await a detailed analysis. To identify interactions between KNU and the genes that were recognized as putative targets, the expression level of some genes was examined in the plants used for microarray analysis (35S_{pro}:KNUamiRNA, FIS). After the flower tissue collection, RNA extraction and cDNA preparation, the qRT-PCR was executed at 6 d time-point (see above).

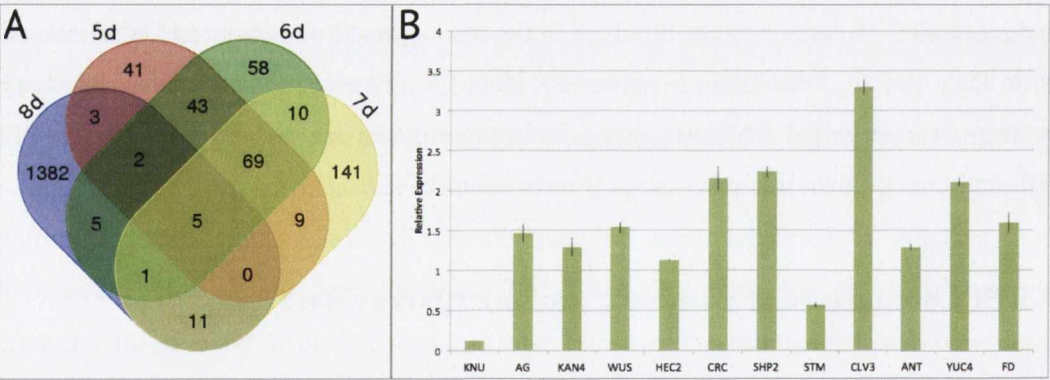


Figure 4.29 Comparison of genome-wide binding data and the results of transcriptomics analysis for KNU. (A) A Venn diagram depicting the overlap in genes associated with a significant binding peak in KNU-1 (8 d) and the overlap with genes differentially expressed (unadjusted $p < 0.01$) in the KNU-amiRNA microarray experiment at days 5, 6 and 7, respectively. **(B)** Genes identified as putative KNU targets by ChIP-seq analysis and as differentially expressed in KNU-amiRNA, FIS plants at 6 d time-point. Expression was monitored by qRT-PCR in the KNU-amiRNA, FIS line used for microarray analysis and

compared to samples with normal *KNU* activity. The expression values were calculated relative to a reference gene (*REF2*). Error bars indicate s.e.m of four biological replicates. Sets for primers used: KK-27/KK-28, DM-355/DM-365, DM-611/DM-612, DM-435/DM-436, DM-551/DM-552, DM-317/DM-318, DM-433/DM-434, DM-218/DM-219, DM-486/DM-487, DM-613/DM-614, DM-232/DM-233, SW-667/SW-668 were used to amplify *KNU*, *AG*, *KAN4* (*KANADI4*), *WUS*, *HEC2* (*HECATE2*), *CRC*, *SHP2*, *STM*, *CLV3*, *ANT* (*AINTEGUMENTA*), *YUC4* (*YUCCA4*), and *FD*, respectively.

This analysis showed that *KNU* affects the expression of genes that were identified as being bound by the transcription factor (Fig. 4.29 B). The levels of *KNU* among four biological replicates were lower than 20%, confirming the knockdown of this gene. Transcript levels of *AG*, which is bound by *KNU*, was increased after *KNU* perturbation, suggesting that *KNU* represses the expression of the C function gene. The level of *WUS* mRNA was also increased ~1.5-fold in this experiment, confirming that *KNU* normally acts to suppress *WUS* expression. Next, the level of *CRC* transcript was analyzed. This gene was also up-regulated after *KNU* perturbation, reaching two-fold increase of the level of expression. The largest increase, a 3.5-fold enrichment was identified for *CLV3* (Fig. 4.29 B). The opposite trend was observed for *SHOOT MERISTEMLESS* (*STM*), where its expression level dropped to 50% in the absence of *KNU* expression, indicating that *KNU* may activate *STM* expression.

Taken together, the comparison of the ChIP-seq and microarray datasets brought some insights into the roles that *KNU* may play during flower development. The confirmation of changes in expression of genes identified as direct targets broadens the list of functions of *KNU* in flower development.

4.4 Discussion

Previous studies on *KNU* have elucidated some of its functions in the control of floral meristem determinacy (Sun et al., 2014; Sun et al., 2009), but did not bring additional information on other functions that this gene may have during the flower development. Taking into consideration the fact that *KNU* is expressed during many stages of development (see Section 1.3.1) reinforces the idea of *KNU* having more roles during floral morphogenesis.

In this thesis, the characterization of various lines that mediate a knockdown of *KNU* function is described. In addition, the effects of over-expression of *KNU* using different promoters is described. By analyzing transcriptomics data derived from microarray analyses and comparing it with the genome-wide binding studies of *KNU*, I directly addressed the key aim of the present thesis. The results of these experiments will be discussed in detail below.

4.4.1 Identification of an amiRNA that efficiently targets *KNU*

The *KNU*-amiRNA was placed under the control of 35S_{pro}:GR-LhG4/6xOP_{pro} dexamethasone inducible system (see Section 4.3.1.1), which has been described as a highly efficient gene expression system (Moore et al., 1998). The pulsed perturbation analysis was performed to allow for the observation of changes in the flower phenotype. The most severe phenotype was observed 11 d after the first dexamethasone treatment. The carpel of the flower was enlarged compared to the wild-type flower (Fig. 4.2). This might suggest that *KNU* could be somehow involved in the development of this organ type, maybe by controlling the proliferation of carpel tissue.

At 16 d after the first dexamethasone treatment, gynoecia became unfused and contained ectopic stamens and carpel tissue. This phenotype was observed just once. To make it more consistent a lower concentration of dexamethasone might be used with the combination of higher frequency of the treatment.

The line that had the strongest phenotype and in which the levels of *KNU* mRNA were the lowest (~20% of wild-type levels) was crossed into the FIS to allow gene profiling experiments after *KNU* perturbation. However, since the FIS used in our laboratory is activated by dexamethasone, it would inevitably cause the induction of both – flower formation and *KNU*-amiRNA expression. This induction of the *KNU* knockdown prior to the normal onset of *KNU* expression would not be ideal because it would not allow a stage-specific analysis of *KNU* function. To circumvent this problem, versions of the FIS which employ other inducible system could be used. In fact, the plant expressing *KNU* at the lowest levels was crossed with AP1_{pro}:AP1-AR in *ap1-1 cal-1* plants (O'Maoileidigh et al., 2015) in which flower development is induced by dihydrotestosterone treatment. These plants await further characterization. mRNA obtained from the inflorescences of these plants will be used in RNA-seq experiment in

the future. The dataset obtained from this experiment could then be compared with the ChIP-seq and microarray data in order to confirm the results obtained.

The line that expressed *KNU*-amiRNA under control of the *AlcR/AlcA_{pro}* ethanol-inducible promoter (Roslan et al., 2001) did not show strong *knu*-like phenotypes. However, these plants exhibited tricarpelloid siliques and some fertility problems (Section 4.3.1.2), but never showed growth of ectopic carpels or stamens. The level of *KNU* mRNA after 24 h ethanol vapor treatment dropped only to 40% of the wild-type expression level. The line that exhibited the strongest phenotype was crossed with the FIS line in order to allow for synchronized flowering and later for transcriptome analysis. In this case, the longer ethanol treatment was needed in order to lower the level of *KNU* expression to a minimum. However, the plants treated with the ethanol vapor for 48 h exhibited problems in growth. That is why the 24 h ethanol treated plants were used in the microarray experiment (for details see below and Section 4.3.9).

4.4.2 Inducible and constitutive over-expression of *KNU* resulted in novel phenotypes

In order to understand in more detail the role of *KNU* in flower development, *Ws* plants were transformed with a *35S_{pro}:KNU* transgene to allow a constitutive over-expression of the gene. This construct did not cause any defects in *L-er* plants that had been transformed in high numbers by Dr Diarmuid O'Maoileidigh. For that, a large number of plants were transformed, 26 independent transformants were identified and only 6 of them produced an abnormal phenotype. The plants with the strongest phenotype displayed changes in the flower architecture and produced trichomes on the carpels (Fig. 4.6). Although the phenotype was strong and was not observed before in *KNU* over-expressing line, only two lines showed this phenotype (Section 4.3.3.2).

To confirm the phenotype obtained with the T-DNA insertion developed earlier, I generated additional constructs that expressed *KNU* containing the *KNU* 5'UTR region and GFP fused at the 3' end that was expressed under *35S* promoter. Although a large number of *L-er* plants were transformed with this construct and 42 transformants were obtained, only 4 out of them exhibited some changes in the phenotype. These plants expressed *KNU-GFP* mRNA at levels that were 2 to 7-fold higher than the wild-type plants (Fig. 4.7). Although, the level of expression was not as high, these plants

displayed phenotypes that confirmed the characteristics described above. One line, that was considered hemizygous, produced flowers with trichomes on the carpels (Fig. 4.8). This phenotype indicates that KNU may be responsible for repressing some genes that are responsible for trichome formation.

Plants harbouring the 35S_{pro}:GR-LhG4/6xOP_{pro}:KNU transgene display different phenotypes after dexamethasone treatment (Section 4.3.3.1) than the plants described above. Although the *KNU* mRNA levels were higher among those plants (Fig. 4.4) and reached 20-fold enrichment over the wild-type plants, the plants never displayed the phenotype with trichomes on carpels. These plants were treated with the hormone-containing solution few times and the strongest phenotype observed included flowers with defects in pollen production and organ elongation problems. In addition, plants produced shorter siliques that had problems with ovule production.

Moreover, none of lines characterized in this work resembled the 5 α -androstan-17 β -ol-3-one (DHT) responsive over-expression line described by Sun et al. (2009). The latter plants produced a *wus-like* phenotype when repeatedly treated with DHT. In addition, this transgene was able to retard meristem growth in *ag-1* mutant plants (Sun et al., 2009). These phenotypes were not observed here for unknown reasons. Further work will aim at constructing the line described by Sun, (2009 #62) and validating the lines that have already been described, in particular, at identifying lines that exhibit an over-expression of the fusion protein.

4.4.3 An activated form of KNU influences floral architecture

In order to change the function of KNU from a repressor to an activator, the coding sequence for the viral transcription factor, VP16, was fused to the 3' end of *KNU* and the resulting fusion proteins expressed under control of the constitutive 35S promoter (Section 4.3.4). In addition, the EAR-like repressive motif was mutated to disrupt the repressor function of KNU (Fig. 4.9). 35S_{pro}:KNU-VP16 plants were much smaller than wild-type plants, suggesting that cell division rates and/or cell elongation was affected. Plants expressing a KNU-VP16 fusion proteins with a mutated version of the EAR-like motif, however, exhibited much stronger phenotypes. The plants with the strongest phenotypes were dwarf and displayed multiple problems in flower architecture (Fig. 4.12). These included organ identity defects such as petal-to-sepal and carpel-to-sepal

transformations. These changes in organ identity suggest that KNU may affect the activity of at least some of the floral homeotic genes. In addition, none of the flowers formed trichomes on carpel valves as observed for the 35S_{pro}:KNU-GFP plants described above. Together these results suggest that KNU may normally repress the expression of repressors of trichome initiation.

To determine what kind of genes KNU may activate, more experiments are needed. Examination of genetic interaction between the activated version of KNU and the selected mutants of the genes responsible for flower formation (i.e., *ap1*, *ap2*, *pi* or *ag*) could bring some insights to understanding the phenotypes observed in this line.

4.4.4 Functionality of the mKNU-GFP fusion protein

A KNU-amiRNA resistant, epitope-tagged version of KNU was constructed to allow the identification of the genomic regions to which the KNU transcription factor binds. To this end, endogenous *KNU* activity was knocked down using the KNU-amiRNA and the amiRNA-resistant version of *KNU* was used to complement for this loss of function. The addition of an epitope tag ensures that commercially available antibodies that are of ChIP-quality can be employed for the global identification of binding sites.

In order to test whether the mKNU-mGFP5 fusion protein could functionally replace endogenous KNU, 35S_{pro}:KNU-amiRNA-containing plants were transformed with a KNU_{pro}:mKNU-mGFP5 construct. Several of the primary transformants obtained showed a wild-type phenotype and the further characterization of these lines revealed that *KNU* mRNA levels were similar or above wild-type levels in the majority of them (Figure 4.13). These results suggest that the mKNU-mGFP5 fusion protein is indeed functional and could be used for the genome-wide localization experiments outlined above.

It remains unclear why the KNU-GFP fusion protein could not be detected by Western blotting (Fig. 4.14). In addition, the GFP signal detected by fluorescence microscopy was only visible in anthers and not in the gynoecium, despite the fact that the KNU-GFP construct rescued the phenotypes of KNU-amiRNA over-expressing lines. A key problem for the detection of the fusion protein may have been the small expression domain of *KNU* and perhaps also low levels of expression. This would explain why an AG-GFP protein, which is expressed in a much broader domain than

KNU and at high levels, could be detected by Western blotting but not *KNU*-GFP. Another possibility is that the fusion protein is short-lived and rapidly removed from cells after synthesis so that it never accumulates to high and readily detectable levels.

4.4.5 Genomic regions bound by *KNU*

KNU appears to initiate transcriptional networks and signaling pathways directly, as shown by chromatin immunoprecipitation combined with ultra high-throughput (Section 4.3.7) in order to regulate some aspects of flower development and to control floral meristem determinacy. In total, more than 1,500 genes were identified to be bound by *KNU*. Gene Ontology terms enriched in the ChIP-seq dataset pointed to likely functions of this transcription factor. In particular, *Regulation of Transcription*, *Regulation of RNA Biosynthetic Processes* and the *Regulation of Meristem Development*, were among the categories enriched in the dataset. Genes that were expected to be regulated by *KNU* were detected in the ChIP-seq experiments. Notably, these included *CRC* and *CLV3*, which play key roles in floral stem cell maintenance. The interaction between those two genes and *KNU* was confirmed by generating double mutants (Sections 3.2.6. and 4.3.8.1). The analysis also confirmed predictions of the feedback loop between the *KNU* and *AG*. As mentioned above (Section 3.1.5), *AG* activates *KNU* at stage 6 of flower development. The data generated as part of this thesis confirm that, at stage 8 of flower development, *KNU* binds regulatory elements of *AG* and represses its activity. What has not been shown by the ChIP-seq analysis is the direct repression of *WUS* by *KNU*. A low enrichment was detected by the ChIP-qPCR (see Section 4.3.7.1), however, no peak was called in the region of the *WUS* locus. In this case the peak score could have been too low for the cut-offs used in the peak calling method.

Moreover, new functions of *KNU* were identified by the ChIP-seq analysis. *KNU* has a role in the *Regulation of Auxin Metabolic Processes* and *Regulation of Hormone Levels* (see Section 4.3.7.3). The *AUXIN RESISTANT1* (*AUX1*) or *AUXIN RESISTANT2* (*AXR2* or *IAA7*) has been identified as putative direct targets of *KNU*. In addition, genes involved in cytokinin metabolism (*CKX3* and *CKX7*) were also identified as genes targeted by *KNU*. An interesting observation was the detection of genomic regions that were associated with genes known to regulate the initiation of trichome formation. *GL3*, *TRY* and *ZFP2*, 5, 7 and 10 were all represented in the

dataset. Moreover, the phenotypes of *KNU* over-expression lines that produced trichomes forming on carpel valves (see Section 4.3.3) indicate that *KNU* is a repressor of some genes acting in the control of trichome formation. Furthermore, the phenotype of the line where *KNU* is turned into an activator through a fusion with the VP16 transactivating domain (see Section 4.3.3), where no trichomes were observed on carpel valves, may indicate that *KNU* is an activator of genes that suppress trichome formation. To analyze that in more detail, qRT-PCR on the *KNU* knockdown line may provide some insights into the roles that *KNU* has in the formation of trichomes. Taken together, the results of the genome-wide binding study provided new insights into the functions of *KNU* during flower development.

4.4.6 Genes acting downstream of *KNU*

To characterize the processes that are dependent on *KNU*, the amiRNA targeting *KNU* was placed under the 35S promoter and expressed in FIS plants. Using those plants I performed a genome-wide expression profiling analysis. More than 300 genes identified as differentially expressed occurred among three time-points. Because of quality issues for two of the hybridizations with RNA from the 7 d time-point, they were excluded from further analysis. The data obtained was analyzed for an enrichment of Gene Ontology (GO) terms. The terms with the highest enrichment included *Pollen Development*, in agreement with the previously reported role of *KNU* in this process (Payne et al., 2004). Although a large number of differentially expressed genes was identified, the overlap between the genomic regions bound by *KNU* and genes acting downstream of *KNU* was small. This effect could be caused by the fact that genes from different stages of development were compared. Also, it is possible that because of stringent cut-offs and not full knockdown of *KNU*, some genes were not detected by the microarray experiment. Indeed, the analysis executed with less stringent *p*-value cut-offs created lists of genes now including the genes identified as direct targets of *KNU* in the ChIP-seq experiment. In addition, qRT-PCR performed with cDNA used for microarray analysis confirmed these predictions. To obtain more precise results, a line that allows a more complete knockdown of *KNU* may be needed. In fact, a second *knu* allele (*knu-2*), which has been described by Sun et al. (2009), could be an alternative to using *knu-1*, which may not constitute a full gene knockdown. The *knu-2* allele contains

a T-DNA insertion towards the 3' end of the coding region, but the exact location has not been described.

In this case the gain-of-function lines would be applicable. The plants that overexpress *KNU* and in the FIS background could be used in the RNA-seq experiment that already has been implemented in the laboratory and yielded good quality datasets. These datasets could then be compared to the available data from microarray analysis and ChIP-seq assays.

4.4.7 Future work

In this study, I have generated several transgenic lines that allowed the role of *KNU* during flower development to be assessed on a genome-wide scale. However, especially the transcriptomics experiments after *KNU* perturbation likely did not yield a comprehensive list of genes that act downstream of this transcription factor. As described above, this was probably due to the fact only a partial knockdown of *KNU* was achieved. Furthermore, because of its relatively small domain of expression, any transcriptional changes that may resulted from a *KNU* knockdown may have not been readily detectable. To possibly circumvent these limitations, it may be necessary to conduct additional experiments using inducible and ectopic *KNU* over-expression.

Additionally, the second biological replicate of the *KNU*-GFP ChIP-seq experiment (*KNU*-2) paled in comparison to the first replicate (*KNU*-1) in that binding enrichments were observed for only a small number of genes. Therefore, a third ChIP-seq replicate will be generated in the near future to confirm the findings made based on the *KNU*-1 dataset. A new line of experimentation will aim at identifying stage-specific differences in the binding patterns of *KNU*. To this end, we will conduct additional ChIP-seq experiments using the *KNU*-GFP, FIS line generated in this study and flowers of different stages of development. The data from these experiments will then be combined with those from the inducible *KNU* perturbation experiments described above and should allow us to correlate changes in gene expression with changes in *KNU* binding patterns. This work should yield new, detailed insights into the dynamic behavior of the gene regulatory network downstream of *KNU*.

CHAPTER 5 – DYNAMIC DISTRIBUTION OF EPIGENETIC MARKS DURING EARLY FLOWER DEVELOPMENT

5.1 Introduction

Over the past few decades, the use of genetic, biochemical and genomic assays yielded detailed insights into the molecular mechanisms that control flower development. This work led to the identification of numerous transcription factors and other regulatory proteins. In recent years, a main focus in the field of flower development has been to understand how these factors act on DNA and how they interact with epigenetic modifiers to control gene expression (Buzas et al., 2012; Carles and Fletcher, 2009; Lu et al., 2011; Smaczniak et al., 2012; Sun et al., 2014). Epigenetic control of genes is often described as heritable changes that are not caused by mutations in the DNA sequences but instead by covalent modification of chromatin such as DNA methylation or the post-translational modification of histones. These changes are known to play key roles in flower development (Guo et al., 2015). In this chapter, I will focus on histone methylation and explain how these modifications contribute to the successful development of a flower.

5.1.1 Histone methylation in plants

In plants and other eukaryotes, DNA is packaged into a compound structure known as chromatin. The basic unit of chromatin is the nucleosome, which is composed of 146 base pairs of DNA wrapped around an octamer of histones. There are two copies of each histone protein: H2A, H2B, H3 and H4 (reviewed in: (Jenuwein and Allis, 2001). The architecture of chromatin is regulated by different epigenetic mechanisms such as DNA methylation, ATP-dependent chromatin remodeling, use of histone variants and also regulation by noncoding RNAs (reviewed in: (Liu et al., 2010). It has been shown that these mechanisms affect the structure of chromatin and thus gene activity. The N-terminal tails of histones are subjected to diverse posttranslational modifications that include methylation, acetylation, ubiquitination, phosphorylation or glycosylation (Fig. 5.1). These modifications influence the binding of specific effector proteins to chromatin and define the functional outcomes (Jenuwein and Allis, 2001).

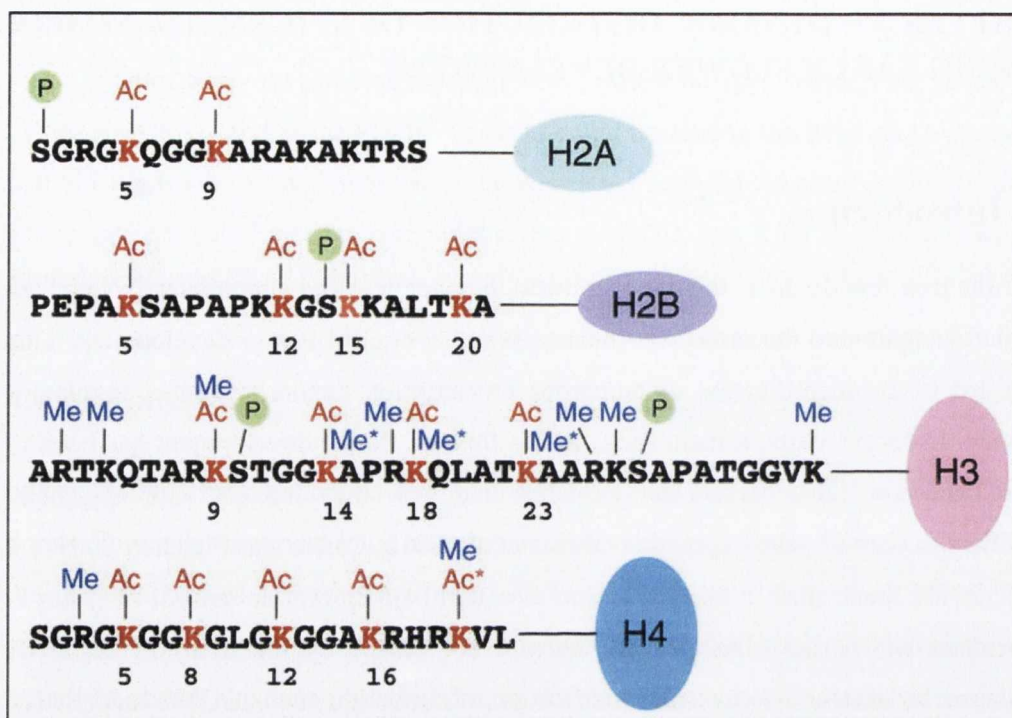


Figure 5.1 Post-translational modifications of core histones. The modifications on N-terminal tails of histones include acetylation (Ac; in red), methylation (Me; in blue), phosphorylation (P, green circles), and ubiquitination (not shown). H2A, Histone 2A; H2B, histone 2B; H3, Histone 3; H4, Histone 4. Figure modified from (Chen and Tian, 2007).

