



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.

The University of Dublin, Trinity College



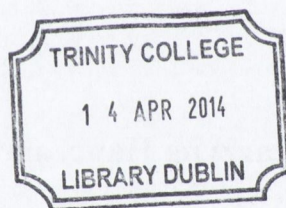
**Transcriptional Regulation by the
Ubiquitin System in the Model Plant
*Arabidopsis thaliana***

Katarzyna Hanczaryk

**A thesis submitted to
The University of Dublin
for the Degree of**

Doctor of Philosophy

**Department of Genetics
Trinity College
University of Dublin
January, 2014**

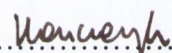


Thesis 10355

Declaration

I hereby declare that this thesis submitted for the degree of Doctor of Philosophy to the University of Dublin, has not been submitted for a degree at this, or any other, university. The work is entirely my own, except where stated.

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 acknowledgement.


.....
Katarzyna Hanczaryk
2014

Motto

“... students arrive from school confident that they know very nearly everything, and they leave years later certain that they know practically nothing. Where did the knowledge go in the meantime? Into the university, of course, where it is carefully dried and stored.”

“The Science of Discworld” Terry Pratchett

Acknowledgements

First and foremost, I would like to thank to my supervisors, Doctor Emmanuelle Graciet and Doctor Frank Wellmer for giving the chance to work on this project and for the valuable guidance, assistance and support over last 4 years.

I would like to express my gratitude to Brenda, Sue, Rachel and Dave for being so helpful and friendly. Paperwork was not so painful with them around.

I convey thanks to Science Foundation of Ireland for providing the financial means and to the Smurfit Institute of Genetics for laboratory facilities.

I thank Dr. Xiang-Wang Deng and Dr. Judy Callis for sharing reagents.

I am so happy that I worked with such great colleagues: Andrea, Bennett, Carol, Darragh, Dearbhaile, Diarmuid, Fran, Gauravi, Kamila, Liina, Sam and Pat. I am grateful for their support and help on daily basis. Special thanks go to: Bennett for helping me with NANNY and GRANNY project; Dearbhaile for being such an excellent student to supervise and for generating some lines which were used in this work; Diarmuid and Sam for very useful comments and suggestions; Fran for our great tea breaks and that due to her warmth I felt like at home in the lab and in Ireland; to Emmanuelle, Kamila and Pat for carrying out the microarray experiments and data analysis.

I would like to thank to Gundi and Agnieszka for interesting chats and to Letal for taking care of my cultural life.

I would like to express my gratitude to my family – beloved Mum, Dad and my brother. I am so grateful that I could always rely on them and that they always believe in me.

Last but not least... Special thanks go to wonderful people without whom I would not go through the most difficult time. To my Mum for all her love, support, patience and being with me whenever I needed her. To Kamila – for her positive energy and fighting back my pessimism. To Ewelina – for her friendship and for our long Skype sessions.

Summary

In eukaryotes, the ubiquitin (Ub) system controls protein stability and regulates many developmental and physiological processes, in part through the regulation of transcription. Recent data show that some components of the Ub system act as *bona fide* transcriptional co-activators by removing from the promoter of target genes ‘spent’ transcription factors that have become inactive after promoting transcription. After degradation of the ‘spent’ transcription factor, a ‘fresh’ and active one binds to the promoter of target genes to maintain transcription. More cases of such regulation by components of the Ub system have been emerged recently, including the first one in plants. Indeed, it has been shown that the protein *UNUSUAL FLORAL ORGANS (UFO)* in the model plant *Arabidopsis thaliana* acts as a transcriptional co-activator of the transcription factor LEAFY (LFY) in the regulation of flower development. *UFO* is an F-box protein, which is part of so-called SCF complexes. The latter are E3 ligases, whose function is to recognize a substrate and target it for degradation in a Ub-dependent manner.