Arguably the best studied epigenetic modification in plants is the methylation of histones (Gan et al., 2013; Pien and Grossniklaus, 2007). Histone methylation can be generally divided into activating marks, for example the methylation of histone H3 at lysine 4 (H3K4) and lysine 36 (H3K36), and repressive marks such as the methylation of histone H3 lysine 9 (H3K9) and lysine 27 (H3K27) (for review, see (Berger, 2007). Mass spectrometry combined with high-performance liquid chromatography has revealed histone modification profiles in *Arabidopsis* to be conserved and in some cases non-conserved, when compared with modification in animals (Johnson et al., 2004; Zhang et al., 2007a).

As described above, the lysine methylations of histone H3 in *Arabidopsis* are the most common and arguably the most important epigenetic marks. These modifications are written by various histone lysine methyltransferases (HKMTs) that often contain SET domains, which function in the addition of the posttranslational

modification. To date, 41 different SET domain proteins have been identified in *Arabidopsis* (Gendler et al., 2008), which can be divided into four distinct groups: SU(VAR)3-9; E(Z) (enhancer of zeste) homologs, TRX (trithorax); and ASH1 (absent, small or homeotic discs 1) (Fig. 5.2) (Baumbusch et al., 2001; Springer et al., 2003; Zhao and Shen, 2004). Apart from the writers of histone methylation there are also readers, proteins that contain recognition domains for methylated histones. In *Arabidopsis* there are at least three distinct families: effectors containing chromolike domains of the royal superfamily; the plant homeodomain finger (PHD) superfamily; and proteins containing the WD40 repeat in WDR5 (Ruthenburg et al., 2007; Taverna et al., 2007).

The regulation of histone methylation is a dynamic process that is controlled by writer and eraser proteins. These proteins are able to add or remove posttranslational modifications to histones. For example, histone demethylases (HDMs) contain two subgroups that represent different mechanisms of activity. Firstly, the lysine-specific demethylase 1 (LSD1) operates through the amine oxidation. Secondly, Jumonji C (JmjC) domain-containing proteins act by hydroxylation of the methyl groups from methylated residues (Shi et al., 2004; Tsukada et al., 2006). In the following sections, the importance of methylation in plant development will be discussed. Proteins that belong to writers and erasers will be described along with their association to the methylation marks, which they are characteristic for.

5.1.1.1 H3K9 methylation is linked to DNA methylation

Methylation of histone H3K9 occurs in plants mostly at mono-(H3K9me1) and di-(H3K9me2) levels, whereas trimethylation (H3K9me3) is only marginally detectable (Johnson et al., 2004). The two first stages are enriched in a highly condensed constitutive heterochromatin region, mainly composed of pericentromeric repeats, transposable elements and ribosomal DNA. In contrast to H3K9me2, H3K9me3 is enriched in euchromatin, where the majority of actively transcribed genes are found (Mathieu et al., 2005; Turck et al., 2007).

One of the histone methyltransferase that was first identified in plants through a suppression screen of a *superman* (*sup*) epi-allele (the *clark kent* (*clk*) allele; silenced by DNA methylation), is KRYPTONITE (KYP) (Jacobsen and Meyerowitz, 1997). It has been shown that the *clk* phenotype was rescued in a *kyp* mutant background. This writer

of histone H3K9 methylation can only add one or two, but not three methyl groups to the H3K9 *in vitro* (Jackson et al., 2004). Moreover, the silencing of *SUP* is correlated with excessive CpG DNA methylation, which is associated with H3K9me2 at heterochromatin (Mathieu et al., 2005). Recent studies have also shown that KYP functions as a repressor of genes involved in seed dormancy. The *kyp-2* mutant shows increased seed dormancy whereas the overexpression of KYP leads to reduced dormancy and abscisic acid (ABA) sensitivity (Zheng et al., 2012).

5.1.1.2 Role of H3K27 methylation in plant development

In both the animal and plant kingdoms, H3K27 methylation is serving as a repressive mark. Similar to H3K9, H3K27 can be mono-, di- and tri- methylated in *Arabidopsis*. Moreover, both of these marks are restricted to euchromatin, but they do not demonstrate significant overlap (Turck et al., 2007). Similar to H3K9me1/2, H3K27me1 is also enriched in silenced heterochromatin (Jackson et al., 2004; Mathieu et al., 2005). While the H3K9me2 is associated with DNA methylation, H3K27me1 seems to be independent of that process (Jacob et al., 2009; Mathieu et al., 2005), which indicates that their maintenance and deposition are controlled by different processes.

Arguably the best studied methylation mark in eukaryotes is H3K27me3. It is often found in the transcribed regions of genes in a close proximity to promoter elements and also the 5' of transcribed regions, which is in agreement with its role in transcriptional repression (Turck et al., 2007; Zhang et al., 2007a). Moreover, around 17% of protein coding genes in *Arabidopsis* are marked with H3K27me3, which implies its role as a main factor in gene silencing (Zhang et al., 2007a). In plants, H3K27me3 silences genes during various developmental stages and is catalyzed by the Polycomb Group protein-containing complex, which is composed of Polycomb Repressive Complex 1 (PRC1) and PRC2 (Zhang et al., 2007a). The *Arabidopsis* genome encodes four homologs of all of the components of the conserved PRC2 complex (Fig. 5.2). These proteins belong to different subfamilies: E(z) homolog proteins are represented by CURLY LEAF (CLF), MEDEA (MEA) and SWINGER (SWN); Su(z)12 homologs are FERTILIZATION-INDEPENDENT SEED2 (FIS2), EMBRYONIC FLOWER2 (EMF2) and VERNALIZATION2 (VRN2). There are five p55 homologs, MULTICOPY SUPPRESSOR OF IRA (MSI) 1-5, and one homolog of ESC FERTILIZATION INDEPENDENT ENDOSPERM (FIE) (reviewed in: Kohler

and Villar, 2008; Pien and Grossniklaus, 2007)). Those proteins act together in different combinations and create three diverse PCR2 complexes.

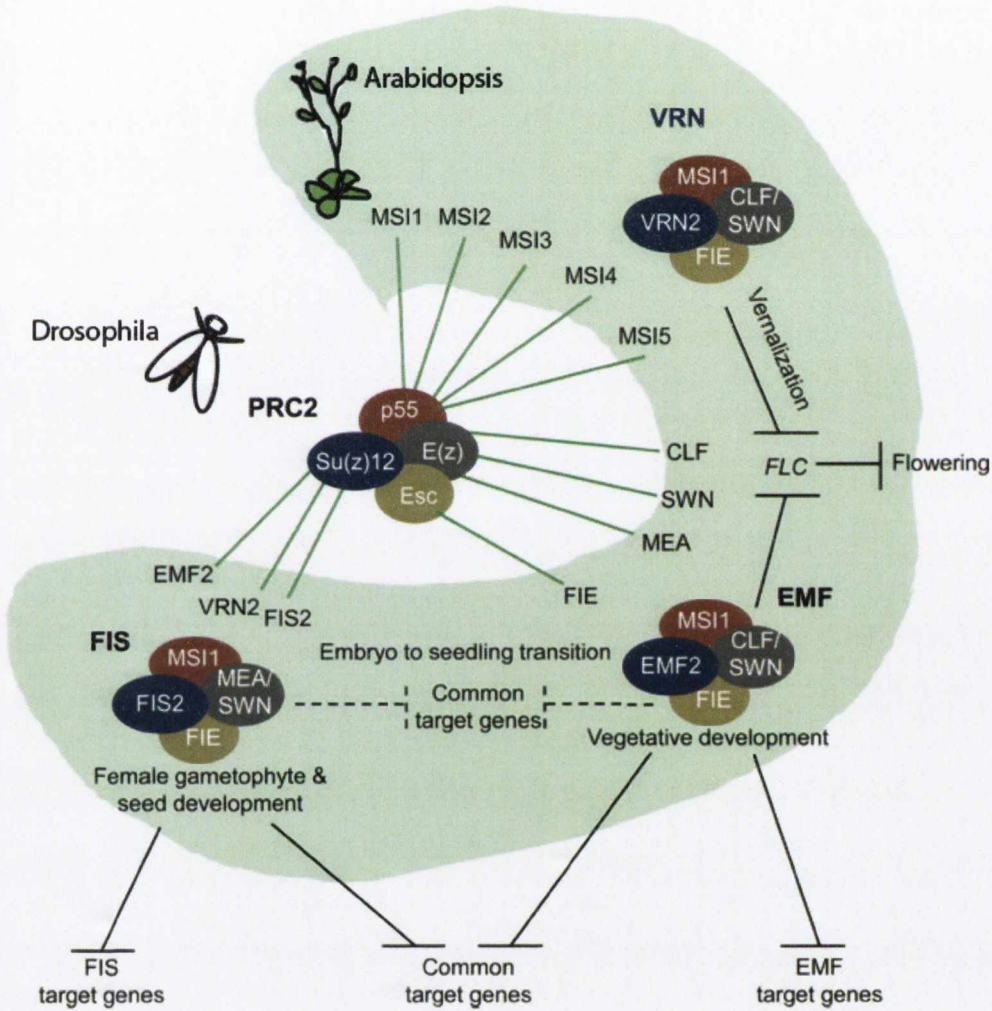


Figure 5.2 POLYCOMB REPRESSIVE COMPLEX2 (PRC2) functions in *Arabidopsis*. *Arabidopsis* homologues of *Drosophila* PRC2 subunits form three PRC2-like complexes, containing either EMBRYONIC FLOWER (EMF), VERNALIZATION (VRN), or FERTILIZATION INDEPENDENT SEED (FIS). These complexes have acquired specialized functions in plant development. Various PRC2-like complexes can regulate the same subset of Polycomb Group targets at different developmental stages. Green lines depict homologous proteins in *Drosophila* and *Arabidopsis*. See main text for a more detailed description. Figure from (Derkacheva and Hennig, 2014).

The FIS-containing PRC2 complex

The first PRC2 complex that was identified in *Arabidopsis* is the FIS-containing complex, which is composed of MEA/FIS1, FIS2, FIE/FIS3 and MSI1 (Chaudhury et al., 1997; Grossniklaus et al., 1998; Kohler and Villar, 2008; Luo et al., 1999; Ohad et al., 1999). Plants with mutations in the corresponding genes exhibit problems in fertilization where self-fertilized plants produce 50% of aborted seeds. This issue appears only when the mutation is inherited through the female gametophyte.

The EMF2-containing PRC2 complex

The EMF2-containing PRC2 complex, which is built from EMF2, CLF/SWN, FIE and MSI1, has been recognized through the functions played in repressing floral transition and floral homeotic gene expression (Fig. 5.2). *emf2* mutant plants produce small and sterile inflorescences with terminal flowers (Sung et al., 1992). Moreover, the floral organ identity genes *PI*, *AG* and *AP1* are over-expressed (Moon et al., 2003) and H3K27me3 levels are decreased at the *AG* locus (Calonje et al., 2008). On the other hand, mutations in *CLF* cause early flowering, partial homeotic transformations and curly leaves (Goodrich et al., 1997). Furthermore, the *ag-10 clf-47* and *ag-10 clf-2* double mutants produce short and bulged siliques with ectopic floral organs inside them. This suggests that CLF has a role in flower meristem determinacy (Liu et al., 2011).

Current studies suggest also that both EMF2 and FIE are bound to specific promoter regions of *KNU* that include binding sites for AG (Sun et al., 2014). As mentioned in Chapter 3, the H3K27me3 mark can be erased by JmjC-domain-containing histone demethylases REF6, JMJ30 or JMJ32 (Gan et al., 2014; Lu et al., 2011). With regards to other processes that are controlled by the members of EMF2-PRC2 complex, EMF2, CLF and FIE are required for the repression of the floral repressor *FLOWERING LOCUS C (FLC)* and the florigen-coding gene *FLOWERING LOCUS (FT)* by directly binding to their chromatin regions (Jiang et al., 2008). Additionally, H3K27me3 levels are decreased at the *AG*, *FLC* and *FT* loci in *clf* mutants. These data strongly suggests that CLF acts as a histone methyltransferase (Jiang et al., 2008; Schonrock et al., 2006; Schubert et al., 2006).

The VRN-containing PRC2 complex

The VRN-containing PRC2 complex contains three additional PHD finger proteins: VERNALIZATION INSENSITIVE3 (VIN3), VERNALIZATION 5/VIN3 LIKE1 (VRN5/VIL1) and VEL1/VIL2, in addition to the four core components, VRN2, FIE, CLF/SWN and MSI1 (Chanvivattana et al., 2004; De Lucia et al., 2008; Wood et al., 2006). This complex is important in vernalization, the process where flowering is activated by the prolonged exposure to cold temperatures (reviewed in: (Henderson and Dean, 2004)). Vernalization is one of the best-studied epigenetically regulated processes that is induced by environmental signals. The two genes *FRIGIDA* (*FRI*) and *FLC* play the main role in the control of flowering time by vernalization. Both of them repress flowering, however *FRI* suppresses it by elevating the expression of *FLC* (Johanson et al., 2000; Michaels and Amasino, 1999; Sheldon et al., 1999), and *FLC* expression is inhibited by vernalization to promote flowering in the spring (for review see (Dennis and Peacock, 2007; Sung and Amasino, 2005)). The VRN2-containing PRC2 complex maintains the repressive state of *FLC* (De Lucia et al., 2008; Wood et al., 2006). Moreover, VRN2 is associated with the *FLC* locus, and in non-vernalized plants *FLC* is not repressed even in the presence of VRN2 (De Lucia et al., 2008). During extended cold conditions *VIN3* expression is induced (Fig. 5.3). Together with VRN5/VIL1 and VEL1/VIL2 they are recruited to the *FLC* locus and form the VRN2-PRC2 complex and all, including the four core members, mediate the deposition of H3K27me3 marks. (De Lucia et al., 2008; Sung and Amasino, 2004). Additionally, *FLC* repression occurs only when *VIN3* is induced, which suggests that *VIN3* is a key factor in the primary establishment of the repressive state of *FLC* (Sung and Amasino, 2004). Overall, the vernalization mediated epigenetic silencing of *FLC* includes active association and unstable composition of Polycomb complexes at different environmental shifts (De Lucia et al., 2008).

Apart from these crucial developmental processes in the early stages of plant growth controlled by trimethylation of lysine 27 on histone 3, genes that act in transcriptional regulation are controlled by H3K27me3 deposition (Zhang et al., 2007a). These include, for example, regulators of flower development such as *AP3*, *PI*, *SUP* or *AG*, as mentioned earlier. As discussed in Chapter 3, *AG* is also involved in the removal of the repressive mark from the *KNU* locus (Sun et al., 2009). Studies have also suggested that

AG represses *WUS*, the main player in stem cell maintenance by the deposition of H3K27me3 repressive marks (Liu et al., 2011). Moreover, it has been reported that various genomic regions containing floral homeotic genes are targeted by the H3K27me3 demethylase REF6 (Lu et al., 2011). Overexpression of *REF6* leads to phenotypes that are similar to those of *clf* or *tfl2* mutant, whereas the *ref6* mutation can partially rescue the curly leaf phenotype in *clf*. In addition, studies on MADS-domain transcription factor complexes suggest that REF6 is able to create a complex with AG (Smaczniak et al., 2012). Likewise, SEP3 and REF6 are also involved in establishment of bigger protein complexes. Another study provided evidence for the physical interaction between PI and ISWI-type chromatin remodelers CHR4, CHR11 and CHR17 (Li et al., 2014). Apart from that interaction, these remodelers are found in different complexes that also contain AG, AP3, SEP3 and AP1 (Smaczniak et al., 2012). Interestingly, AP1 is also implicated in H3K27me3 removal from the *SEP3* locus during early stages of flower development, probably through the interplay with REF6. A study by (Pajoro et al., 2014) discusses chromatin availability by MADS-domain transcription factors during flower development and suggests that AP1 and SEP3 possibly bind their target genes before chromatin decondenses locally. Furthermore, these two proteins are able to adjust the chromatin accessibility of their targets and therefore facilitate access to those genes by different transcription factors (Pajoro et al., 2014).

Taken together, the H3K27 mark correlated with gene repression plays a major role during many stages of plant development, including floral transition and the vernalization response. The trimethylation of lysine 27 has a dominant function during the early stages of flower development and this phenomenon was used in the experiments described in results section.

5.1.1.3 H3K4 methylation and Trithorax Group Proteins

Proteins acting in opposition to the PcG complexes to release genes from H3K27me3 mediated repression are the Trithorax group (TrxG) protein. These proteins accumulate at promoter and 5' genomic regions (Zhang et al., 2009). *ARABIDOPSIS* TRITHORAX1 (ATX1) is an active histone methyltransferase that is specific for histone H3K4 methylation. Plants with a mutation in the corresponding gene exhibit abnormal floral organ identity and early flowering (Alvarez-Venegas et al., 2003).

ATX1 interacts with the *FLC* promoter at its first exon and catalyzes the H3K4me3 modification. The levels of H3K4me3 at the *FLC* locus are reduced, however, H3K4me2 levels remains unchanged in the *atx1* mutant, indicating that ATX1 is an H3K4me3 methyltransferase (Pien et al., 2008). Additionally, *atx1* mutants show reduction in H3K4me2 (6-8%) and H3K4me3 (around 15%) at a global level, suggesting that other methyltransferases also catalyze H3K4 methylation (Alvarez-Venegas and Avramova, 2005). In fact, the *Arabidopsis* genome encodes five ATX proteins and seven ATX related proteins (Baumbusch et al., 2001; Saleh et al., 2008).

One of the most studied TrxG protein ULT1, functions to induce *AG* in floral stem cells (Carles and Fletcher, 2009). It has been reported that ULT1 posses a SAND domain, which directly binds to the *AG* regulatory sequences. Through the direct interaction with ATX1, ULT1 acts antagonistically to PcG-activity to harmonize the H3K4me3 methylation status at the *AG* locus (Fig. 5.3) (Alvarez-Venegas et al., 2003; Carles and Fletcher, 2009). As mentioned earlier, *atx1* mutant plants display abnormalities in floral organs and the expression levels of homeotic genes such as *API*, *AP2*, *AP3*, *PI* and *AG* is lower than normal. This suggests that *trxG* has a function in the control of flower development (Alvarez-Venegas et al., 2003).

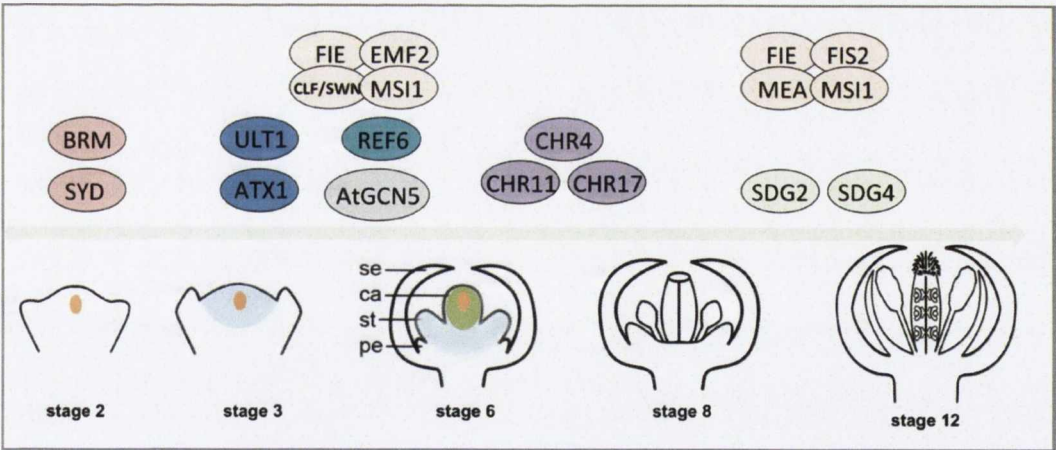


Figure 5.3 Chromatin-remodeling factors in flower development. During floral stages 1–2 (Smyth et al. 1990), chromatin-remodeling factors SYD and BRM are recruited to the *AG* and *AP3* loci for their activation. The *trxG* protein ULT1 interacts with ATX1 for the activation of *AG* and other MADS-box genes. Commencing at floral stage 3, the chromatin-remodeling factors CHR4, 11 and 17 interact and form large complexes with *API*, *PI*, *AP3*, *AG* and SEPs. The JmjC-domain protein REF6 interacts with *AG* and *SEP3* to promote chromatin opening leading to the transcription of many downstream targets for the proper development of

stamens and carpels. Later in the development, during male and female gametophyte growth, SDG2 may function in gene activation. In contrast, the FIS–PRC2 complex plays a role in gene silencing during gametogenesis and seed development. In addition, SDG4, an H3K36 methyltransferase, is involved in pollen tube formation. Figure modified from (Guo et al., 2015).

Further studies identified others proteins reported to be H3K4 methyltransferases (Berr et al., 2010; Berr et al., 2009; Saleh et al., 2008; Tamada et al., 2009). Mutations in these genes, and in particular in *SET DOMAIN GROUP 2* (*SDG2*), effect pollen and ovule sterility, but no other phenotypes in floral organs are observed (Berr et al., 2010). Moreover, the levels of H3K4me3 in the *sdg2* mutant are decreased at the *SPOROCTELESS* (*SPL*), *BTB AND TAZ DOMAIN PROTEIN 3* (*BT3*) and *MSII* loci, which implies a function of the trxG protein in gametophytic development. AG directly induces *SPL* (Ito et al., 2004) and this suggests that AG takes part in the changes of H3K4me3 deposition at the *SPL* locus (reviewed in (Guo et al., 2015)). Other *SDG* genes are implicated in the proper development of the pollen tube (Thorstensen et al., 2008). In *sdg4* mutants, the pollen tubes grow much slower than in wild-type plants, which causes male sterility. This demonstrates that SDG4 is able to adjust the expression of genes that are main players of pollen tube growth (Cartagena et al., 2008).

In summary, the H3K4me3 activation mark acts in some processes of flower development, however, it does not appear to be as commonly distributed as the repressive H3K27me3 methylation mark.

5.1.1.4 H3K36 methylation and ASH-related Group proteins

The active H3K36 methylation mark is enriched in the 5' regions of transcribed regions of genes. ASH1-related protein (ASHH2)/ SDG8, SDG7 and SDG26 have been identified to play a role as a methyltransferases (Roudier et al., 2011). Mutations in *SDG8* and *SDG26* cause early and late flowering phenotypes, respectively (Dong et al., 2008; Xu et al., 2008b). Moreover, defects in floral organs and abnormalities in gametophyte development occur when these genes are mutated (Berr et al., 2010; Cazzonelli et al., 2009; Grini et al., 2009; Xu et al., 2008b). *sdg8* mutant plants exhibit homeotic transformations in flowers. Sepals are converted to carpels and petals to

stamens or carpeloid organs. These effects in flowers could be due to the expanded expression domain of *AG* in the first and second floral whorls. This is in an agreement with the down-regulation of the negative regulators of *AG* in *sdg8* mutants (for review see (Guo et al., 2015). Other phenotypes of *sdg8* mutants include abnormal ovule and stamen development (Grini et al., 2009). This is thought to be because of a strong decrease in H3K36me3 marks at the *MYB99* and *AtDMC1* loci, which encode transcription factors involved in embryo sac and pollen development, accordingly. SDG8 activity is also characteristic to H3K36me2/3, in order to activate the expression of *FLC* and *MADS AFFECTING FLOWERING (MAF)* genes. On the other hand, SDG26 repressed the same genes but possibly acts in an indirect manner (Xu et al., 2008b).

5.1.2 Histone acetylation in plants

In addition to protein complexes that act in histone methylation, there is another group of proteins that promotes histone acetylation. Acetylated histones mark active chromatin and there are numerous histone acetyltransferases (HATs) and histone deacetylases (HDAs) that add and remove acetyl group to histones, respectively (Pandey et al., 2002). The *Arabidopsis* HAT, *AtGCN5*, is involved in different developmental processes including floral stem cell regulation (Bertrand et al., 2003). *atgcn5* mutant plants exhibit an expansion of the *WUS* expression domain beyond the organizing center. This results in defects in vegetative development. Moreover, these plants produce terminal flowers with homeotic transformations such as petals converted into stamens and sepals into filamentous structures. In addition, ectopic carpels are produced in *atgcn5* mutant flowers, suggesting an expansion of the *AG* expression domain. Thus, *AtGCN5* appears to be necessary for the activation of repressors of both *AG* and *WUS* expression (Bertrand et al., 2003).

One particular deacetylase, HDA19, is known to associate with the transcriptional co-repressors complex LUG-SEU in order to remove the acetylation mark from the *AG* locus (Chen and Tian, 2007; Tian and Chen, 2001). The repression of *AG* expression in the first and second whorls by the LEU-SEU-HDA19 complex ensures its expression in the proper domain. In the *lug seu* double mutant, *AG* is expressed in all floral organs, which causes homeotic transformation of sepals and petals into reproductive organs (Tian and Chen, 2001).

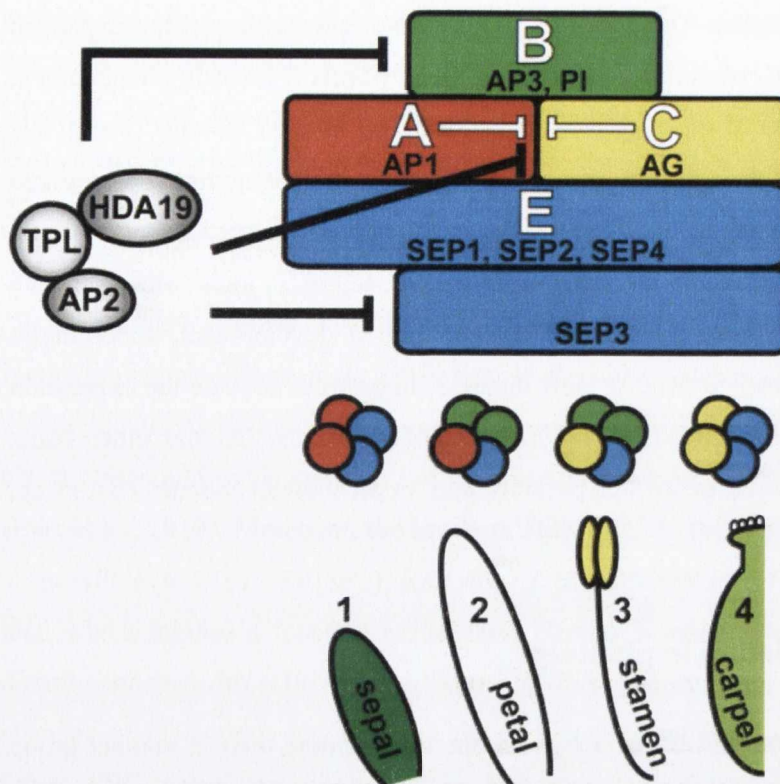


Figure 5.4 Model of AP2 function during flower development. AP2 regulates the outer boundaries of B-, C- and E-class genes by recruiting TPL and HDA19. This complex directly represses *AG* in sepals and petals and *AP3* and *SEP3* in sepals. Figure from (Krogan et al., 2012).

Recent studies have shown that HDA19 interacts with AP2 and the transcriptional co-repressor protein TOPLESS (TPL) to repress floral homeotic genes (Fig. 5.4) (Krogan et al., 2012). It has been reported that the WD40 domain protein TPL and TPL-related (TPR) interact with transcription factors that are involved in auxin and jasmonate signal transduction, meristem maintenance and immune response (Pauwels et al., 2010; Szemenyei et al., 2008; Zhu et al., 2010). In *tpl-1* mutant plants the PLETHORA1/2 (PLT1/2) transcription factor is upregulated, which causes the transformation of shoot tissue to root tissue in high temperatures. This conversion takes place with the support of the HD-ZIPIII transcription factor that specifies apical fate (Smith and Long, 2010). It has been shown that in the shoot apical meristem, WUS recruits TPL/TPR co-repressors to suppress the genes responsible for differentiation.

Therefore, stem cell specification is guaranteed (Kieffer et al., 2006). In addition, TPL plays a pivotal role in floral organ specification. As mentioned earlier, AP2 and HDA19 recruit TPL/TPR that is essential to determine the boundary of whorls through spatial repression of the floral homeotic genes *AP3*, *PI*, *SEP3* and *AG* (Fig. 5.4) (Dinh et al., 2012; Krogan et al., 2012). In *tpl-1* single mutants and the double mutant *tpl-1 ap2-2* /+, expression of *AP3* and *SEP3* are identified in sepals. The AP2-TPL-HDA19 complex binds to the second intron of *AG* and also the promoters of *AP3* and *SEP3*. This binding by the repressive complex restricts the petal fate by maintaining the boundaries between organs during their development (Krogan et al., 2012).

5.2 Aim of the project

While the genetic mechanisms that control flower development have been elucidated in some detail over the past 30 years (Becker et al., 2002; Bowman et al., 1993; Bowman et al., 1991; Bowman and Smyth, 1999; Jack et al., 1994; Kaufmann et al., 2009; Kaufmann et al., 2010; O'Maoileidigh et al., 2013), our understanding of the epigenetic control of flower formation is still vastly limited. To address this knowledge gap, I aimed at generating genome-wide datasets to characterize the relationship between the master regulator of floral meristem identity specification AP1 and the dynamic distribution of selected epigenetic marks (H3K27me3, H3K4me3, H3K9me3 and AcH3K9) during early flower development. The results of these experiments would give insights in the relationship between flower regulators and epigenetic control proteins. Because of time constraints, I performed only part of the planned experiments. The results of this work are described below.

5.3 Results

5.3.1 Genome-wide H3K27me3 distribution at early stages of flower development

It has been shown that AP1 activates *SEP3* (*SEPALLATA3*) at early stages of flower development and that this activation is associated with the removal of the repressive H3K27me3 mark (Kaufmann et al., 2010; Smaczniak et al., 2012). Because of this specific interaction, an experiment was designed where the effects of a specific

activation of AP1 (see Section 1.4.2) on *SEP3* could be measured and the levels of the repressive H3K27me3 mark could be determined.

In order to analyze the distribution of H3K27 trimethylation marks at early stages of flower development, inflorescence tissue of AP1_{pro}:AP1-GR *ap1-1 cal-1* plants (referred as FIS hereafter) was collected 0, 2, 4 and 6 d after treatment of the plants with dexamethasone. Approximately 500 µL of tissue was used for ChIP-qPCR analysis (see section 2.12) with a specific H3K27me3 antiserum from Millipore (see Section 2.12.3). The enrichment level of the repressive mark was very high at the 0 d time-point, especially in the *SEP3* intronic region (Fig. 5.5). Two days after the dexamethasone treatment, the level of enrichment dropped dramatically. This drop continued at 4 and 6 d after the AP1-GR activation. These data are consistent with results previously published (Smaczniak et al., 2012), and suggested that the experimental set-up used may be suitable for the global analysis of H3K27me3 distribution during early flower development. I therefore conducted a similar experiment and prepared the ChIP libraries for ultra high-throughput sequencing.

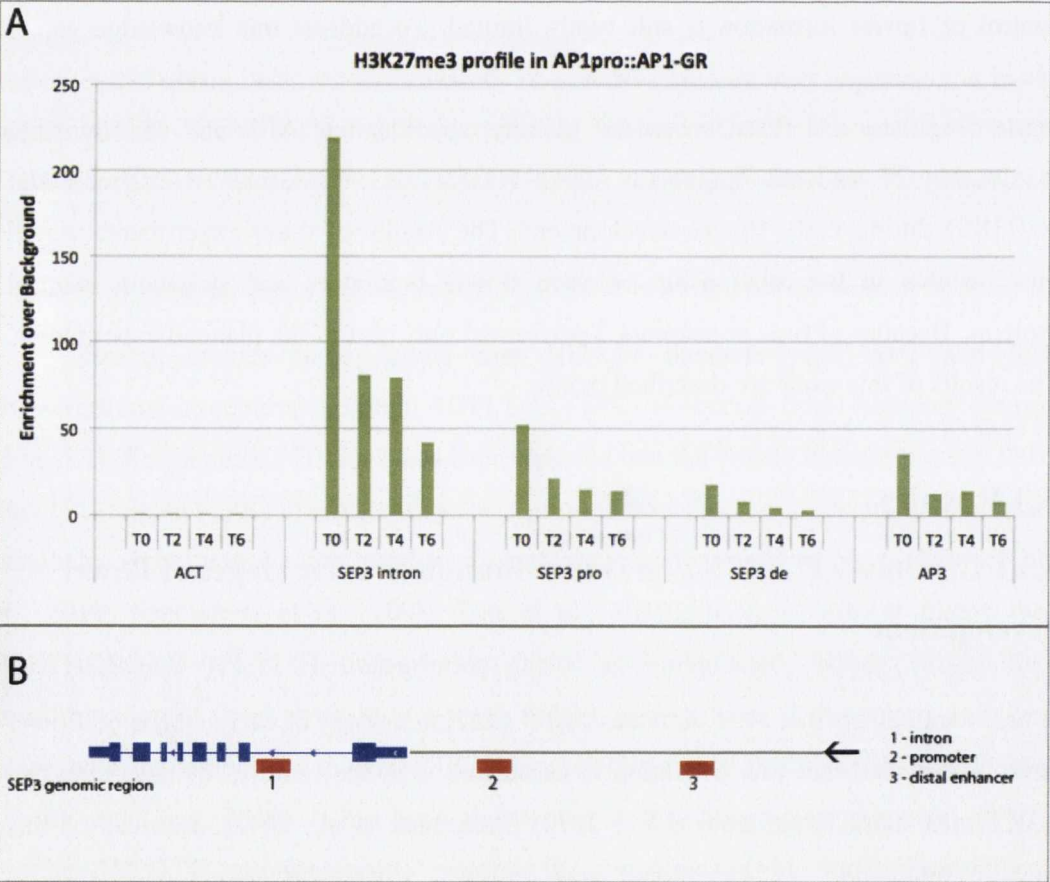


Figure 5.5 Accumulation of H3K27me3 marks at selected loci. (A) ChIP-qPCR was performed using tissue from AP1_{pro}:AP1-GR *ap1-1 cal-1* plants at different time-points (0, 2, 4, and 6 d) after activation of AP1-GR. For all selected regions (with the exception of ACT), enrichment values for H3K27me3 were high at the 0 d time-point but decreased after induction of flower formation, in agreement with the known activation of *AP3* and *SEP3* at early floral stages. (B) *SEP3* locus map with indicated regions of primers binding. 1 - region in the second intron, 2 - promoter region, 3 - distal enhancer. ACT, *ACTIN*; SEP3 intron, intronic region of *SEP3*; SEP3 pro, *SEP3* promoter, SEP3 de, *SEP3* distal enhancer; AP3, *APETALA3*. Primers pairs KK-65/KK-66, KK-67/KK-68, KK-69/KK-70, KK-71/KK-72, and KK-77/KK-78 were used to amplify *ACT*, *SEP3* intron, *SEP3* pro, *SEP3* de and *AP3*, respectively.

5.3.1.1 Generation of ChIP-seq libraries from chromatin associated with repressive H3K27me3 marks

In order to map genomic regions that are associated with the repressive H3K27me3 mark and to identify loci that exhibit a change in the levels of the mark during the onset of flower development, precipitated chromatin from 0 and 2 d time-points was used to generate Diagenode ChIP libraries (described in Section 2.12.6; libraries referred to as AP1-K27-0d and AP1-K27-2d hereafter). In brief, the ends of the ChIP-enriched DNA fragments were ligated with oligonucleotide adapter sequences and PCR-amplified. Next, the adapter-DNA mixture was purified using magnetic beads and size distribution of the library was examined by electrophoresis (Fig. 5.6 B-C). The average size of the generated libraries was 338 bp for AP1-K27-0d and 370 bp for AP1-K27-2d. An aliquot of these libraries was taken and qPCR analysis was performed to ensure that enrichment values were maintained after library construction. Input DNA from each ChIP experiment was used to normalize the data, as described above. For both libraries, the same positive regions were tested and the level of enrichment was found to be lower (Fig. 5.6 A) when compared to the enrichment value obtained in the initial ChIP-qPCR analysis. In the case of the *SEP3* intronic region, the enrichment at 0 d dropped from over 200-fold to 120-fold, suggesting a decreased signal-to-noise ratio after library construction. Although the enrichment values decreased overall, they appeared to be high enough to attempt the genome-wide localization of H3K27me3 marks. Consequently, the libraries (as well as libraries constructed from ChIP input DNA) were sent for ultra high-throughput sequencing, which was carried out by the Beijing Genomics Institute using an Illumina Genome Analyzer.

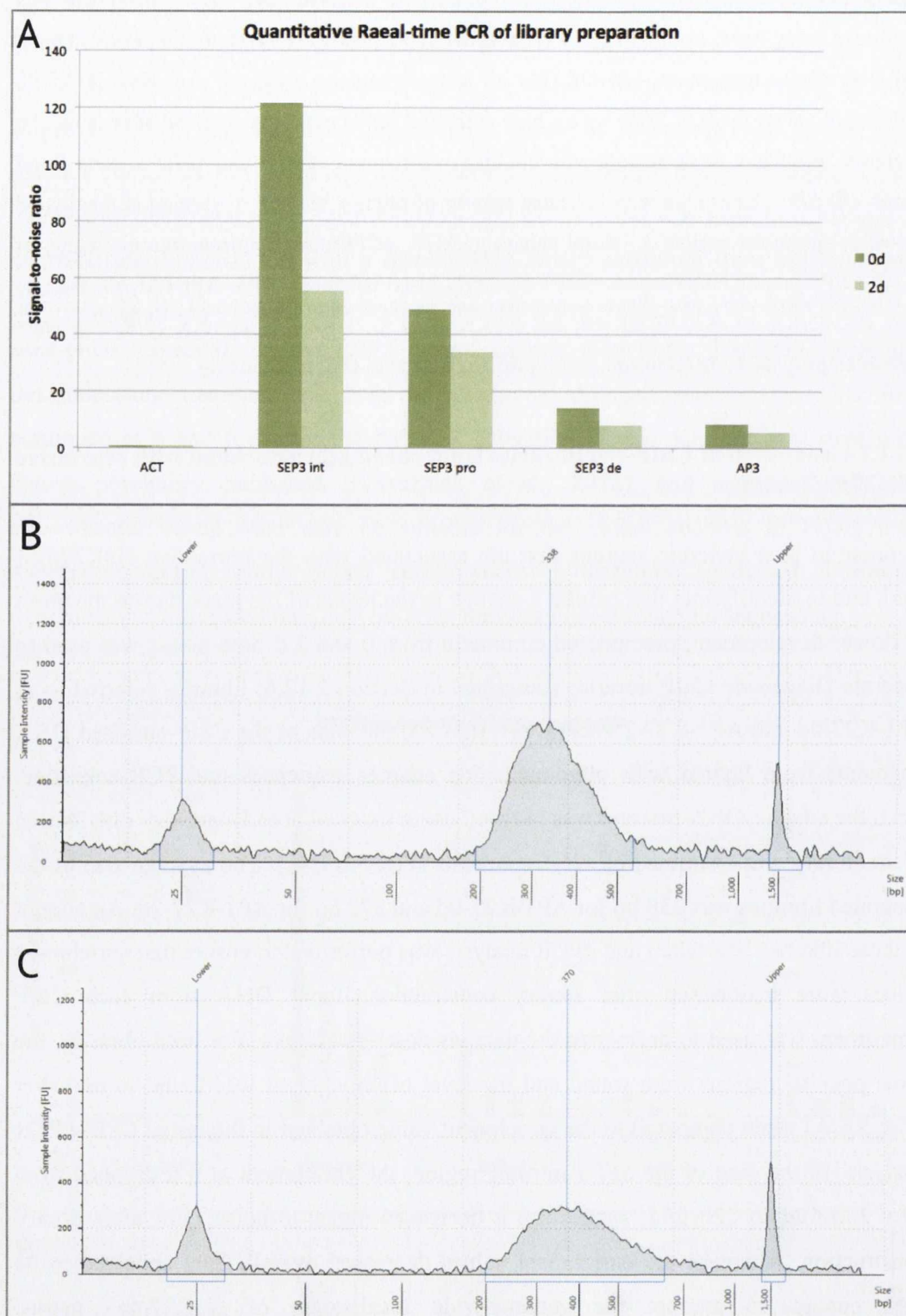


Figure 5.6 Preparation of ChIP-seq libraries for the analysis of the genome-wide distribution of H3K27me3 marks. (A) qPCR was performed for selected loci (as indicated) using DNA from the 0-d and 2-d ChIP-seq libraries. The signal-to-noise ratio was calculated as

outlined in Section 2.9. **(B, C)** DNA size distribution (analyzed with an Agilent Tape-Station) of the 0-d (B) and 2-d (C) libraries used for ultra high-throughput sequencing. Primer pairs KK-65/KK-66, KK-67/KK-68, KK-69/KK-70, KK-71/KK-72, and KK-77/KK-78 were used to amplify *ACT*, *SEP3* intron, *SEP3* pro, *SEP3* de, and *AP3*, respectively.

5.3.1.2 Quality assessment of data from the H3K27me3 ChIP-seq experiments

The libraries generated from genomic DNA isolated by ChIP and control libraries derived from chromatin not subjected to immunoprecipitation (denoted AP1-K27-INPUT-0 and AP1-K27-INPUT-2 hereafter) were analyzed by Illumina next-generation sequencing. The analysis of the sequencing data obtained was done with the help of Dr Beibei Zheng in the laboratory. In brief, the data produced by the Illumina sequencing pipeline included the sequence string output and a quality score. The quality score reflects the probability that a given base call at a given position is correct. These quality scores and sequence strings were uploaded to the online quality control tool, *FastQC* (www.bioinformatics.bbsrc.ac.uk).

As described in Section 4.3.6.2, the *FastQC* software generates quality analysis for sequenced libraries where the distribution of per-base quality reads is illustrated in a box plot (Fig. 5.7 A; the results for the AP-K27-2d library are shown as an example). The green blocks indicate very good quality calls, calls of reasonable quality (orange), and calls of poor quality (red). The box-plots produced for all sequenced samples indicated that the sequencing data were generally of very high quality. The *FastQC* software analyses various other parameters of a sequencing run, including the number of duplicated sequences. A high level of duplication indicates that there may be some enrichment bias present (Fig. 5.7 B). The AP1-K27-2d library contained a substantial number (64.5%) of duplicated reads. Given the large data volume resulting from ChIP-seq experiments, these sequences can be readily removed without affecting the overall sequencing coverage.

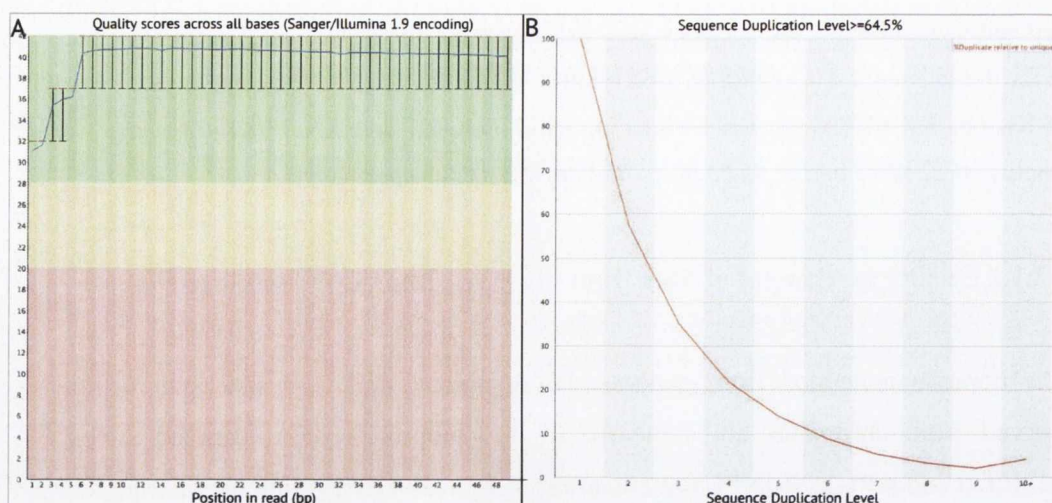


Figure 5.7 Results of ChIP-seq quality controls. (A) Quality scores at a given position within the sequence of all reads from the AP1-K27-2d ChIP-seq experiment, as determined by the FastQC software. The box-plots show the distribution of per-base qualities for all reads in the AP1-K27-2d library. The average quality score is high, indicating a good quality dataset. **(B)** Duplication levels of the reads from the AP1-K27-2d library. The number of single reads is represented as 100% and duplicated sequences are represented as a fraction of this. A duplication level of 10 is equivalent to the percentage of sequences that were present 10 or more times. The sequence duplication level reached ~65%.

Next, the *HOMER* (<http://homer.ucsd.edu/>) software was used to align the unique short read sequences obtained to the *Arabidopsis* genome (TAIR release 10, www.arabidopsis.org). For the AP1-K27-0d and AP1-K27-2d libraries, ~113.7 million and ~39.8 million unique reads were obtained and ~92% and ~93% of these reads aligned to the *Arabidopsis* genome, respectively (Fig. 5.8 A). As already described in Section 4.3.6.2, the software (*MACS*) used for the ChIP-seq peak calling can identify genomic regions that may be far away from the regulatory regions of their associated gene, and also it calls peaks associated with pseudogenes, transposable elements and small ORFs, which could be in the regulatory regions of other genes. For the sake of simplicity, the latter regions were removed from the list of genes with significant enrichment peaks. For example, for the (6,287) peaks identified for the AP1-K27-0d library, 5% associated with pseudogenes and ~16% with hypothetical ORFs (Fig. 5.8 B). For the AP1-K27-2d library the percentages of pseudogenes and hypothetical proteins was nearly identical. After removing the pseudogenes and hypothetical

proteins, 4,723 unique regions were defined as enriched above background in the AP1-K27-0d library, and 5,354 unique regions were identified for AP1-K27-2d (Figure 5.8 C). As expected, the overlap between both samples was found to be high (4,511 unique regions). Taken together, the analysis of the datasets showed that they were of suitable quality and sufficient coverage for further analysis.

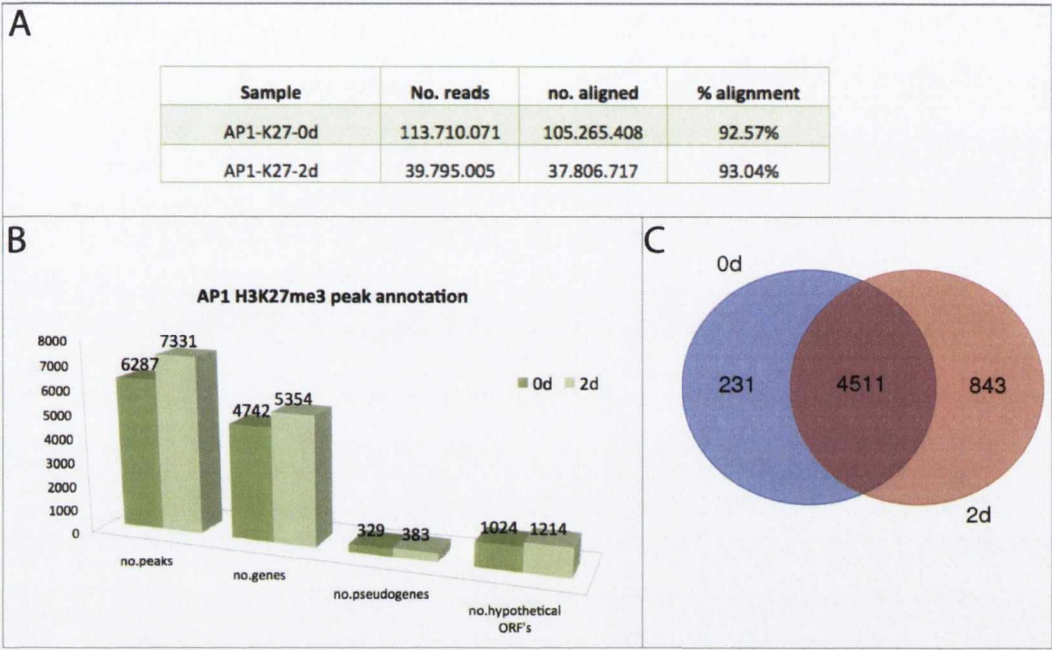


Figure 5.8 Analysis of ChIP-seq datasets from H3K27me3 experiments. (A) Number of unique reads obtained in ChIP-seq experiments and their alignment to the *Arabidopsis* genome. **(B)** Number of peaks identified in the *Arabidopsis* genome for the two ChIP-seq libraries. The number of (protein-coding) genes, pseudogenes and hypothetical ORFs associated with the peaks are shown. **(C)** A Venn diagram of the overlap in genes associated with a binding peak in the AP1-K27-0d and AP1-K27-2d experiments. The number of genes was compared in this calculation (<http://bioinformatics.psb.ugent.be>).

5.3.1.3 H3K27me3 distribution in the *Arabidopsis* genome

In order to identify genomic regions associated with the H3K27me3 mark, enrichment values were examined. To this end, the *IGV* genome viewer (www.software.broadinstitute.org) was used to display genomic regions identified by the *MACS* peak calling software as enriched over background. As expected, strong enrichment was detected for example in the *SEP3* genomic region (Fig. 5.9). H3K27me3 mark covered the gene body as well as the promoter and distal enhancer

regions at 0 and 2 d after dexamethasone treatment (green track). In contrast, in the control sample (in this case the AP1-K27-INPUT-0/2) (in red), this enrichment was undetectable, indicating that the signal in the samples from the ChIP assay were specific. Furthermore, the observed enrichment at the 0 d time-point (fold-enrichment: 13.31) was higher than at the 2 d time-point (fold-enrichment: 10.22), in agreement with the known reduction of H3K27me3 marks at the *SEP3* locus upon floral induction (see Figure 5.6). This positive control suggests that the analysis was performed correctly and that the data obtained may accurately describe the genome-wide H3K27me3 distribution during early flower development.

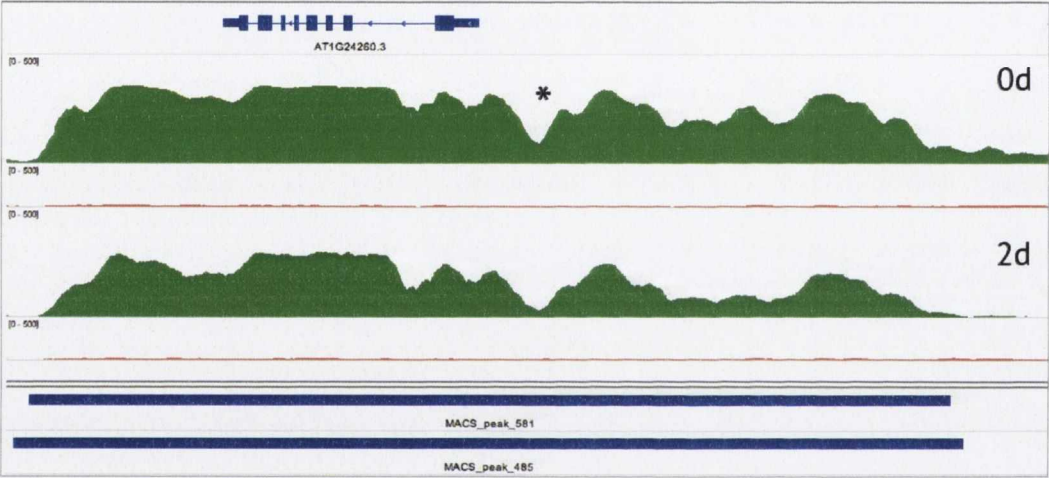


Figure 5.9 Distribution of H3K27me3 at the *SEP3* locus. The entire genomic region surrounding *SEP3* is covered with the repressive mark. However, some parts of the promoter show lower coverage (indicated by an asterisk). Peaks identified in the AP1-K27-0d and AP1-K27-2d libraries (as indicated) are marked in green. Gene models are shown at the top of each graph (in blue). The rectangular blue boxes at the bottom track represent peaks called by the *MACS* software. The red tracks represent the results for the control library (AP1-INPUT-1). The peaks were visualized using the *IGV* genome viewer.

In a next step, the genome-wide binding data were analyzed for other regions known to be covered with the H3K27me3 repressive mark. These included the *KNU* locus. It has been shown that H3K27me3 marks are enriched specifically around the transcription start site and the gene body of *KNU* (Sun et al., 2009). This study investigated the interaction between *KNU*, *AG* and *WUS* in floral stem cell maintenance. As described in Section 3.1.6, *AG* represses *WUS* weakly after stage 3 of flower development. *KNU*

then terminates the floral stem cells by repressing *WUS* expression at stage 6 of flower development (Payne et al., 2004; Sun et al., 2009). PcG-mediated H3K27me3 covers the *KNU* locus while AG is involved in the removal of the repressive mark. The level of the H3K27me3 mark at the *KNU* locus decreases during the 2 days that it takes to induce its expression. This might indicate the relationship between the transcriptional activation of *KNU* by AG and the possible removal of the H3K27me3 mark from *KNU* locus in the AG-dependent manner (Sun et al., 2009).

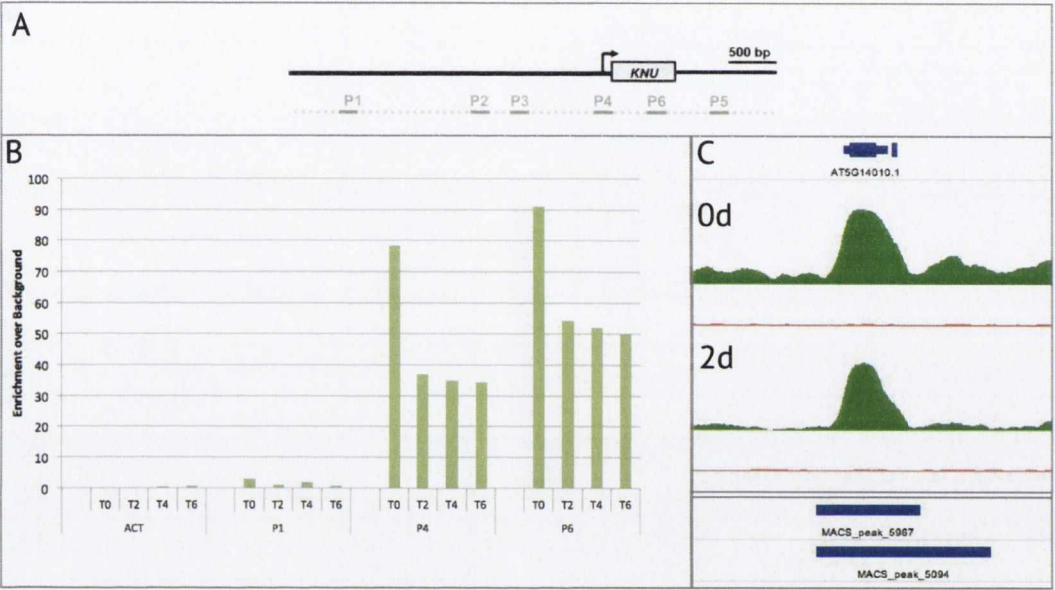


Figure 5.10 Distribution of H3K27me3 marks at the *KNU* locus. (A) Amplicons used for the ChIP-qPCR assays shown in (B). Image from (Sun et al., 2009). (B) ChIP-qPCR was performed using tissue from AP1_{pro}:AP1-GR *apl-1 cal-1* plants at different time-points (0, 2, 4, and 6 d) after activation of AP1-GR. The transcription start site (P4) and the gene body (P6) of *KNU* contained higher levels of the repressive mark, which were reduced 2 days after dexamethasone treatment. (C) Genomic region covered with repressive mark around *KNU* locus. Peaks identified in the AP1-K27-0d and AP1-K27-2d libraries (as indicated) are marked in green. Gene models are shown at the top of each graph (in blue). The rectangular blue boxes at the bottom track represent peaks called by the MACS software. The red tracks represent the results for the control library (AP1-INPUT-1). The peaks were visualized using the *IGV* genome viewer.

To analyze where and when the repressive mark is present, Sun et al. (2009) examined different regions in the promoter and gene body of *KNU* (Fig. 5.10 A). Using the sets of

primers described in this paper, I determined the levels of methylation by ChIP-qPCR in the samples used for next-generation sequencing. The region P1 did not yield any enrichment in agreement with what Sun et al. had described. In contrast, two regions (P4, P6) with reported H3K27me3 enrichment showed high enrichment values in the experiment (Fig. 5.10 B). The level in both cases was the highest at the 0 d time-point and then dropped to around 50% at 2 d after induction of flowering, suggesting the removal of the repressive H3K27me3 mark. The levels of enrichment were the same in the later stages of development (4 d) or decreased slightly at 6 d (Fig. 5.10 B). The ChIP-seq experiment confirmed the reduction in level of H3K27me3 mark at 2 d relative to the 0 d time-point (Fig. 5.10 C). A peak was visible only in the transcribed region of the *KNU* locus, whereas the promoter region was not covered by the repressive mark. These results confirm those by Sun et al. (2009) and indicate the validity of the data obtained.

It has been shown that AP1 and LFY act in a positive feedback loop (Kaufmann et al., 2010). Furthermore, LFY together with WUS activate *AG* at stage 3 of flower development (Lohmann et al., 2001). This indirect activation of *AG* through LFY could lead to the removal of the repressive mark from the *AG* locus after AP1 activation. Analysis of the ChIP-seq data I obtained revealed that the transcribed region of the *AG* locus and the promoter is covered with the repressive mark at the 0 d time-point (fold-enrichment: 16.15) and that enrichment levels decrease slightly at 2 d after induction of flowering (fold-enrichment: 15.15) (Fig. 5.11 A).

It was of interest to analyze other regions in the genome that are regulated by AP1 directly and may be associated with the H3K27me3 mark. One candidate gene that is directly activated by AP1 is *LFY* (Liljegren et al., 1999). It has been shown that AP1 binds to regulatory elements in the *LFY* promoter and activates its expression (Kaufmann et al., 2010). As described above, these two transcription regulators act directly on each other, activating each others expression.

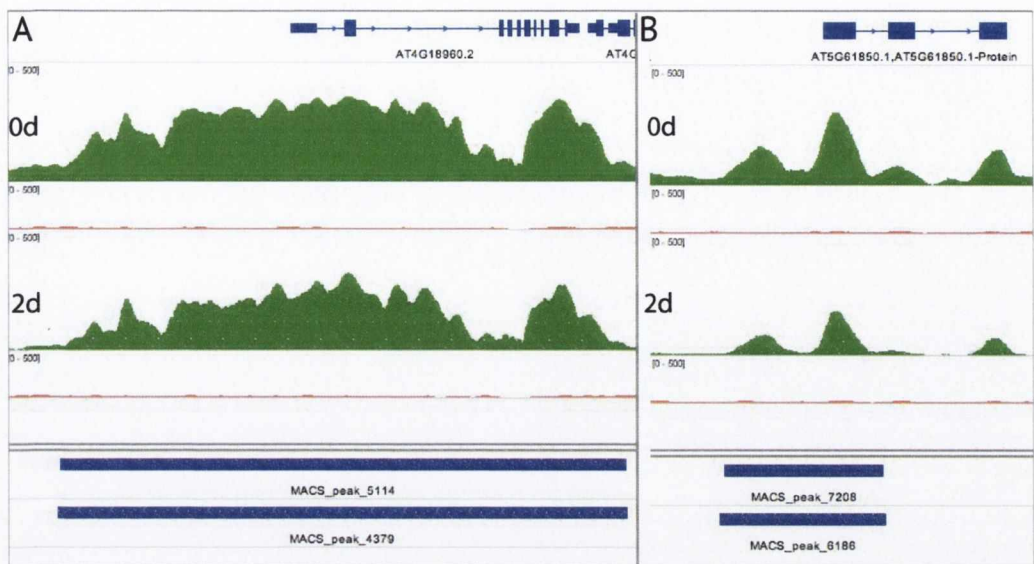


Figure 5.11 Distribution of H3K27me3 marks at the *AG* and *LFY* loci. (A) Distribution of the repressive mark around the *AG* locus. **(B)** Distribution of the repressive mark around the *LFY* locus. Peaks identified in the AP1-K27-0d and AP1-K27-2d libraries (as indicated) are marked in green. Gene models are shown at the top of each graph (in blue). The rectangular blue boxes at the bottom track represent peaks called by the *MACS* software. The red tracks represent the results for the control library (AP1-INPUT-1). The peaks were visualized using the *IGV* genome viewer.

Is the AP1-dependent up-regulation of *LFY* during floral initiation associated with a removal of the H3K27me3 repressive mark? The ChIP-seq data I obtained suggest that this may indeed be the case. The promoter and transcribed region of *LFY* at 0 d was covered with the H3K27me3 mark (fold-enrichment: 11.89), while at the 2 d time-point after AP1 activation, the level of the enrichment had substantially decreased (fold-enrichment: 7.21) (Fig. 5.11 B).

Taken together, the genome-wide analysis of H3K27me3 marks confirmed existing data on the Polycomb-mediated repression of key floral regulators genes. In this case, only the genes that were activated at 2 d after flowering were discussed. Further analysis will include the genes that are repressed by AP1 after the induction of flowering.

5.3.2 Genome-wide distribution of activating H3K4me3 marks at early stages of flower development

In order to determine the genome-wide distribution of H3K4me3 marks at early stages of flower development, inflorescence tissue of FIS plants was collected 0 and 2 d after activation of the AP1-GR fusion protein. The experimental set-up for the subsequent ChIP-seq analysis was similar to that used for the H3K27me3 mark described above. Briefly, ~500 µL of inflorescence tissue was used as starting material for ChIP analysis (see Section 2.12) with an H3K4me3-specific antiserum (see Section 2.12.3). The genomic DNA obtained was subjected to library preparation as described below.

5.3.2.1 Generation of ChIP-seq libraries from chromatin associated with H3K4me3

To identify the genomic regions that are associated with the activating H3K4me3 mark, the precipitated chromatin from the 0 and 2 d time-points (referred to as AP1-K4-0d and AP1-K4-2d) was used to generate ChIP-seq libraries as described in Section 2.12.6. The average fragment size was 379 bp and 299 bp for the AP1-K4-0d and AP1-K4-2d libraries, respectively (Fig. 5.12 B, C). Aliquots of these libraries were subjected to qPCR analysis for selected control regions (Fig. 5.12 A). Regions selected for closer examination are associated with genes known to be directly regulated by AP1. These contained several flowering time genes, such as *SHORT VEGETATIVE PHASE (SVP)*, *AGAMOUS-LIKE24 (AGL24)* and *FLOWERING LOCUS D (FD)* (Kaufmann et al., 2010; Liu et al., 2007), as well as *LFY*.

Input DNA from each ChIP was used to normalize the data, as described above. For both libraries the same positive regions were tested. The highest enrichment was detected in *SVP* region at 0 d time-point, whereas the level of the activating H3K4me3 mark decrease 2 d after AP1-GR activation. The enrichment for *LFY*, which is activated in emerging floral primordia by AP1, was the lowest at the 0 d time-point, however, at 2 d after AP1-GR activation, a 15-fold enrichment over background was observed. Although this enrichment was not as high as observed for the repressive H3K27me3 mark (see Section 5.3.1.1), it was deemed sufficient to be sent for ultra high-throughput sequencing.

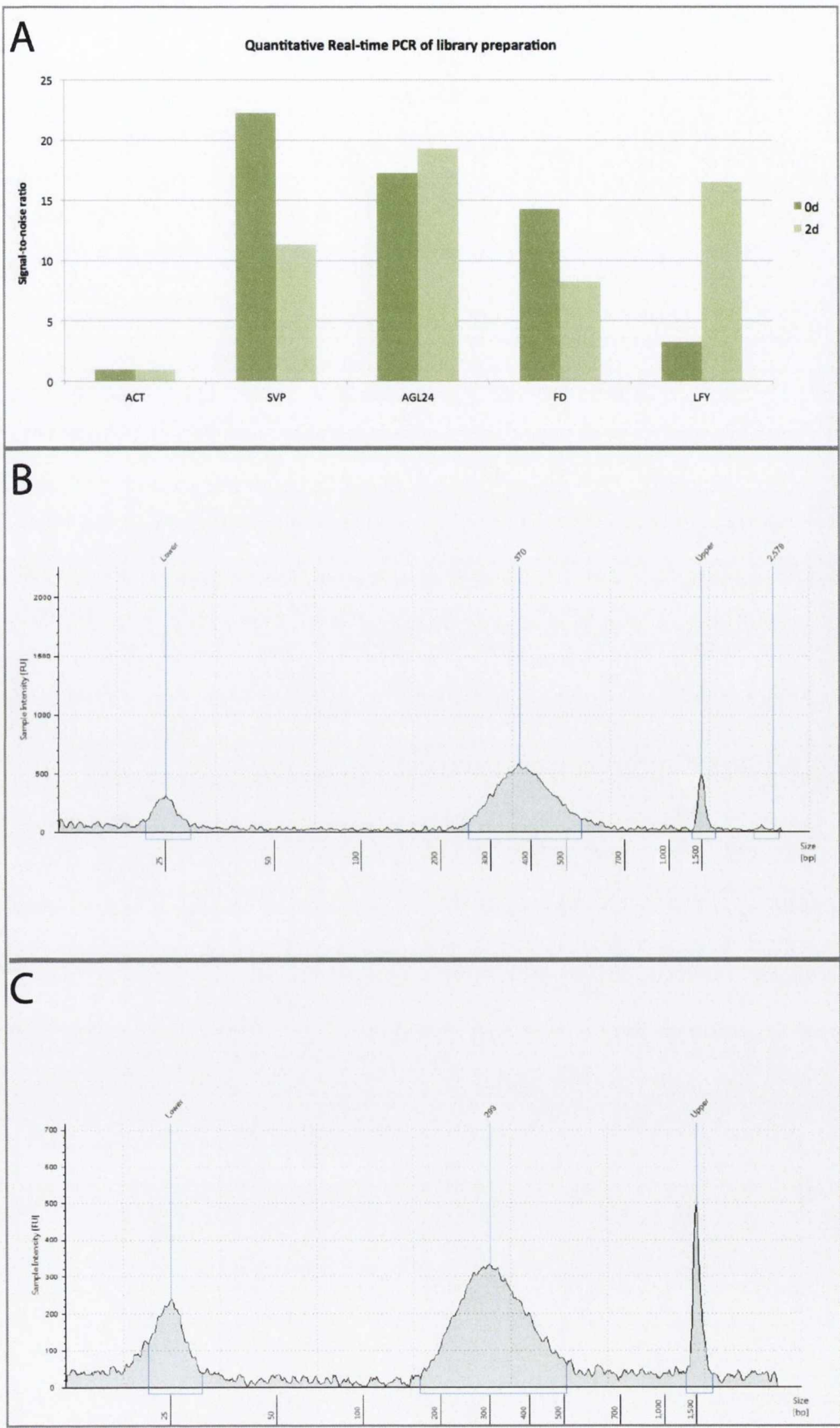


Figure 5.12 Preparation of ChIP-seq libraries for the analysis of the genome-wide distribution of H3K4me3. (A) qPCR was performed for selected loci (as indicated) using DNA from the 0-d and 2-d ChIP-seq libraries. The signal-to-noise ratio was calculated as outlined in Section 2.9. (B, C) DNA size distribution (analyzed with an Agilent Tape-Station) of the 0-d (B) and 2-d (C) libraries used for ultra high-throughput sequencing. Primers pairs KK-65/KK-66, KK-268/KK-269, KK-266/KK-267, KK-270/KK-271, and KK-272/KK-273 were used to amplify *ACT*, *SVP*, *AGL24*, *FD* and *LFY*, respectively.

5.3.2.2 Quality assessment of data from the H3K4me3 ChIP-seq experiment

The libraries generated from genomic DNA isolated by ChIP and control libraries derived from chromatin that was not subjected to immunoprecipitation (denoted AP1-K27-INPUT-0 and AP1-K27-INPUT-2 hereafter) were analyzed by Illumina next-generation sequencing. Dr. Beibei Zheng provided assistance with the analysis of the data obtained from Illumina sequencing. In brief, the data produced by the Illumina sequencing pipeline included the sequence string output and a quality score. The quality score reflects the probability that a given base call at a given position is correct. These quality scores and sequence strings were uploaded to the online quality control tool, *FastQC* (www.bioinformatics.bbsrc.ac.uk). The distribution of per-base quality reads was illustrated in a box plot, where the green blocks indicate good quality calls (Fig. 5.13 A; the results for the AP1-K4-2d library are shown as an example). The *FastQC* software analyses various other parameters of the sequencing run including the number of duplicated sequences. A high level of duplication indicates that there is some enrichment bias present (Fig. 5.13 B). The AP1-K4-2d library exhibited a 76% duplication level, which is a substantial amount of multiple reads.

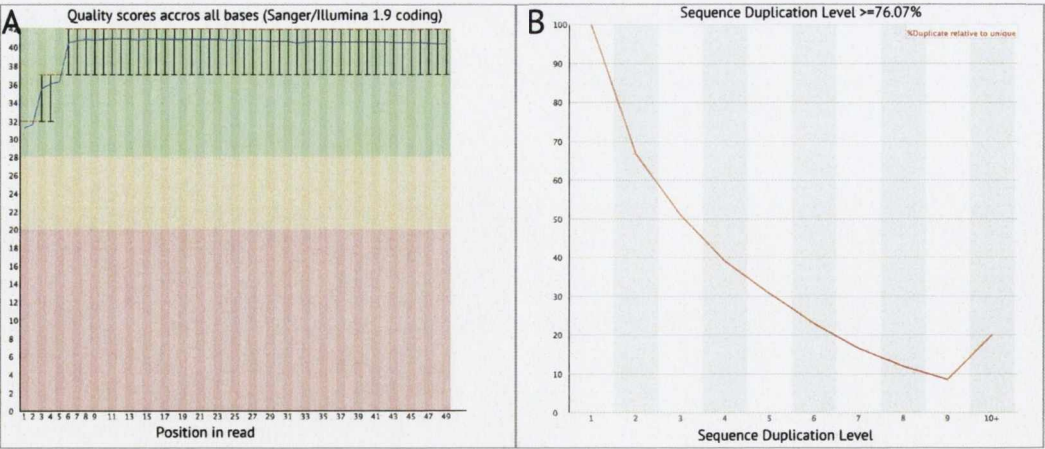


Figure 5.13 Results of quality controls for the H3K4me3 ChIP-seq experiment. (A) Quality scores at a given position within the sequence of all reads from the AP1-K4-2d ChIP-seq experiment, as determined by the FastQC software. The box-plots show the distribution of per-base qualities for all reads in the AP1-K4-2d library. The average quality score is high, indicating a good quality dataset. (B) Duplication levels of the reads from the AP1-K4-2d library. The number of single reads is represented as 100% and duplicated sequences are represented as a fraction of this. A duplication level of 10 is equivalent to the percentage of sequences that were present 10 or more times. The sequence duplication level reached ~76%.

Next, the *HOMER* (<http://homer.ucsd.edu/>) software was used to align the unique short read sequences obtained to the *Arabidopsis* genome (TAIR release 10; www.arabidopsis.org). For the AP1-K4-0d and AP1-K4-2d libraries, ~55 million and ~38 million unique reads were obtained and ~94% and ~78% of these reads aligned to the *Arabidopsis* genome, respectively (Fig. 5.14 A). Although the percentage of reads aligned for the AP1-K4-2d library was much lower than for the 0 d library, the range above 70% uniquely mapped reads is normal (Bailey et al., 2013). The total number of regions associated with the H3K4me3 mark for library AP1-K4-0d reached 14,757, including pseudogenes (1% of all peaks) and hypothetical protein (15% of peaks) (Fig. 5.14 B). The number of protein-coding genes identified with significant enrichment was 12,245. For the AP1-K4-2d library, the percentages of pseudogenes and hypothetical proteins remained at the same level with 1% and 15%, respectively, from the total number of 14,969 peaks. Using the *MACS* peak calling software outlined in Section 2.14.5, 12,358 unique elements were defined as being enriched above background. The overlap between both time-points came to 11,966 of unique regions. Both datasets were considered of suitable quality and sufficient coverage for further analysis.

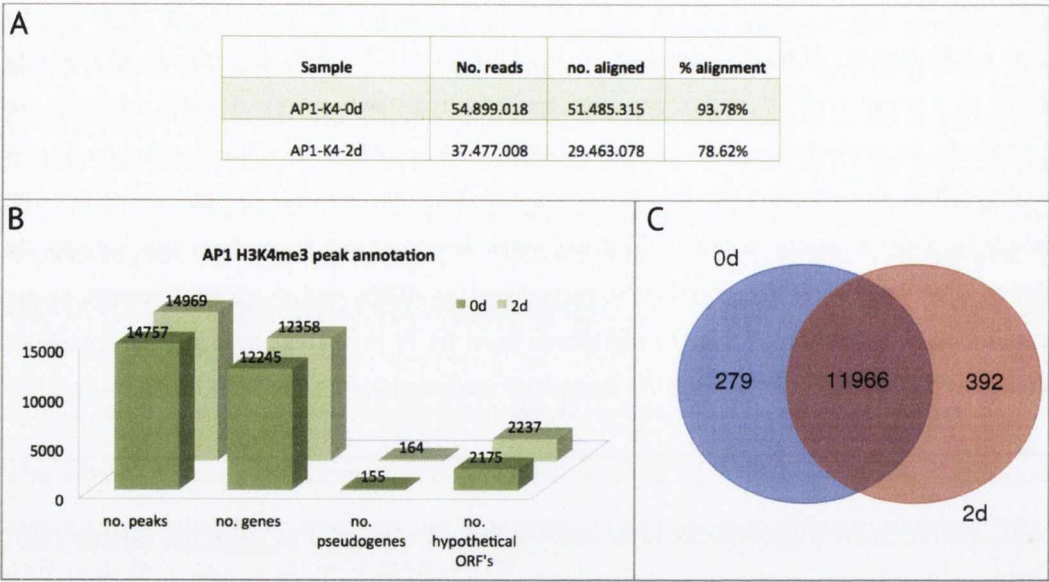


Figure 5.14 Analysis of data from the H3K4me3 ChIP-seq experiment. (A) Number of unique reads obtained from two ChIP-seq experiments and their alignment to the *Arabidopsis* genome. **(B)** Number of peaks identified in the *Arabidopsis* genome for the two ChIP-seq libraries. The number of (protein-coding) genes, pseudogenes and hypothetical ORFs associated with the peaks are shown. **(C)** A Venn diagram of the overlap in genes associated with a binding peak in the AP1-K4-0d and AP1-K4-2d experiments. The number of genes, not peaks, was compared in this case (<http://bioinformatics.psb.ugent.be>).

5.3.2.3 H3K4me3 distribution in the *Arabidopsis* genome

In order to identify genomic fragments associated with the H3K4me3 mark, the enrichments of reads that were aligned to the binding sites were investigated. The *IGV* viewer (www.software.broadinstitute.org) was used to display genomic regions found to be enriched for the H3K4me3 mark. Genomic regions associated with the activating mark were enriched at different levels to what was indicated by the peak score. As predicted, peaks were detected in the regions of genes that were found to be enriched in the ChIP-qPCR assays (Fig. 5.12 A). As mentioned earlier, the level of enrichment for *SVP* was the highest at the 0 d time-point. The fold enrichment established by the *MACS* peak calling method reached 11.63 and declined to 9.27, 2 d after the induction of flowering. The decrease in the levels of the activation was visualized in the *IGV* genome viewer (Fig. 5.15 A). The detected peak is only present in the first exon, spanning the first intron.

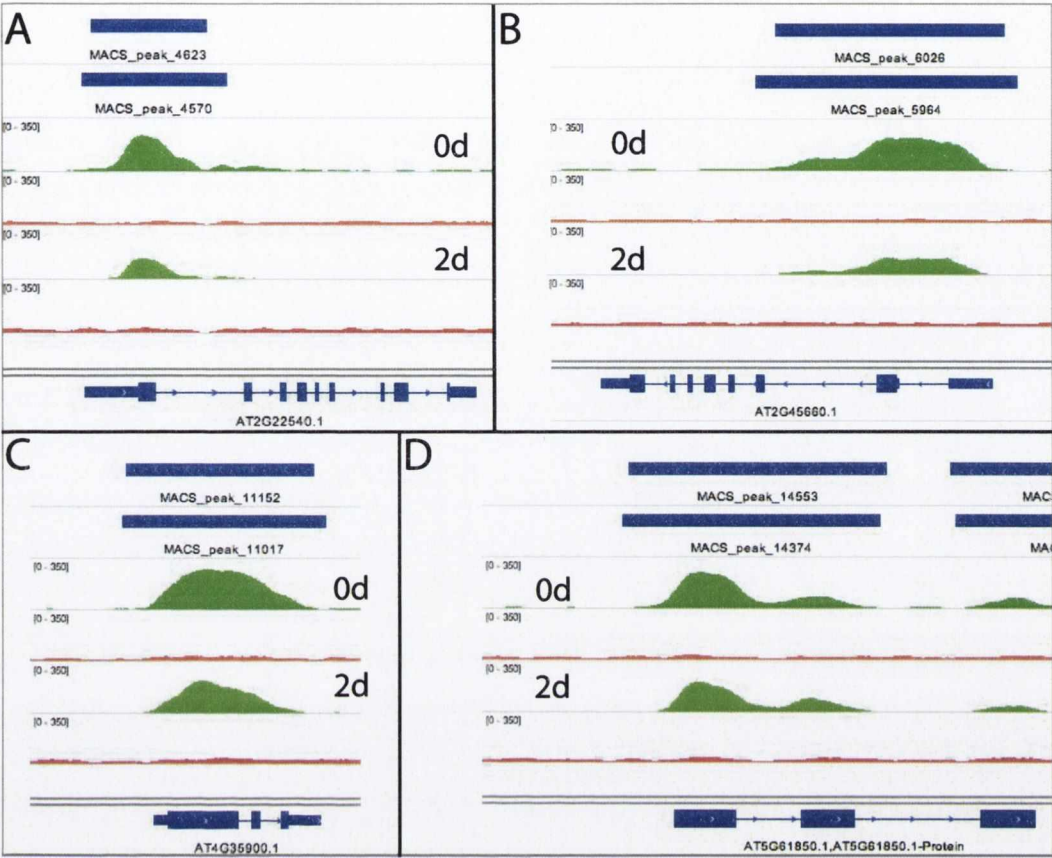


Figure 5.15 Genomic regions associated with H3K4me3 mark. Distribution of H3K4me3 marks at the *SVP* (A); *AGL24* (B); *FD* (C) and *LFY* (D) loci. Distribution of the activating mark around the *LFY* locus. Peaks identified in the AP1-K4-0d and AP1-K4-2d libraries (as indicated) are marked in green. Gene models are shown at the bottom of each graph (in blue). The rectangular blue boxes at the top track represent peaks called by the MACS software. The red tracks represent the results for the control library (AP1-INPUT-1). The peaks were visualized using the *IGV* genome viewer.

In case of *AGL24*, which is also directly repressed in floral primordia by AP1 (Liu et al., 2007), the fold-enrichment level at the 0-d time-point was 9.71 and increased to over 13-fold 2 d after the dexamethasone treatment. This increase in enrichment was also detected by ChIP-qPCR assays (Fig. 5.12 A). Another gene that is repressed by AP1 during early stages of flower development is *FD* (Wigge et al., 2005). The enrichment detected by ChIP-qPCR at the 0-d time-point was about 15-fold and decreased to 7, 2 d after AP1-GR activation (Fig. 5.12 A). The peak calling method confirmed this decline in the levels of the activation mark, where fold-enrichment from

11.58 dropped to 10.42. Furthermore, the *IGV* genome viewer detected a high peak in the gene body at 0 d time-point, and lower peak at 2-d time-point (Fig. 5.15 C). The last of the analyzed genes is directly activated by the AP1 transcription factor. The level of enrichment detected with the qPCR (Fig. 5.12 A) was low at 0 d, whereas it increased dramatically 2 d after AP1-GR activation. However, in this case, the *IGV* viewer did not showed the increase in the level of the activation mark at the *LFY* locus at 2 d (Fig. 5.15 D).

Because of time constraints, I was not able to analyze the data in more detail. However, the preliminary data obtained in this study suggests that the generated dataset is of a sufficient quality and can be used for further investigation of the genes associated with the H3K4me3 activation mark.

5.4 Discussion and future work

The preliminary data generated in this study confirm previous reports on the epigenetic silencing and activation of genes during early flower development (Smaczniak et al., 2012; Sun et al., 2014; Zhang et al., 2009). Although, the datasets from the experiments were not analyzed in much detail, the quality of the data was deemed sufficient and found to reproduce results previously described for several key floral regulators. Thus, further analysis of the data obtained should yield interesting insights into the interactions between the AP1 master regulator and changes in the floral epigenome.

The analysis of the epigenetic state of the *Arabidopsis* genome at early stages of flower development showed that almost 17% of the genes are covered with the repressive H3K27me3 mark. On the other hand, more then 40% of transcribed regions were covered with the activating H3K4me3 mark. The analysis confirmed the removal of the H3K27me3 repressive mark from the *SEP3* locus 2 d after AP1-GR activation (see Section 5.3.1). Furthermore, the studies by (Sun et al., 2014) showing that *KNU* activation is associated with the removal of the repressive mark, probably via AG activity, was also confirmed (see Section 5.3.1.3). Moreover, the analysis of the *AG* locus showed that 2 d after the onset of flower formation, the level of the repressive mark is reduced, likely indicating the beginning of *AG* expression. Additionally, the level of the same mark declined at the *LFY* locus, which may indicate that this gene is activated in the same manner as *SEP3*, namely by removing the repressive H3K27me3 mark. The *LFY* locus is also associated with the H3K4me3 activation mark (see section

above). The level of enrichment increases 2 d after AP1-GR activation, indicating the interplay of the two marks on the *LFY* locus. Genes repressed by AP1 (*SVP*, *AGL24* or *FD*) showed a high enrichment at the 0 d time-point indicating that the activation mark was still surrounding the loci of the transcribed regions of those genes (see section above). Two days after the induction of flowering the level of the activation mark decreased in case of *SVP* and *FD*. It was unclear, however, why the level of the activating mark was increased for *AGL24* at the 2-d time-point. This would suggest an activation of the gene during early stages of flower development, which is not in agreement with previous findings (Liu et al., 2007).

The examined epigenetic marks are known to play a role in flower development and act antagonistically in the control of gene expression. However, it has been reported that specific genomic regions are covered with both repressive and activating marks (Ha et al., 2011). The number of genes shared between the two different time-points was >1,000 (Fig. 5.16 A). Taking into account the nature of the occurrence of these marks this is considered a high number. Further analysis will aim at detecting those genomic regions and analyzing their relationship with the AP1 transcription factor and other regulators that play roles in the induction of flower formation.

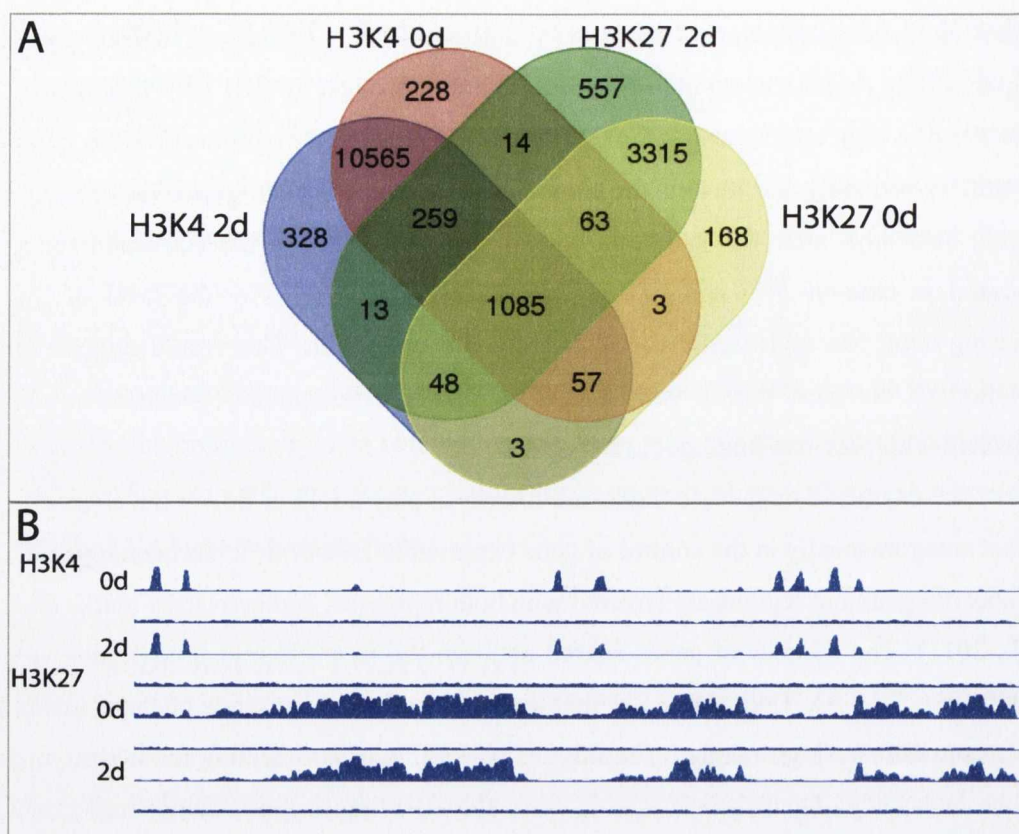


Figure 5.16 The antagonistic character of H3K27me3 and H3K4me3 marks. (A) A Venn diagram depicting the overlap in genes associated with peaks identified in the four ChIP-seq libraries, AP1-K27-0d (H3K27 0d), AP1-K27-2d (H3K27 2d), AP1-K4-0d (H3K4 0d) and AP1-K4-2d (H3K4 2d). **(B)** Distribution of H3K4me3 (top tracks) and H3K27me3 (bottom tracks) marks at randomly selected genomic region. The activating mark is enriched in transcribed regions whereas the repressive mark covers long regions of promoters and gene bodies.

The two marks are enriched at different regions of the genome, acting in antagonistic manner. As described above, the H3K4me3 mark is enriched at the 5' of the transcribed regions, creating sharp peaks of enrichment along the *Arabidopsis* genome (Fig. 5.16 B, top two tracks). On the contrary, the H3K27me3 mark covers large regions of the promoter and gene body regions (Fig. 5.16 B, bottom tracks). The results presented above suggest that the experiment works properly as the correct genomic regions were identified, covered with the specific methylation mark.

Additional experiments are planned in the future to further characterize the importance of epigenetic control mechanisms during early stages of flower development. The H3K9me3 repressive mark and AcH3K9 activation mark will be studied and data compared with the genome-wide analysis obtained in this study. The advanced study could also include the comparison of the data obtained with the published genome-wide transcriptomics studies (Ryan et al., 2015). The observation made based on the analogy of those two datasets could answer the questions regarding the relationship between the genes activated or repressed by AP1 and the epigenomic changes that occur at these genomic regions. Furthermore, the genome-wide transcriptomic studies of LFY generated recently in the laboratory could also be included in the analysis. The study by (Goslin et al., 2017) included monitoring LFY activity in the absence of functional AP1/CAL. The regulation of several known LFY target genes is unaffected by AP1 perturbation, while others appear to require AP1 activity. Furthermore, they obtained evidence that LFY and AP1 control the expression of some genes in an antagonistic manner.

Additionally, it would be of interest to study the epigenome in a line that allows the activation of both LFY and AP1 at the same time or in the line with the over-expression of one of them. The ChIP-seq data that have been generated by Dr. Beibei Zheng in the laboratory included the analysis of regions that are bound by LFY in the presence or absence of AP1 master regulator. Further analysis could also include these datasets and the comparison would give insights into the interaction between the AP1 and LFY and the genes that act downstream of both factors, including the changes of the epigenome at those genomic regions.

REFERENCES

- Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., Ichinoki, H., Notaguchi, M., Goto, K., and Araki, T. (2005). FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *Science* 309, 1052-1056.
- Alvarez-Venegas, R., and Avramova, Z. (2005). Methylation patterns of histone H3 Lys 4, Lys 9 and Lys 27 in transcriptionally active and inactive Arabidopsis genes and in *atx1* mutants. *Nucleic Acids Res* 33, 5199-5207.
- Alvarez-Venegas, R., Pien, S., Sadler, M., Witmer, X., Grossniklaus, U., and Avramova, Z. (2003). ATX-1, an Arabidopsis homolog of trithorax, activates flower homeotic genes. *Current biology : CB* 13, 627-637.
- Amasino, R. (2010). Seasonal and developmental timing of flowering. *Plant J* 61, 1001-1013.
- Bailey, T., Krajewski, P., Ladunga, I., Lefebvre, C., Li, Q., Liu, T., Madrigal, P., Taslim, C., and Zhang, J. (2013). Practical guidelines for the comprehensive analysis of ChIP-seq data. *PLoS Comput Biol* 9, e1003326.
- Bartrina, I., Otto, E., Strnad, M., Werner, T., and Schmulling, T. (2011). Cytokinin regulates the activity of reproductive meristems, flower organ size, ovule formation, and thus seed yield in *Arabidopsis thaliana*. *The Plant cell* 23, 69-80.
- Baum, and Hileman (2006). A developmental genetic model for the origin of the flower. In *Flowering and its Manipulation*, C. Ainsworth, ed. (UK), pp. 3-27.
- Baumbusch, L.O., Thorstensen, T., Krauss, V., Fischer, A., Naumann, K., Assalkhou, R., Schulz, I., Reuter, G., and Aalen, R.B. (2001). The *Arabidopsis thaliana* genome contains at least 29 active genes encoding SET domain proteins that can be assigned to four evolutionarily conserved classes. *Nucleic Acids Res* 29, 4319-4333.
- Becker, A., Kaufmann, K., Freialdenhoven, A., Vincent, C., Li, M.A., Saedler, H., and Theissen, G. (2002). A novel MADS-box gene subfamily with a sister-group relationship to class B floral homeotic genes. *Mol Genet Genomics* 266, 942-950.
- Benjamini, Y., and Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society* 57, 289-300.
- Berger, S.L. (2007). The complex language of chromatin regulation during transcription. *Nature* 447, 407-412.
- Bernhardt, C., Zhao, M., Gonzalez, A., Lloyd, A., and Schiefelbein, J. (2005). The bHLH genes GL3 and EGL3 participate in an intercellular regulatory circuit that controls cell patterning in the *Arabidopsis* root epidermis. *Development* 132, 291-298.

Berr, A., McCallum, E.J., Menard, R., Meyer, D., Fuchs, J., Dong, A., and Shen, W.H. (2010). Arabidopsis SET DOMAIN GROUP2 is required for H3K4 trimethylation and is crucial for both sporophyte and gametophyte development. *The Plant cell* 22, 3232-3248.

Berr, A., Xu, L., Gao, J., Cognat, V., Steinmetz, A., Dong, A., and Shen, W.H. (2009). SET DOMAIN GROUP25 encodes a histone methyltransferase and is involved in FLOWERING LOCUS C activation and repression of flowering. *Plant Physiol* 151, 1476-1485.

Bertrand, C., Bergounioux, C., Domenichini, S., Delarue, M., and Zhou, D.X. (2003). Arabidopsis histone acetyltransferase AtGCN5 regulates the floral meristem activity through the WUSCHEL/AGAMOUS pathway. *J Biol Chem* 278, 28246-28251.

Blazquez, M.A., Green, R., Nilsson, O., Sussman, M.R., and Weigel, D. (1998). Gibberellins promote flowering of arabidopsis by activating the LEAFY promoter. *The Plant cell* 10, 791-800.

Bleckmann, A., Weidtkamp-Peters, S., Seidel, C.A., and Simon, R. (2010). Stem cell signaling in Arabidopsis requires CRN to localize CLV2 to the plasma membrane. *Plant Physiol* 152, 166-176.

Bock, K.W., Honys, D., Ward, J.M., Padmanaban, S., Nawrocki, E.P., Hirschi, K.D., Twell, D., and Sze, H. (2006). Integrating membrane transport with male gametophyte development and function through transcriptomics. *Plant Physiol* 140, 1151-1168.

Bowman, Alvarez, Weigel, Meyerowitz, E.M., and Smith (1993). Control of flower development in Arabidopsis thaliana by APETALA1 and interacting genes. *Development* 119, 721-743.

Bowman, J.L., Baum, S.F., Eshed, Y., Putterill, J., and Alvarez, J. (1999). Molecular genetics of gynoecium development in Arabidopsis. *Curr Top Dev Biol* 45, 155-205.

Bowman, J.L., Drews, G.N., and Meyerowitz, E.M. (1991). Expression of the Arabidopsis floral homeotic gene AGAMOUS is restricted to specific cell types late in flower development. *The Plant cell* 3, 749-758.

Bowman, J.L., Sakai, H., Jack, T., Weigel, D., Mayer, U., and Meyerowitz, E.M. (1992). SUPERMAN, a regulator of floral homeotic genes in Arabidopsis. *Development* 114, 599-615.

Bowman, J.L., and Smyth, D.R. (1999). CRABS CLAW, a gene that regulates carpel and nectary development in Arabidopsis, encodes a novel protein with zinc finger and helix-loop-helix domains. *Development* 126, 2387-2396.

Bowman, J.L., Smyth, D.R., and Meyerowitz, E.M. (1989). Genes directing flower development in Arabidopsis. *The Plant cell* 1, 37-52.

Brand, U., Fletcher, J.C., Hobe, M., Meyerowitz, E.M., and Simon, R. (2000). Dependence of stem cell fate in Arabidopsis on a feedback loop regulated by CLV3 activity. *Science* 289, 617-619.

Brand, U., Grunewald, M., Hobe, M., and Simon, R. (2002). Regulation of CLV3 expression by two homeobox genes in Arabidopsis. *Plant Physiol* 129, 565-575.

Buzas, D.M., Tamada, Y., and Kurata, T. (2012). FLC: a hidden polycomb response element shows up in silence. *Plant Cell Physiol* 53, 785-793.

Calonje, M., Sanchez, R., Chen, L., and Sung, Z.R. (2008). EMBRYONIC FLOWER1 participates in polycomb group-mediated AG gene silencing in Arabidopsis. *The Plant cell* 20, 277-291.

Cao, X., He, Z., Guo, L., and Liu, X. (2015). Epigenetic Mechanisms Are Critical for the Regulation of WUSCHEL Expression in Floral Meristems. *Plant Physiol* 168, 1189-1196.

Carles, C.C., and Fletcher, J.C. (2009). The SAND domain protein ULTRAPETALA1 acts as a trithorax group factor to regulate cell fate in plants. *Genes Dev* 23, 2723-2728.

Carles, C.C., Lertpiriyapong, K., Reville, K., and Fletcher, J.C. (2004). The ULTRAPETALA1 gene functions early in Arabidopsis development to restrict shoot apical meristem activity and acts through WUSCHEL to regulate floral meristem determinacy. *Genetics* 167, 1893-1903.

Cartagena, J.A., Matsunaga, S., Seki, M., Kurihara, D., Yokoyama, M., Shinozaki, K., Fujimoto, S., Azumi, Y., Uchiyama, S., and Fukui, K. (2008). The Arabidopsis SDG4 contributes to the regulation of pollen tube growth by methylation of histone H3 lysines 4 and 36 in mature pollen. *Dev Biol* 315, 355-368.

Casamitjana-Martinez, E., Hofhuis, H.F., Xu, J., Liu, C.M., Heidstra, R., and Scheres, B. (2003). Root-specific CLE19 overexpression and the *sol1/2* suppressors implicate a CLV-like pathway in the control of Arabidopsis root meristem maintenance. *Current biology : CB* 13, 1435-1441.

Causier, B., Schwarz-Sommer, Z., and Davies, B. (2010). Floral organ identity: 20 years of ABCs. *Semin Cell Dev Biol* 21, 73-79.

Cazzonelli, C.I., Cuttriss, A.J., Cossetto, S.B., Pye, W., Crisp, P., Whelan, J., Finnegan, E.J., Turnbull, C., and Pogson, B.J. (2009). Regulation of carotenoid composition and shoot branching in Arabidopsis by a chromatin modifying histone methyltransferase, SDG8. *The Plant cell* 21, 39-53.

Chanvivattana, Y., Bishopp, A., Schubert, D., Stock, C., Moon, Y.H., Sung, Z.R., and Goodrich, J. (2004). Interaction of Polycomb-group proteins controlling flowering in Arabidopsis. *Development* 131, 5263-5276.

Chaudhury, A.M. (1993). Nuclear Genes Controlling Male Fertility. *The Plant cell* 5, 1277-1283.

- Chaudhury, A.M., Ming, L., Miller, C., Craig, S., Dennis, E.S., and Peacock, W.J. (1997). Fertilization-independent seed development in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* *94*, 4223-4228.
- Chen, X. (2004). A microRNA as a translational repressor of *APETALA2* in *Arabidopsis* flower development. *Science* *303*, 2022-2025.
- Chen, X. (2005). MicroRNA biogenesis and function in plants. *FEBS Lett* *579*, 5923-5931.
- Chen, Z.J., and Tian, L. (2007). Roles of dynamic and reversible histone acetylation in plant development and polyploidy. *Biochim Biophys Acta* *1769*, 295-307.
- Clark et al., R.M., Meyerowitz EM (1995). *CLAVATA3* is a specific regulator of shoot and floral meristem development affecting the same processes as *CLAVATA1*. *Development* *121*, 2057-2067.
- Clark, S.E., Running, M.P., and Meyerowitz, E.M. (1993). *CLAVATA1*, a regulator of meristem and flower development in *Arabidopsis*. *Development* *119*, 397-418.
- Clark, S.E., Williams, R.W., and Meyerowitz, E.M. (1997). The *CLAVATA1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. *Cell* *89*, 575-585.
- Coen, E.S., and Meyerowitz, E.M. (1991). The war of the whorls: genetic interactions controlling flower development. *Nature* *353*, 31-37.
- Colombo, L., Franken, J., Koetje, E., van Went, J., Dons, H.J., Angenent, G.C., and van Tunen, A.J. (1995). The petunia MADS box gene *FBP11* determines ovule identity. *The Plant cell* *7*, 1859-1868.
- Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., and Scheible, W.R. (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiol* *139*, 5-17.
- Das, P., Ito, T., Wellmer, F., Vernoux, T., Dedieu, A., Traas, J., and Meyerowitz, E.M. (2009). Floral stem cell termination involves the direct regulation of *AGAMOUS* by *PERIANTHIA*. *Development* *136*, 1605-1611.
- De Lucia, F., Crevillen, P., Jones, A.M., Greb, T., and Dean, C. (2008). A PHD-polycomb repressive complex 2 triggers the epigenetic silencing of *FLC* during vernalization. *Proceedings of the National Academy of Sciences of the United States of America* *105*, 16831-16836.
- Dennis, E.S., and Peacock, W.J. (2007). Epigenetic regulation of flowering. *Curr Opin Plant Biol* *10*, 520-527.

- Derkacheva, M., and Hennig, L. (2014). Variations on a theme: Polycomb group proteins in plants. *J Exp Bot* 65, 2769-2784.
- DeYoung, B.J., Bickle, K.L., Schrage, K.J., Muskett, P., Patel, K., and Clark, S.E. (2006). The CLAVATA1-related BAM1, BAM2 and BAM3 receptor kinase-like proteins are required for meristem function in Arabidopsis. *Plant J* 45, 1-16.
- Dinh, T.T., Girke, T., Liu, X., Yant, L., Schmid, M., and Chen, X. (2012). The floral homeotic protein APETALA2 recognizes and acts through an AT-rich sequence element. *Development* 139, 1978-1986.
- Dinneny, J.R., Weigel, D., and Yanofsky, M.F. (2005). A genetic framework for fruit patterning in Arabidopsis thaliana. *Development* 132, 4687-4696.
- Dinneny, J.R., Yadegari, R., Fischer, R.L., Yanofsky, M.F., and Weigel, D. (2004). The role of JAGGED in shaping lateral organs. *Development* 131, 1101-1110.
- Ditta, G., Pinyopich, A., Robles, P., Pelaz, S., and Yanofsky, M.F. (2004). The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity. *Current biology : CB* 14, 1935-1940.
- Dong, Z, H., M, S., M, M., and DP, V. (2005). Callose synthase (CalS5) is required for exine formation during microgametogenesis and for pollen viability in Arabidopsis. *The Plant Journal* 42, 315-328.
- Dong, G., Ma, D.P., and Li, J. (2008). The histone methyltransferase SDG8 regulates shoot branching in Arabidopsis. *Biochem Biophys Res Commun* 373, 659-664.
- Doyle (2008). Integrating molecular phylogenetic and paleobotanical evidence on origin of the flower. *International Journal of Plant Sciences*, 169, 816-843.
- Doyle (2012). Molecular and fossil evidence on the origin of Angiosperms. *Annual Review of Earth and Planetary Sciences* 40, 301-326.
- Doyle, and Donoghue (1986). Seed plant phylogeny and the origin of Angiosperms: an experimental cladistic approach. *The Botanical Review* 52, 321-425.
- Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C., and Lepiniec, L. (2010). MYB transcription factors in Arabidopsis. *Trends Plant Sci* 15, 573-581.
- Edwards, K., Johnstone, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res* 19, 1349.
- Engelhorn, J., Moreau, F., Fletcher, J.C., and Carles, C.C. (2014). ULTRAPETALA1 and LEAFY pathways function independently in specifying identity and determinacy at the Arabidopsis floral meristem. *Ann Bot* 114, 1497-1505.
- Englbrecht, C.C., Schoof, H., and Bohm, S. (2004). Conservation, diversification and expansion of C2H2 zinc finger proteins in the Arabidopsis thaliana genome. *BMC Genomics* 5, 39.

Esau (1977). *Anatomy of Seed Plants*, 2ND 77 Wiley Student Edition edn (John Wiley and Sons).

Favaro, R., Pinyopich, A., Battaglia, R., Kooiker, M., Borghi, L., Ditta, G., Yanofsky, M.F., Kater, M.M., and Colombo, L. (2003). MADS-box protein complexes control carpel and ovule development in *Arabidopsis*. *The Plant cell* 15, 2603-2611.

Feldmann, and Marks (1987). *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana*: A non-tissue culture approach. *Molecular Genetics and Genomics* 208, 1-9.

Ferrandiz, C., Gu, Q., Martienssen, R., and Yanofsky, M.F. (2000). Redundant regulation of meristem identity and plant architecture by FRUITFULL, APETALA1 and CAULIFLOWER. *Development* 127, 725-734.

Ferrandiz, C., Pelaz, S., and Yanofsky, M.F. (1999). Control of carpel and fruit development in *Arabidopsis*. *Annu Rev Biochem* 68, 321-354.

Fletcher, J.C. (2001). The ULTRAPETALA gene controls shoot and floral meristem size in *Arabidopsis*. *Development* 128, 1323-1333.

Fletcher, J.C. (2002). Shoot and floral meristem maintenance in *arabidopsis*. *Annu Rev Plant Biol* 53, 45-66.

Fletcher, J.C., Brand, U., Running, M.P., Simon, R., and Meyerowitz, E.M. (1999). Signaling of cell fate decisions by CLAVATA3 in *Arabidopsis* shoot meristems. *Science* 283, 1911-1914.

Franco-Zorrilla, J.M., Lopez-Vidriero, I., Carrasco, J.L., Godoy, M., Vera, P., and Solano, R. (2014). DNA-binding specificities of plant transcription factors and their potential to define target genes. *Proceedings of the National Academy of Sciences of the United States of America* 111, 2367-2372.

Friedman, W.E. (2009). The meaning of Darwin's 'abominable mystery'. *Am J Bot* 96, 5-21.

Frohlich M.W. (2006). Recent Developments Regarding the Evolutionary Origin of Flowers. *Advances in Botanical Research* 44, 63-127.

Frohlich M.W. and Parker (2000). The Mostly Male theory of flower evolutionary origins: from genes to fossils. *Systematic Botany* 25, 155-170.

Frohlich, M.W. (2003). An evolutionary scenario for the origin of flowers. *Nat Rev Genet* 4, 559-566.

Frohlich, M.W., and Chase, M.W. (2007). After a dozen years of progress the origin of angiosperms is still a great mystery. *Nature* 450, 1184-1189.

- Gaiser, J.C., Robinson-Beers, K., and Gasser, C.S. (1995). The Arabidopsis SUPERMAN Gene Mediates Asymmetric Growth of the Outer Integument of Ovules. *The Plant cell* 7, 333-345.
- Gan, E.S., Huang, J., and Ito, T. (2013). Functional roles of histone modification, chromatin remodeling and microRNAs in Arabidopsis flower development. *Int Rev Cell Mol Biol* 305, 115-161.
- Gan, E.S., Xu, Y., Wong, J.Y., Goh, J.G., Sun, B., Wee, W.Y., Huang, J., and Ito, T. (2014). Jumonji demethylases moderate precocious flowering at elevated temperature via regulation of FLC in Arabidopsis. *Nat Commun* 5, 5098.
- Gendler, K., Paulsen, T., and Napoli, C. (2008). ChromDB: the chromatin database. *Nucleic Acids Res* 36, D298-302.
- Glover, J., Grelon, M., Craig, S., Chaudhury, A., and Dennis, E. (1998). Cloning and characterization of MS5 from Arabidopsis: a gene critical in male meiosis. *Plant J* 15, 345-356.
- Gomez-Mena, C., de Folter, S., Costa, M.M., Angenent, G.C., and Sablowski, R. (2005). Transcriptional program controlled by the floral homeotic gene AGAMOUS during early organogenesis. *Development* 132, 429-438.
- Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E.M., and Coupland, G. (1997). A Polycomb-group gene regulates homeotic gene expression in Arabidopsis. *Nature* 386, 44-51.
- Goslin K, Zheng B, Serrano-Mislata A, Rae L, Ryan, P.T., Kwasniewska, K., Thomson B, O'Maoileidigh, D.S., Madueno F, Wellmer, F., *et al.* (2017). Transcription factor interplay between LEAFY and APETALA1/CAULIFLOWER during floral initiation. *Plant Physiology* 174(2), 1097-1109.
- Graciet, E., O'Maoileidigh, D.S., and Wellmer, F. (2014). Next-generation sequencing applied to flower development: ChIP-Seq. *Methods Mol Biol* 1110, 413-429.
- Grini, P.E., Thorstensen, T., Alm, V., Vizcay-Barrena, G., Windju, S.S., Jorstad, T.S., Wilson, Z.A., and Aalen, R.B. (2009). The ASH1 HOMOLOG 2 (ASHH2) histone H3 methyltransferase is required for ovule and anther development in Arabidopsis. *PLoS One* 4, e7817.
- Gross-Hardt, R., Lenhard, M., and Laux, T. (2002). WUSCHEL signaling functions in interregional communication during Arabidopsis ovule development. *Genes Dev* 16, 1129-1138.
- Grossniklaus, U., Vielle-Calzada, J.P., Hoepfner, M.A., and Gagliano, W.B. (1998). Maternal control of embryogenesis by MEDEA, a polycomb group gene in Arabidopsis. *Science* 280, 446-450.

- Guo, S., Sun, B., Looi, L.S., Xu, Y., Gan, E.S., Huang, J., and Ito, T. (2015). Co-ordination of Flower Development Through Epigenetic Regulation in Two Model Species: Rice and Arabidopsis. *Plant Cell Physiol* 56, 830-842.
- Guo, Y., and Clark, S.E. (2010). Membrane distributions of two ligand-binding receptor complexes in the CLAVATA pathway. *Plant Signal Behav* 5, 1442-1445.
- Ha, M., Ng, D.W., Li, W.H., and Chen, Z.J. (2011). Coordinated histone modifications are associated with gene expression variation within and between species. *Genome Res* 21, 590-598.
- Heim, M.A., Jakoby, M., Werber, M., Martin, C., Weisshaar, B., and Bailey, P.C. (2003). The basic helix-loop-helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. *Mol Biol Evol* 20, 735-747.
- Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y.C., Laslo, P., Cheng, J.X., Murre, C., Singh, H., and Glass, C.K. (2010). Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol Cell* 38, 576-589.
- Hempel, and Feldman (1994). Bi-directional inflorescence development in *Arabidopsis thaliana*: Acropetal initiation of flowers and basipetal initiation of paracletes. *Planta*, 276-286.
- Henderson, I.R., and Dean, C. (2004). Control of Arabidopsis flowering: the chill before the bloom. *Development* 131, 3829-3838.
- Hiratsu, K., Mitsuda, N., Matsui, K., and Ohme-Takagi, M. (2004). Identification of the minimal repression domain of SUPERMAN shows that the DLELRL hexapeptide is both necessary and sufficient for repression of transcription in Arabidopsis. *Biochem Biophys Res Commun* 321, 172-178.
- Hiratsu, K., Ohta, M., Matsui, K., and Ohme-Takagi, M. (2002). The SUPERMAN protein is an active repressor whose carboxy-terminal repression domain is required for the development of normal flowers. *FEBS Lett* 514, 351-354.
- Hobe, M., Muller, R., Grunewald, M., Brand, U., and Simon, R. (2003). Loss of CLE40, a protein functionally equivalent to the stem cell restricting signal CLV3, enhances root waving in Arabidopsis. *Dev Genes Evol* 213, 371-381.
- Honma, T., and Goto, K. (2001). Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. *Nature* 409, 525-529.
- Huang, T., Bohlenius, H., Eriksson, S., Parcy, F., and Nilsson, O. (2005). The mRNA of the Arabidopsis gene FT moves from leaf to shoot apex and induces flowering. *Science* 309, 1694-1696.
- Ito, T., and Sun, B. (2009). Epigenetic regulation of developmental timing in floral stem cells. *Epigenetics* 4, 564-567.

Ito, T., Wellmer, F., Yu, H., Das, P., Ito, N., Alves-Ferreira, M., Riechmann, J.L., and Meyerowitz, E.M. (2004). The homeotic protein AGAMOUS controls microsporogenesis by regulation of SPOROCTELESS. *Nature* 430, 356-360.

Jack, T. (2004). Molecular and genetic mechanisms of floral control. *The Plant cell* 16 Suppl, S1-17.

Jack, T., Fox, G.L., and Meyerowitz, E.M. (1994). Arabidopsis homeotic gene APETALA3 ectopic expression: transcriptional and posttranscriptional regulation determine floral organ identity. *Cell* 76, 703-716.

Jackson, J.P., Johnson, L., Jasencakova, Z., Zhang, X., PerezBurgos, L., Singh, P.B., Cheng, X., Schubert, I., Jenuwein, T., and Jacobsen, S.E. (2004). Dimethylation of histone H3 lysine 9 is a critical mark for DNA methylation and gene silencing in Arabidopsis thaliana. *Chromosoma* 112, 308-315.

Jacob, Y., Feng, S., LeBlanc, C.A., Bernatavichute, Y.V., Stroud, H., Cokus, S., Johnson, L.M., Pellegrini, M., Jacobsen, S.E., and Michaels, S.D. (2009). ATXR5 and ATXR6 are H3K27 monomethyltransferases required for chromatin structure and gene silencing. *Nat Struct Mol Biol* 16, 763-768.

Jacobsen, S.E., and Meyerowitz, E.M. (1997). Hypermethylated SUPERMAN epigenetic alleles in arabidopsis. *Science* 277, 1100-1103.

Jenuwein, T., and Allis, C.D. (2001). Translating the histone code. *Science* 293, 1074-1080.

Jeong, S., Trotochaud, A.E., and Clark, S.E. (1999). The Arabidopsis CLAVATA2 gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. *The Plant cell* 11, 1925-1934.

Ji, L., Liu, X., Yan, J., Wang, W., Yumul, R.E., Kim, Y.J., Dinh, T.T., Liu, J., Cui, X., Zheng, B., *et al.* (2011). ARGONAUTE10 and ARGONAUTE1 regulate the termination of floral stem cells through two microRNAs in Arabidopsis. *PLoS Genet* 7, e1001358.

Jiang, D., Wang, Y., and He, Y. (2008). Repression of FLOWERING LOCUS C and FLOWERING LOCUS T by the Arabidopsis Polycomb repressive complex 2 components. *PLoS One* 3, e3404.

Jin, and Martin (1999). Multifunctionality and diversity within the plant MYB-gene family. *Plant Molecular Biology* 41, 577-585.

Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R., and Dean, C. (2000). Molecular analysis of FRIGIDA, a major determinant of natural variation in Arabidopsis flowering time. *Science* 290, 344-347.

Johnson, L., Mollah, S., Garcia, B.A., Muratore, T.L., Shabanowitz, J., Hunt, D.F., and Jacobsen, S.E. (2004). Mass spectrometry analysis of Arabidopsis histone H3 reveals

distinct combinations of post-translational modifications. *Nucleic Acids Res* 32, 6511-6518.

Kagale, S., Links, M.G., and Rozwadowski, K. (2010). Genome-wide analysis of ethylene-responsive element binding factor-associated amphiphilic repression motif-containing transcriptional regulators in *Arabidopsis*. *Plant Physiol* 152, 1109-1134.

Kanaoka, S, U., M, F., and K, O. (2005). An *Arabidopsis* Rhomboid homolog is an intramembrane protease in plants. *FEBS letters* 579, 5723-5728.

Kaufmann, K., Melzer, R., and Theissen, G. (2005). MIKC-type MADS-domain proteins: structural modularity, protein interactions and network evolution in land plants. *Gene* 347, 183-198.

Kaufmann, K., Muino, J.M., Jauregui, R., Airoidi, C.A., Smaczniak, C., Krajewski, P., and Angenent, G.C. (2009). Target genes of the MADS transcription factor SEPALLATA3: integration of developmental and hormonal pathways in the *Arabidopsis* flower. *PLoS Biol* 7, e1000090.

Kaufmann, K., Wellmer, F., Muino, J.M., Ferrier, T., Wuest, S.E., Kumar, V., Serrano-Mislata, A., Madueno, F., Krajewski, P., Meyerowitz, E.M., *et al.* (2010). Orchestration of floral initiation by APETALA1. *Science* 328, 85-89.

Kempin, S.A., Savidge, B., and Yanofsky, M.F. (1995). Molecular basis of the cauliflower phenotype in *Arabidopsis*. In *Science*, pp. 522-525.

Kieffer, M., Stern, Y., Cook, H., Clerici, E., Maulbetsch, C., Laux, T., and Davies, B. (2006). Analysis of the transcription factor WUSCHEL and its functional homologue in *Antirrhinum* reveals a potential mechanism for their roles in meristem maintenance. *The Plant cell* 18, 560-573.

Kim, D.H., Doyle, M.R., Sung, S., and Amasino, R.M. (2009). Vernalization: winter and the timing of flowering in plants. *Annu Rev Cell Dev Biol* 25, 277-299.

Kinoshita, A., Betsuyaku, S., Osakabe, Y., Mizuno, S., Nagawa, S., Stahl, Y., Simon, R., Yamaguchi-Shinozaki, K., Fukuda, H., and Sawa, S. (2010). RPK2 is an essential receptor-like kinase that transmits the CLV3 signal in *Arabidopsis*. *Development* 137, 3911-3920.

Kobayashi, Y., and Weigel, D. (2007). Move on up, it's time for change--mobile signals controlling photoperiod-dependent flowering. *Genes Dev* 21, 2371-2384.

Kohler, C., and Villar, C.B. (2008). Programming of gene expression by Polycomb group proteins. *Trends Cell Biol* 18, 236-243.

Komeda, Y. (2004). Genetic regulation of time to flower in *Arabidopsis thaliana*. *Annu Rev Plant Biol* 55, 521-535.

Kondo, T., Sawa, S., Kinoshita, A., Mizuno, S., Kakimoto, T., Fukuda, H., and Sakagami, Y. (2006). A plant peptide encoded by CLV3 identified by in situ MALDI-TOF MS analysis. *Science* 313, 845-848.

Krizek, B.A., and Fletcher, J.C. (2005). Molecular mechanisms of flower development: an armchair guide. *Nat Rev Genet* 6, 688-698.

Krizek, B.A., Lewis, M.W., and Fletcher, J.C. (2006). RABBIT EARS is a second-whorl repressor of AGAMOUS that maintains spatial boundaries in Arabidopsis flowers. *Plant J* 45, 369-383.

Krogan, N.T., Hogan, K., and Long, J.A. (2012). APETALA2 negatively regulates multiple floral organ identity genes in Arabidopsis by recruiting the co-repressor TOPLESS and the histone deacetylase HDA19. *Development* 139, 4180-4190.

Lafos, M., Kroll, P., Hohenstatt, M.L., Thorpe, F.L., Clarenz, O., and Schubert, D. (2011). Dynamic regulation of H3K27 trimethylation during Arabidopsis differentiation. *PLoS Genet* 7, e1002040.

Laux, T., Mayer, K.F., Berger, J., and Jurgens, G. (1996). The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. *Development* 122, 87-96.

Lee, J.Y., Baum, S.F., Alvarez, J., Patel, A., Chitwood, D.H., and Bowman, J.L. (2005). Activation of CRABS CLAW in the Nectaries and Carpels of Arabidopsis. *The Plant cell* 17, 25-36.

Lee, T.I., Johnstone, S.E., and Young, R.A. (2006). Chromatin immunoprecipitation and microarray-based analysis of protein location. *Nat Protoc* 1, 729-748.

Lenhard, M., Bohnert, A., Jurgens, G., and Laux, T. (2001). Termination of stem cell maintenance in Arabidopsis floral meristems by interactions between WUSCHEL and AGAMOUS. *Cell* 105, 805-814.

Lenhard, M., and Laux, T. (2003). Stem cell homeostasis in the Arabidopsis shoot meristem is regulated by intercellular movement of CLAVATA3 and its sequestration by CLAVATA1. *Development* 130, 3163-3173.

Li, G., Liu, S., Wang, J., He, J., Huang, H., Zhang, Y., and Xu, L. (2014). ISWI proteins participate in the genome-wide nucleosome distribution in Arabidopsis. *Plant J* 78, 706-714.

Li, H., and Durbin, R. (2010). Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* 26, 589-595.

Liljegren, S.J., Gustafson-Brown, C., Pinyopich, A., Ditta, G.S., and Yanofsky, M.F. (1999). Interactions among APETALA1, LEAFY, and TERMINAL FLOWER1 specify meristem fate. *The Plant cell* 11, 1007-1018.

Litt (2007). An Evaluation of A-Function: Evidence from the APETALA1 and APETALA2 Gene Lineages. *International Journal of Plant Sciences* 168, 73-91.

Liu, C., Lu, F., Cui, X., and Cao, X. (2010). Histone methylation in higher plants. *Annu Rev Plant Biol* 61, 395-420.

Liu, C., Thong, Z., and Yu, H. (2009). Coming into bloom: the specification of floral meristems. *Development* 136, 3379-3391.

Liu, C., Zhou, J., Bracha-Drori, K., Yalovsky, S., Ito, T., and Yu, H. (2007). Specification of Arabidopsis floral meristem identity by repression of flowering time genes. *Development* 134, 1901-1910.

Liu, X., Dinh, T.T., Li, D., Shi, B., Li, Y., Cao, X., Guo, L., Pan, Y., Jiao, Y., and Chen, X. (2014a). AUXIN RESPONSE FACTOR 3 integrates the functions of AGAMOUS and APETALA2 in floral meristem determinacy. *Plant J* 80, 629-641.

Liu, X., Gao, L., Dinh, T.T., Shi, T., Li, D., Wang, R., Guo, L., Xiao, L., and Chen, X. (2014b). DNA topoisomerase I affects polycomb group protein-mediated epigenetic regulation and plant development by altering nucleosome distribution in Arabidopsis. *The Plant cell* 26, 2803-2817.

Liu, X., Kim, Y.J., Muller, R., Yumul, R.E., Liu, C., Pan, Y., Cao, X., Goodrich, J., and Chen, X. (2011). AGAMOUS terminates floral stem cell maintenance in Arabidopsis by directly repressing WUSCHEL through recruitment of Polycomb Group proteins. *The Plant cell* 23, 3654-3670.

Lohmann, J.U., Hong, R.L., Hobe, M., Busch, M.A., Parcy, F., Simon, R., and Weigel, D. (2001). A molecular link between stem cell regulation and floral patterning in Arabidopsis. *Cell* 105, 793-803.

Long, J.A., Moan, E.I., Medford, J.I., and Barton, M.K. (1996). A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. *Nature* 379, 66-69.

Long, J.A., Ohno, C., Smith, Z.R., and Meyerowitz, E.M. (2006). TOPLESS regulates apical embryonic fate in Arabidopsis. *Science* 312, 1520-1523.

Lu, F., Cui, X., Zhang, S., Jenuwein, T., and Cao, X. (2011). Arabidopsis REF6 is a histone H3 lysine 27 demethylase. *Nat Genet* 43, 715-719.

Luo, M., Bilodeau, P., Koltunow, A., Dennis, E.S., Peacock, W.J., and Chaudhury, A.M. (1999). Genes controlling fertilization-independent seed development in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America* 96, 296-301.

Mallory, A.C., Elmayan, T., and Vaucheret, H. (2008). MicroRNA maturation and action--the expanding roles of ARGONAUTES. *Curr Opin Plant Biol* 11, 560-566.

- Mandel, T., Moreau, F., Kutsher, Y., Fletcher, J.C., Carles, C.C., and Eshed Williams, L. (2014). The ERECTA receptor kinase regulates Arabidopsis shoot apical meristem size, phyllotaxy and floral meristem identity. *Development* *141*, 830-841.
- Mardis, E.R. (2007). ChIP-seq: welcome to the new frontier. *Nat Methods* *4*, 613-614.
- Marsch-Martinez, N., Ramos-Cruz, D., Irepan Reyes-Olalde, J., Lozano-Sotomayor, P., Zuniga-Mayo, V.M., and de Folter, S. (2012). The role of cytokinin during Arabidopsis gynoecia and fruit morphogenesis and patterning. *Plant J* *72*, 222-234.
- Mathieu, O., Probst, A.V., and Paszkowski, J. (2005). Distinct regulation of histone H3 methylation at lysines 27 and 9 by CpG methylation in Arabidopsis. *EMBO J* *24*, 2783-2791.
- McBride, K.E., and Summerfelt, K.R. (1990). Improved binary vectors for Agrobacterium-mediated plant transformation. *Plant Mol Biol* *14*, 269-276.
- Mi, S., Cai, T., Hu, Y., Chen, Y., Hodges, E., Ni, F., Wu, L., Li, S., Zhou, H., Long, C., *et al.* (2008). Sorting of small RNAs into Arabidopsis argonaute complexes is directed by the 5' terminal nucleotide. *Cell* *133*, 116-127.
- Michaels, S.D., and Amasino, R.M. (1999). FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. *The Plant cell* *11*, 949-956.
- Mizukami, Y., and Ma, H. (1992). Ectopic expression of the floral homeotic gene AGAMOUS in transgenic Arabidopsis plants alters floral organ identity. *Cell* *71*, 119-131.
- Mizuno, S., Osakabe, Y., Maruyama, K., Ito, T., Osakabe, K., Sato, T., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2007). Receptor-like protein kinase 2 (RPK 2) is a novel factor controlling anther development in Arabidopsis thaliana. *Plant J* *50*, 751-766.
- Moon, Y.H., Chen, L., Pan, R.L., Chang, H.S., Zhu, T., Maffeo, D.M., and Sung, Z.R. (2003). EMF genes maintain vegetative development by repressing the flower program in Arabidopsis. *The Plant cell* *15*, 681-693.
- Moore, I., Galweiler, L., Grosskopf, D., Schell, J., and Palme, K. (1998). A transcription activation system for regulated gene expression in transgenic plants. *Proceedings of the National Academy of Sciences of the United States of America* *95*, 376-381.
- Mravec, J., Skupa, P., Bailly, A., Hoyerova, K., Krecek, P., Bielach, A., Petrasek, J., Zhang, J., Gaykova, V., Stierhof, Y.D., *et al.* (2009). Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter. *Nature* *459*, 1136-1140.
- Muller, R., Bleckmann, A., and Simon, R. (2008). The receptor kinase CORYNE of Arabidopsis transmits the stem cell-limiting signal CLAVATA3 independently of CLAVATA1. *The Plant cell* *20*, 934-946.

- Nimchuk, Z.L., Zhou, Y., Tarr, P.T., Peterson, B.A., and Meyerowitz, E.M. (2015). Plant stem cell maintenance by transcriptional cross-regulation of related receptor kinases. *Development* 142, 1043-1049.
- O'Maoileidigh, D.S., Thomson, B., Raganelli, A., Wuest, S.E., Ryan, P.T., Kwasniewska, K., Carles, C.C., Graciet, E., and Wellmer, F. (2015). Gene network analysis of *Arabidopsis thaliana* flower development through dynamic gene perturbations. *Plant J* 83, 344-358.
- O'Maoileidigh, D.S., and Wellmer, F. (2014). A floral induction system for the study of early *Arabidopsis* flower development. *Methods Mol Biol* 1110, 307-314.
- O'Maoileidigh, D.S., Wuest, S.E., Rae, L., Raganelli, A., Ryan, P.T., Kwasniewska, K., Das, P., Lohan, A.J., Loftus, B., Graciet, E., *et al.* (2013). Control of Reproductive Floral Organ Identity Specification in *Arabidopsis* by the C Function Regulator AGAMOUS. *The Plant cell*.
- O'Maoileidigh (2012). Specification of Floral Reproductive Organs: The Role of AGAMOUS. In Smurfit Institute of Genetics (Dublin, Trinity College Dublin).
- Ogawa, M., Shinohara, H., Sakagami, Y., and Matsubayashi, Y. (2008). *Arabidopsis* CLV3 peptide directly binds CLV1 ectodomain. *Science* 319, 294.
- Ohad, N., Yadegari, R., Margossian, L., Hannon, M., Michaeli, D., Harada, J.J., Goldberg, R.B., and Fischer, R.L. (1999). Mutations in FIE, a WD polycomb group gene, allow endosperm development without fertilization. *The Plant cell* 11, 407-416.
- Pajoro, A., Madrigal, P., Muino, J.M., Matus, J.T., Jin, J., Mecchia, M.A., Debernardi, J.M., Palatnik, J.F., Balazadeh, S., Arif, M., *et al.* (2014). Dynamics of chromatin accessibility and gene regulation by MADS-domain transcription factors in flower development. *Genome Biol* 15, R41.
- Palatnik, J.F., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J.C., and Weigel, D. (2003). Control of leaf morphogenesis by microRNAs. *Nature* 425, 257-263.
- Pandey, R., Muller, A., Napoli, C.A., Selinger, D.A., Pikaard, C.S., Richards, E.J., Bender, J., Mount, D.W., and Jorgensen, R.A. (2002). Analysis of histone acetyltransferase and histone deacetylase families of *Arabidopsis thaliana* suggests functional diversification of chromatin modification among multicellular eukaryotes. *Nucleic Acids Res* 30, 5036-5055.
- Parcy, F. (2005). Flowering: a time for integration. *The International journal of developmental biology* 49, 585-593.
- Parcy, F., Bomblies, K., and Weigel, D. (2002). Interaction of LEAFY, AGAMOUS and TERMINAL FLOWER1 in maintaining floral meristem identity in *Arabidopsis*. *Development* 129, 2519-2527.
- Park, P.J. (2009). ChIP-seq: advantages and challenges of a maturing technology. *Nat Rev Genet* 10, 669-680.

Pauwels, L., Barbero, G.F., Geerinck, J., Tilleman, S., Grunewald, W., Perez, A.C., Chico, J.M., Bossche, R.V., Sewell, J., Gil, E., *et al.* (2010). NINJA connects the co-repressor TOPLESS to jasmonate signalling. *Nature* 464, 788-791.

Payne, T., Johnson, S.D., and Koltunow, A.M. (2004). KNUCKLES (KNU) encodes a C2H2 zinc-finger protein that regulates development of basal pattern elements of the Arabidopsis gynoecium. *Development* 131, 3737-3749.

Pelaz, S., Ditta, G.S., Baumann, E., Wisman, E., and Yanofsky, M.F. (2000). B and C floral organ identity functions require SEPALLATA MADS-box genes. *Nature* 405, 200-203.

Pelaz, S., Tapia-Lopez, R., Alvarez-Buylla, E.R., and Yanofsky, M.F. (2001). Conversion of leaves into petals in Arabidopsis. *Current biology : CB* 11, 182-184.

Pien, S., Fleury, D., Mylne, J.S., Crevillen, P., Inze, D., Avramova, Z., Dean, C., and Grossniklaus, U. (2008). ARABIDOPSIS TRITHORAX1 dynamically regulates FLOWERING LOCUS C activation via histone 3 lysine 4 trimethylation. *The Plant cell* 20, 580-588.

Pien, S., and Grossniklaus, U. (2007). Polycomb group and trithorax group proteins in Arabidopsis. *Biochim Biophys Acta* 1769, 375-382.

Pinyopich, A., Ditta, G.S., Savidge, B., Liljegren, S.J., Baumann, E., Wisman, E., and Yanofsky, M.F. (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. *Nature* 424, 85-88.

Poethig, R.S. (1987). Clonal Analysis of Cell Lineage Patterns in Plant Development. *American Journal of Botany* vol. 74, 581-594.

Poethig, R.S., and Sussex, I.M. (1985a). The cellular parameters of leaf development in tobacco: a clonal analysis. *Planta* 165, 170-184.

Poethig, R.S., and Sussex, I.M. (1985b). The developmental morphology and growth dynamics of the tobacco leaf. *Planta* 165, 158-169.

Prigge, M.J., Otsuga, D., Alonso, J.M., Ecker, J.R., Drews, G.N., and Clark, S.E. (2005). Class III homeodomain-leucine zipper gene family members have overlapping, antagonistic, and distinct roles in Arabidopsis development. *The Plant cell* 17, 61-76.

Prunet, N., Morel, P., Thierry, A.M., Eshed, Y., Bowman, J.L., Negrutiu, I., and Trehin, C. (2008). REBELOTE, SQUINT, and ULTRAPETALA1 function redundantly in the temporal regulation of floral meristem termination in Arabidopsis thaliana. *The Plant cell* 20, 901-919.

Puranik, S., Acajjaoui, S., Conn, S., Costa, L., Conn, V., Vial, A., Marcellin, R., Melzer, R., Brown, E., Hart, D., *et al.* (2014). Structural basis for the oligomerization of the MADS domain transcription factor SEPALLATA3 in Arabidopsis. *The Plant cell* 26, 3603-3615.

Ritchie, M.E., Silver, J., Oshlack, A., Holmes, M., Diyagama, D., Holloway, A., and Smyth, G.K. (2007). A comparison of background correction methods for two-colour microarrays. *Bioinformatics* 23, 2700-2707.

Roslan, H.A., Salter, M.G., Wood, C.D., White, M.R., Croft, K.P., Robson, F., Coupland, G., Doonan, J., Laufs, P., Tomsett, A.B., *et al.* (2001). Characterization of the ethanol-inducible alc gene-expression system in *Arabidopsis thaliana*. *Plant J* 28, 225-235.

Roudier, F., Ahmed, I., Berard, C., Sarazin, A., Mary-Huard, T., Cortijo, S., Bouyer, D., Caillieux, E., Duvernois-Berthet, E., Al-Shikhley, L., *et al.* (2011). Integrative epigenomic mapping defines four main chromatin states in *Arabidopsis*. *EMBO J* 30, 1928-1938.

Running, M.P., and Meyerowitz, E.M. (1996). Mutations in the *PERIANTHIA* gene of *Arabidopsis* specifically alter floral organ number and initiation pattern. *Development* 122, 1261-1269.

Ruthenburg, A.J., Allis, C.D., and Wysocka, J. (2007). Methylation of lysine 4 on histone H3: intricacy of writing and reading a single epigenetic mark. *Mol Cell* 25, 15-30.

Ryan, P.T., O'Maoileidigh, D.S., Drost, H.G., Kwasniewska, K., Gabel, A., Grosse, I., Graciet, E., Quint, M., and Wellmer, F. (2015). Patterns of gene expression during *Arabidopsis* flower development from the time of initiation to maturation. *BMC Genomics* 16, 488.

Sablowski, R. (2007). Flowering and determinacy in *Arabidopsis*. *J Exp Bot* 58, 899-907.

Sakai, H., Krizek, B.A., Jacobsen, S.E., and Meyerowitz, E.M. (2000). Regulation of *SUP* expression identifies multiple regulators involved in *arabidopsis* floral meristem development. *The Plant cell* 12, 1607-1618.

Sakai, H., Medrano, L.J., and Meyerowitz, E.M. (1995). Role of *SUPERMAN* in maintaining *Arabidopsis* floral whorl boundaries. *Nature* 378, 199-203.

Saleh, A., Alvarez-Venegas, R., Yilmaz, M., Le, O., Hou, G., Sadler, M., Al-Abdallat, A., Xia, Y., Lu, G., Ladunga, I., *et al.* (2008). The highly similar *Arabidopsis* homologs of trithorax *ATX1* and *ATX2* encode proteins with divergent biochemical functions. *The Plant cell* 20, 568-579.

Schiessl, K., Muino, J.M., and Sablowski, R. (2014). *Arabidopsis* *JAGGED* links floral organ patterning to tissue growth by repressing Kip-related cell cycle inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* 111, 2830-2835.

Schonrock, N., Bouveret, R., Leroy, O., Borghi, L., Kohler, C., Grissem, W., and Hennig, L. (2006). Polycomb-group proteins repress the floral activator AGL19 in the FLC-independent vernalization pathway. *Genes Dev* 20, 1667-1678.

Schoof, H., Lenhard, M., Haecker, A., Mayer, K.F., Jurgens, G., and Laux, T. (2000). The stem cell population of Arabidopsis shoot meristems is maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. *Cell* 100, 635-644.

Schubert, D., Primavesi, L., Bishopp, A., Roberts, G., Doonan, J., Jenuwein, T., and Goodrich, J. (2006). Silencing by plant Polycomb-group genes requires dispersed trimethylation of histone H3 at lysine 27. *EMBO J* 25, 4638-4649.

Schwab, R., Ossowski, S., Riester, M., Warthmann, N., and Weigel, D. (2006). Highly specific gene silencing by artificial microRNAs in Arabidopsis. *The Plant cell* 18, 1121-1133.

Schwab, R., Palatnik, J.F., Riester, M., Schommer, C., Schmid, M., and Weigel, D. (2005). Specific effects of microRNAs on the plant transcriptome. *Dev Cell* 8, 517-527.

Sharma, V.K., Ramirez, J., and Fletcher, J.C. (2003). The Arabidopsis CLV3-like (CLE) genes are expressed in diverse tissues and encode secreted proteins. *Plant Mol Biol* 51, 415-425.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J., and Dennis, E.S. (1999). The FLM MADS box gene: a repressor of flowering in Arabidopsis regulated by vernalization and methylation. *The Plant cell* 11, 445-458.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstone, J.R., Cole, P.A., and Casero, R.A. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 119, 941-953.

Shinohara, H., and Matsubayashi, Y. (2015). Reevaluation of the CLV3-receptor interaction in the shoot apical meristem: dissection of the CLV3 signaling pathway from a direct ligand-binding point of view. *Plant J* 82, 328-336.

Silver, J.D., Ritchie, M.E., and Smyth, G.K. (2009). Microarray background correction: maximum likelihood estimation for the normal-exponential convolution. *Biostatistics* 10, 352-363.

Simon, R., Igeno, M.I., and Coupland, G. (1996). Activation of floral meristem identity genes in Arabidopsis. *Nature* 384, 59-62.

Simpson, G.G. (2004). The autonomous pathway: epigenetic and post-transcriptional gene regulation in the control of Arabidopsis flowering time. *Curr Opin Plant Biol* 7, 570-574.

Simpson, G.G., and Dean, C. (2002). Arabidopsis, the Rosetta stone of flowering time? *Science* 296, 285-289.

- Smaczniak, C., Immink, R.G., Muino, J.M., Blanvillain, R., Busscher, M., Busscher-Lange, J., Dinh, Q.D., Liu, S., Westphal, A.H., Boeren, S., *et al.* (2012). Characterization of MADS-domain transcription factor complexes in Arabidopsis flower development. *Proceedings of the National Academy of Sciences of the United States of America* 109, 1560-1565.
- Smith, Z.R., and Long, J.A. (2010). Control of Arabidopsis apical-basal embryo polarity by antagonistic transcription factors. *Nature* 464, 423-426.
- Smyth (2005). Individual channel analysis of two-colour microarrays. In 55th Session of the International Statistics Institute (Sydney).
- Smyth, D.R., Bowman, J.L., and Meyerowitz, E.M. (1990). Early flower development in Arabidopsis. *The Plant cell* 2, 755-767.
- Smyth, G.K. (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 3, Article3.
- Smyth, G.K., and Speed, T. (2003). Normalization of cDNA microarray data. *Methods* 31, 265-273.
- Somssich, M., Je, B.I., Simon, R., and Jackson, D. (2016). CLAVATA-WUSCHEL signaling in the shoot meristem. *Development* 143, 3238-3248.
- Song, S.K., and Clark, S.E. (2005). POL and related phosphatases are dosage-sensitive regulators of meristem and organ development in Arabidopsis. *Dev Biol* 285, 272-284.
- Song, S.K., Lee, M.M., and Clark, S.E. (2006). POL and PLL1 phosphatases are CLAVATA1 signaling intermediates required for Arabidopsis shoot and floral stem cells. *Development* 133, 4691-4698.
- Springer, N.M., Napoli, C.A., Selinger, D.A., Pandey, R., Cone, K.C., Chandler, V.L., Kaeppler, H.F., and Kaeppler, S.M. (2003). Comparative analysis of SET domain proteins in maize and Arabidopsis reveals multiple duplications preceding the divergence of monocots and dicots. *Plant Physiol* 132, 907-925.
- Steeves T.A. and Sussex I.M. (1989). *Patterns in Plant Development*. University of Cambridge.
- Stewart, D. (2016). Exploring the function of SUPERMAN, and the repression of trichome initiation pathway, during Arabidopsis flower development. In Smurfit Institute of Genetics (Dublin, Trinity College Dublin).
- Sun, B., and Ito, T. (2010). Floral stem cells: from dynamic balance towards termination. *Biochem Soc Trans* 38, 613-616.
- Sun, B., and Ito, T. (2015). Regulation of floral stem cell termination in Arabidopsis. *Front Plant Sci* 6, 17.

- Sun, B., Looi, L.S., Guo, S., He, Z., Gan, E.S., Huang, J., Xu, Y., Wee, W.Y., and Ito, T. (2014). Timing mechanism dependent on cell division is invoked by Polycomb eviction in plant stem cells. *Science* 343, 1248559.
- Sun, B., Xu, Y., Ng, K.H., and Ito, T. (2009). A timing mechanism for stem cell maintenance and differentiation in the *Arabidopsis* floral meristem. *Genes Dev* 23, 1791-1804.
- Sung, S., and Amasino, R.M. (2004). Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. *Nature* 427, 159-164.
- Sung, S., and Amasino, R.M. (2005). Remembering winter: toward a molecular understanding of vernalization. *Annu Rev Plant Biol* 56, 491-508.
- Sung, Z.R., Belachew, A., Shunong, B., and Bertrand-Garcia, R. (1992). EMF, an *Arabidopsis* Gene Required for Vegetative Shoot Development. *Science* 258, 1645-1647.
- Szemenyei, H., Hannon, M., and Long, J.A. (2008). TOPLESS mediates auxin-dependent transcriptional repression during *Arabidopsis* embryogenesis. *Science* 319, 1384-1386.
- Szymkowiak, E.J., and Sussex, I.M. (1992). The internal meristem layer (L3) determines floral meristem size and carpel number in tomato periclinal chimeras. *The Plant Cell* 4, 1089-1100.
- Takeda, S., Matsumoto, N., and Okada, K. (2004). RABBIT EARS, encoding a SUPERMAN-like zinc finger protein, regulates petal development in *Arabidopsis thaliana*. *Development* 131, 425-434.
- Tamada, Y., Yun, J.Y., Woo, S.C., and Amasino, R.M. (2009). ARABIDOPSIS TRITHORAX-RELATED7 is required for methylation of lysine 4 of histone H3 and for transcriptional activation of FLOWERING LOCUS C. *The Plant cell* 21, 3257-3269.
- Taverna, S.D., Li, H., Ruthenburg, A.J., Allis, C.D., and Patel, D.J. (2007). How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers. *Nat Struct Mol Biol* 14, 1025-1040.
- Theissen, and Becker (2004). Gymnosperm orthologues of class B floral homeotic genes and their impact on understanding flower origin. *Critical Reviews in Plant Sciences* 23, 129-148.
- Theissen, Becker, Winter, Munster, Kirchner, and Saedler (2002). How the land plants learned their floral ABCs: the role of MADS-box domain genes in the evolutionary origin of flowers. *Developmental Geneics and Plant Evolution*, 173-206.
- Theissen, G. (2001). Development of floral organ identity: stories from the MADS house. *Curr Opin Plant Biol* 4, 75-85.

Theissen, G., Kim, J.T., and Saedler, H. (1996). Classification and phylogeny of the MADS-box multigene family suggest defined roles of MADS-box gene subfamilies in the morphological evolution of eukaryotes. *J Mol Evol* 43, 484-516.

Theissen, G., and Melzer, R. (2007). Molecular mechanisms underlying origin and diversification of the angiosperm flower. *Ann Bot* 100, 603-619.

Theissen, G., Melzer, R., and Rümpler, F. (2016). MADS-domain transcription factors and the floral quartet model of flower development: linking plant development and evolution. *Development* 143, 3259-3271.

Theissen, G., and Saedler, H. (2001). Plant biology. Floral quartets. *Nature* 409, 469-471.

Thomas, P.D., Kejariwal, A., Guo, N., Mi, H., Campbell, M.J., Muruganujan, A., and Lazareva-Ulitsky, B. (2006). Applications for protein sequence-function evolution data: mRNA/protein expression analysis and coding SNP scoring tools. *Nucleic Acids Res* 34, W645-650.

Thorstensen, T., Grini, P.E., Mercy, I.S., Alm, V., Erdal, S., Aasland, R., and Aalen, R.B. (2008). The Arabidopsis SET-domain protein ASHR3 is involved in stamen development and interacts with the bHLH transcription factor ABORTED MICROSPORES (AMS). *Plant Mol Biol* 66, 47-59.

Tian, L., and Chen, Z.J. (2001). Blocking histone deacetylation in Arabidopsis induces pleiotropic effects on plant gene regulation and development. *Proceedings of the National Academy of Sciences of the United States of America* 98, 200-205.

Triezenberg, S.J., Kingsbury, R.C., and McKnight, S.L. (1988). Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. *Genes Dev* 2, 718-729.

Trotochaud, A.E., Hao, T., Wu, G., Yang, Z., and Clark, S.E. (1999). The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a Rho-related protein. *The Plant cell* 11, 393-406.

Tsukada, Y., Fang, J., Erdjument-Bromage, H., Warren, M.E., Borchers, C.H., Tempst, P., and Zhang, Y. (2006). Histone demethylation by a family of JmjC domain-containing proteins. *Nature* 439, 811-816.

Turck, F., Roudier, F., Farrona, S., Martin-Magniette, M.L., Guillaume, E., Buisine, N., Gagnot, S., Martienssen, R.A., Coupland, G., and Colot, V. (2007). Arabidopsis TFL2/LHP1 specifically associates with genes marked by trimethylation of histone H3 lysine 27. *PLoS Genet* 3, e86.

Voinnet, O. (2009). Origin, biogenesis, and activity of plant microRNAs. *Cell* 136, 669-687.

- Wang, J.W., Czech, B., and Weigel, D. (2009). miR156-regulated SPL transcription factors define an endogenous flowering pathway in *Arabidopsis thaliana*. *Cell* 138, 738-749.
- Warnes, G., Bolker, B., Bonebakker, L., Gentleman, R., Liaw, W., Lumley, T., Maechler, M., Magnusson, A., Moeller, S., Schwartz, M., *et al.* (2011). gplots: Various R programming tools for plotting data. In <http://CRAN.R-project.org/package=gplots>.
- Weigel, D., Alvarez, J., Smyth, D.R., Yanofsky, M.F., and Meyerowitz, E.M. (1992). LEAFY controls floral meristem identity in *Arabidopsis*. *Cell* 69, 843-859.
- Wellmer, F., Alves-Ferreira, M., Dubois, A., Riechmann, J.L., and Meyerowitz, E.M. (2006). Genome-wide analysis of gene expression during early *Arabidopsis* flower development. *PLoS Genet* 2, e117.
- Wellmer, F., Riechmann, J.L., Alves-Ferreira, M., and Meyerowitz, E.M. (2004). Genome-wide analysis of spatial gene expression in *Arabidopsis* flowers. *The Plant cell* 16, 1314-1326.
- Wigge, P.A., Kim, M.C., Jaeger, K.E., Busch, W., Schmid, M., Lohmann, J.U., and Weigel, D. (2005). Integration of spatial and temporal information during floral induction in *Arabidopsis*. *Science* 309, 1056-1059.
- Williams, L., Grigg, S.P., Xie, M., Christensen, S., and Fletcher, J.C. (2005). Regulation of *Arabidopsis* shoot apical meristem and lateral organ formation by microRNA miR166g and its AtHD-ZIP target genes. *Development* 132, 3657-3668.
- Wood, C.C., Robertson, M., Tanner, G., Peacock, W.J., Dennis, E.S., and Helliwell, C.A. (2006). The *Arabidopsis thaliana* vernalization response requires a polycomb-like protein complex that also includes VERNALIZATION INSENSITIVE 3. *Proceedings of the National Academy of Sciences of the United States of America* 103, 14631-14636.
- Wuest, S.E., O'Maoileidigh, D.S., Rae, L., Kwasniewska, K., Raganelli, A., Hanczaryk, K., Lohan, A.J., Loftus, B., Graciet, E., and Wellmer, F. (2012). Molecular basis for the specification of floral organs by APETALA3 and PISTILLATA. *Proceedings of the National Academy of Sciences of the United States of America* 109, 13452-13457.
- Wynn, A.N., Seaman, A.A., Jones, A.L., and Franks, R.G. (2014). Novel functional roles for PERIANTHIA and SEUSS during floral organ identity specification, floral meristem termination, and gynoecial development. *Front Plant Sci* 5, 130.
- Xu, B., Li, Z., Zhu, Y., Wang, H., Ma, H., Dong, A., and Huang, H. (2008a). *Arabidopsis* genes AS1, AS2, and JAG negatively regulate boundary-specifying genes to promote sepal and petal development. *Plant Physiol* 146, 566-575.
- Xu, L., Zhao, Z., Dong, A., Soubigou-Taconnat, L., Renou, J.P., Steinmetz, A., and Shen, W.H. (2008b). Di- and tri- but not monomethylation on histone H3 lysine 36 marks active transcription of genes involved in flowering time regulation and other processes in *Arabidopsis thaliana*. *Mol Cell Biol* 28, 1348-1360.

Yang, Y., Fanning, L., and Jack, T. (2003). The K domain mediates heterodimerization of the Arabidopsis floral organ identity proteins, APETALA3 and PISTILLATA. *Plant J* 33, 47-59.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A., and Meyerowitz, E.M. (1990). The protein encoded by the Arabidopsis homeotic gene *agamous* resembles transcription factors. *Nature* 346, 35-39.

Yu, L.P., Miller, A.K., and Clark, S.E. (2003). POLTERGEIST encodes a protein phosphatase 2C that regulates CLAVATA pathways controlling stem cell identity at Arabidopsis shoot and flower meristems. *Current biology : CB* 13, 179-188.

Yu, L.P., Simon, E.J., Trotochaud, A.E., and Clark, S.E. (2000). POLTERGEIST functions to regulate meristem development downstream of the CLAVATA loci. *Development* 127, 1661-1670.

Zhang, K., Sridhar, V.V., Zhu, J., Kapoor, A., and Zhu, J.K. (2007a). Distinctive core histone post-translational modification patterns in Arabidopsis thaliana. *PLoS One* 2, e1210.

Zhang, L., Dai, Y., Peng, W., Lu, J., Zhang, Y., and Wang, L. (2009). Genome-wide analysis of histone H3 lysine 4 trimethylation in peripheral blood mononuclear cells of minimal change nephrotic syndrome patients. *Am J Nephrol* 30, 505-513.

Zhang, X., Clarenz, O., Cokus, S., Bernatavichute, Y.V., Pellegrini, M., Goodrich, J., and Jacobsen, S.E. (2007b). Whole-genome analysis of histone H3 lysine 27 trimethylation in Arabidopsis. *PLoS Biol* 5, e129.

Zhang, X., Henriques, R., Lin, S.S., Niu, Q.W., and Chua, N.H. (2006). Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. *Nat Protoc* 1, 641-646.

Zhang, Y., Liu, T., Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nusbaum, C., Myers, R.M., Brown, M., Li, W., *et al.* (2008). Model-based analysis of ChIP-Seq (MACS). *Genome Biol* 9, R137.

Zhao, L., Kim, Y., Dinh, T.T., and Chen, X. (2007). miR172 regulates stem cell fate and defines the inner boundary of APETALA3 and PISTILLATA expression domain in Arabidopsis floral meristems. *Plant J* 51, 840-849.

Zhao, Z., and Shen, W.H. (2004). Plants contain a high number of proteins showing sequence similarity to the animal SUV39H family of histone methyltransferases. *Ann N Y Acad Sci* 1030, 661-669.

Zheng, J., Chen, F., Wang, Z., Cao, H., Li, X., Deng, X., Soppe, W.J., Li, Y., and Liu, Y. (2012). A novel role for histone methyltransferase KYP/SUVH4 in the control of Arabidopsis primary seed dormancy. *New Phytol* 193, 605-616.

Zhu, Z., Xu, F., Zhang, Y., Cheng, Y.T., Wiermer, M., and Li, X. (2010). Arabidopsis resistance protein SNC1 activates immune responses through association with a transcriptional corepressor. *Proceedings of the National Academy of Sciences of the United States of America* *107*, 13960-13965.

Zuniga-Mayo, V.M., Marsch-Martinez, N., and de Folter, S. (2012). The class II HD-ZIP JAIBA gene is involved in meristematic activity and important for gynoecium and fruit development in Arabidopsis. *Plant Signal Behav* *7*, 1501-1503.

Appendix 1

List of oligonucleotides used in this study:

Name	Sequence
DM-31	GGTAAGGATCTGAGCTACACATGCTC
DM-57	gaACACGTATGTGTAGCCTTTTTtcacaggtcgtgatatg
DM-59	CTTGACATAGCTAGTTATCGTACG
DM-86	GTCCCGCAATTATACATTTAATACGCG
DM-87	CTCCGTCATCACTCTCGCTAGG
DM-90	GAATCTCGAAGACACTAACTGGAGAGCGGTTT
DM-91	CTCTAGAGATCAAAAATGGGAAGGGGTAGGGTTC
DM-92	CGGATCCTGCGGCGAAGCAGCCAAG
DM-93	TGTTAGCAAGCTAGTTACGTTAGG
DM-319	CGAGCATGGAAACCTGACTAAACCC
DM-320	GACTGTGAGAGATGGGACGATTGC
DM-323	CGTTTCGCTTTCCTTAGTGTTAGCT
DM-324	AGCGAACGGATCTAGAGACTCACCTTG
DM-325	ATCCGTGAAGAGTACCCAGAT
DM-326	AAGAACCATGCACTCATCAGC
DM-339	TCCAAGAAGTAATGTGAGGAAAGGC
DM-340	TCCAAGATGAAATTGCCAAACATGG
DM-347	TTGGCAGAGTGTGGACATAACATG
DM-348	GACTTGTCTTTGACCTTCTCCACC
DM-400	TCCGACAAGATCCTTTGAGCAC
DM-523	TCCATCGAGAAGGTTGAGAGTTCAGAC
DM-524	TGTCCGAGTAACATCACAACGTTCC
DM-563	TCTCTTTACTACCAGCAAGTTG
DM-564	GATCAAGACTTAGAGAGGAACAAG
DM-565	GCAACCTCGAACCCTTTATTG
DM-566	TGGCCTACTTTGCTGTAGG
DM-664	GTAGGTCCTGCAAAAACCTCGATC
DM-647	GGATTTGAGGAAGAGATTTCTGG
DS-51	GTACCACAAATCATTACTAGTACTAT
DS-52	CGGTAACAAGCGCATACATATTAT
KK-26	GTACTCACTGGCCTATACTG
KK-27	ACTTCTGGAAACGAACCGCTTCAG
KK-28	CGGATGAAACGGATCGTAGCCATC
KK-29	CACTCTTCTATGTTGGGTCACACCAG
KK-30	TTATCGCCATCGCCTTGCTGCTGC
KK-31 (DM-55)	gaTAAAAGGGTACACATACGGGTtctctctttgtattcc
KK-32 (DM-56)	gaACCCGTATGTGTACCCTTTTAtcaaagagaatcaatga
KK-33 (DM-57)	gaACACGTATGTGTAGCCTTTTTtcacaggtcgtgatatg
KK-34 (DM-58)	gaAAAAAGGCTACACATACGTGTtctacatatattcct
KK-35	GGTAAGGATCTGAGCTACACATGCTC
KK-47	CCGCATAGCTGAACATCTATATAA
KK-48	gaACGACCCATTTTCATCCGTATAtctctctttgtattcc

KK-49	gaTATACGGATGAAATGGGTCGTtcaaagagaatcaatga
KK-50	gaTACACGGATGAAAAGGGTCGTtcacaggtcgtgatatg
KK-51	gaACGACCCTTTTCATCCGTGTAtctacatatattcct
KK-52	gaAAGTACTGTCCTCGAAAGTTAtctctcttttgattcc
KK-53	gaTAACTTTCGAGGACAGTACTTtcaaagagaatcaatga
KK-54	gaTACCTTTCGAGGAGAGTACTTtcacaggtcgtgatatg
KK-55	gaAAGTACTCTCCTCGAAAGGTAtctacatatattcct
KK-59	ATCTTAAGATGGCTAGTGAAGCTCGA
KK-61	GGAGCAAAGCAGAGCAGG
KK-65	CGTTTCGCTTTCCTTAGTGTTAGCT
KK-66	AGCGAACGGATCTAGAGACTCACCTT
KK-67	gatattgttccacgacaatcc
KK-68	ccattaatcttactcatcaagttc
KK-69	TCATGTCTGACCGAAAGTCTTAGTGC
KK-70	TGAATCATACCTTTGTGTCCATCACC
KK-71	CGTGAAGGGTCGAAGAAAGAATAAGA
KK-72	CACTCAATAAACCCAGACGTGACTTG
KK-73	tgggtactgagaggaaagtgaga
KK-74	cattaacacattgggtataaatcc
KK-75	aaagccaaccaaatccacctgcac
KK-76	ttgaagagatttggaggagaggacaag
KK-77	TCCATCGAGAAGGTTGAGAGTTCAGAC
KK-78	TGTCCGAGTAACATCACAACGTTCC
KK-79	caattgatttaagcagtgtc
KK-80	gttgaagaagtaaagggtccact
KK-95	GCGGATAACAATTTACACACAGGAA
KK-126	GTAGTATGTGTGCCAACAAAAAGGT
KK-127	ATAGGATGCACAGAACAAATCTACC
KK-128	GGGCCTTAAAATAGGTATGTGTG
KK-129	GAAGGTTTCTATGTTTCTCTTACCC
KK-130	AGTGTTTCATTGATTCACTCGTACTG
KK-131	GATGGGATCACAGTACTTCTCTCTC
KK-132	CACATCCTTCACTCTTCTCTCTCTC
KK-133	AGGACCTACGAAATGAAGGTAAGAC
KK-134	CGTTACGAGTGATAATCGGAATGT
KK-135	GTACCAACTTTGTAGAGGCGGTAAG
KK-136	CCTTACCCTTGCTTTTACCAAAAC
KK-137	GGATAATGCAAAAGGGTACACATAC
KK-138	GATTTACAAGGAATCTGTTGGTGGT
KK-139	CATAACATAGGTTTAGAGCATCTGC
KK-140	TTGAGAATGCTGATGAGTGCATGG
KK-141	AGAGTTGAGTTGACCAGGGAACC
KK-142	ACTCGTTTCGCTTTCCTTAGTGTTAGCTG
KK-143	AGCGAACGGATCTAGAGACTCACCTTG
KK-148	TGCTTTGAAGACGTGGTTGGA
KK-180	aaaaGAATTCATGGCGGAACCAC
KK-196	GTACCGAATTCCGTgcACGGgcAGAAgcGTCTgc
KK-198	aaaaGAATTCTAAACGGAGAGA

KK-202	ATCGGTAAACATCTGCTCAAACCTC
KK-233	CGATGCGGTCACTCATTACG
KK-234	CCCAGTCGAGCATCTCTTC
KK-247	CGTTATGGAAGAAGACGAGCCTC
KK-248	TTCCGTATGTTGCATCACCTTCACC
KK-262	GTCTTACCTTCATTTCGTAGGTCCTGC
KK-263	GCAAAAGGGTACACATACGGATACGG
KK-266	CTTTGCGATGCTGATGTTGCTCTC
KK-267	accatattctcaaaaacacaaacatg
KK-268	CAGGAAGATCGACAACGCAACG
KK-269	gagagagaagaaagacCTGGAGCT
KK-270	CATCAACCTTGCTTCCATCCACC
KK-271	GCTGTGTCGGTTTAGGTGGTG
KK-272	AGTGGCTTATTCCGGTGGAAC
KK-273	CTAACTCCGCTATCTTCGCCG
KK-280	ACTAAGCAGATGCAGAGCTTTGG
KK-281	TCCGGATTGGCACTTTTGATGCGTTGGATCTGAGCC
KK-282	GCATACAACCGCTTCATGTG
KK-283	CAATGTCTCCCAAAGAGCCCAGGACTT
KK-284	GCAACAAGGCATATAGATTTAATTTG
KK-285	TGGGTATAATTCGCCCCGAG
KK-286	GATGTGGAACACTGTCAGCG
KK-287	GTGAGGGTGGCGATTAAACTTC
KK-288	GGAGCAAGCGTCACAATTCA
pMLBART	AGTACATTAAAAACGTCC GCAATGTG
SW-369	GATTTACAAGGAATCTGTTGGTGGT
SW-370	CATAACATAGGTTTAGAGCATCTG C
SW-506	aactcaagagcatatagaagggtg
SW-507	cagtgagcgagtgaaaactacag
SW-540	aaatgggagaaaggaacatccac
SW-541	aggaaagagtgatgggtgaatatg
SW-627	accagcactaaacagaagatcag
SW-628	cccaaccacctatctcttgattctc
SW-631	TCTCCGACCTTTCTTCACACCCATTCC
SW-632	GTCTCCGCTTAGGAGCACGAAAGCTATC
SW-633	cgttatgccttctttgcctttagtg
SW-634	tttatcgattatcgaccgtccag

Appendix 2

Buffers used in this work:

Name	Composition
2xSDS loading buffer	25% (v/v) 4x stacking buffer, 20% (v/v) glycerol, 4% SDS (v/v), 2% (v/v) b-mercaptoethanol, 1%(w/v) bromphenol blue
Edward's extraction buffer	200mM Tris pH 7.5, 10 mM NaCl, 10mM MgCl ₂ , 10% (v/v) glycerol
FAA	50% ethanol, 3.7% formaldehyde, 5% acetic acid
Farmer's fixative	75mL ethanol, 25mL glacial acetic acid
GUS staining buffer	10% Triton, 100mM NaPO ₄ Buffer, 100mM potassium ferrocyanide, 100mM potassium ferricyanide, 100mM X-Gluc (5-bromo-4chloro-3-indyl b-D-glucuronide cyclohexamine salt
LB	1% (w/v) tryptone, 0.5 % (w/v) yeast extract, 1% (w/v) NaCl
LB agar	1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 4.5% (w/v agar)
Low TE buffer	10mM Tris-HCl pH 8.0, 0.1mM EDTA
PBS-T	32 mM NA ₂ PO ₄ , 5 mM KH ₂ PO ₄ , 13 mM KCl, 1.35 M NaCl, 0.05% (v/v) Tween-20
Separating buffer	1.5 M Tris pH 8.8, 0.4% (w/v) SDS
Stacking buffer	0.5 M Tris pH 6.8, 0.4% (w/v) SDS
Transfer buffer	10 mM Tris base, 0.1 M glycine, 10% methanol