The aim of my Ph.D. was to determine if this type of transcriptional co-regulation by components of the Ub system is widespread, and to identify which biological processes and genes are transcriptionally co-regulated by components of the Ub system. However, before answering these questions, I sought to validate our experimental approach using *UFO* as a proof of concept. In doing so, I also aimed at gaining further insights into the roles of *UFO* as a transcriptional co-regulator. In the first part of my Ph.D., I hence validated the use of two dominant repressor domains (termed SRDX and WUSB) by characterizing plants that expressed *UFO* fused to the SRDX or WUSB domains under the control of the constitutive 35S promoter. The plants I obtained showed phenotypic defects similar to those previously described for plants expressing *UFO*-SRDX under the control of the same promoter. Moreover, plants expressing *UFO* fused to mutated and inactive versions of the dominant repressor domains confirmed that the phenotypic defects were due to the activity of the SRDX or WUSB domain. To identify, at a genome-wide scale, genomic regions bound by *UFO*, I generated a *UFO*-GFP rescue line that will be used for chromatin immunoprecipitation coupled to next-generation sequencing (ChIP-Seq).

To determine if transcriptional co-regulation by components of the Ub system is

a widespread mechanism to control gene expression, I focused on a protein, ASK1, which is part of numerous SCF complexes. I expressed a fusion of ASK1 to the SRDX domain (noted SRDX-ASK1) under the control of the 35S promoter. The phenotypic defects observed indicated that ASK1, and likely ASK1-containing SCF complexes, control diverse aspects of plant development throughout the life cycle of the plant by acting as transcriptional co-regulators. This conclusion was further supported by control experiments that included the expression of ASK1 fused to an inactive version of the SRDX domain, as well as measurements of SCF complex activity that indicated that these complexes remained active upon expression of SRDX-ASK1. In order to gain further insights into the biological pathways that were transcriptionally co-regulated by ASK1, I carried out a preliminary microarray analysis using lines that conditionally express SRDX-ASK1. However, the results of this analysis revealed that the inducible system used led to large artefactual gene expression changes, which I am currently investigating. Finally, to determine regions bound by ASK1 at the genome-wide scale, a ChIP-Seq experiment was carried out and data is currently being analyzed.

Another aim of my Ph.D. was to gain novel insights into the role of two F-box proteins (At4g19865 and At4g19870, termed *NANNY* or *NAN* and *GRANNY* or *GRA*, respectively) that were hypothesized to act redundantly with *UFO*. I generated plants with reduced levels of *NAN* and *GRA* mRNAs, but the resulting mutant did not display any phenotypic abnormalities. In order to test if *NAN* and *GRA* indeed act redundantly with *UFO*, I carried out a genetic interaction analysis after crossing plants with reduced levels of *NAN* and *GRA* mRNAs with different mutant alleles of *UFO*. Contrary to my expectations, the *ufo* mutant phenotype was partially rescued, indicating that *NAN* and *GRA* act as negative regulators of petal and stamen development, and act antagonistically to *UFO*. This was further confirmed by ectopic expression of *GRA*, which resulted in flowers that lacked petals. Because of the potential role of *NAN/GRA* in petal and stamen development, I also analyzed the genetic interaction between plants with reduced levels of *NAN* and *GRA* and plants deficient for the activities of transcription factors that are known to specify these organs, namely *APETALA3* and *PISTILLATA*. The results of this interaction revealed roles of *NAN/GRA* not only in petals and stamens, but also in carpel development.

Abbreviations

3'OCS	octopine synthase terminator sequence
A	adenine
AFB	Auxin Receptor F-box protein
AG	AGAMOUS
AGI	<i>Arabidopsis</i> Genome Initiative
<i>Agrobacterium</i>	<i>Agrobacterium tumefaciens</i>
Ala	alanine
ALF	ABERRANT LEAF AND FLOWER
(a)miRNA	(artificial) microRNA
AP1	APETALA1
AP2	APETALA2
AP3	APETALA3
<i>Arabidopsis</i>	<i>Arabidopsis thaliana</i>
ARF	Auxin Response Factor
Arg	arginine
Asp	aspartic acid
ATGTF2H2	GENERAL TRANSCRIPTION FACTOR II H2
ATOF7	ARABIDOPSIS THALIANA OVATE FAMILY PROTEIN 7
ATORC4	ORIGIN RECOGNITION COMPLEX SUBUNIT 4
ATP	adenosine triphosphate
ATTERT	TELOMERASE REVERSE TRANSCRIPTASE
AUX/IAA	auxin/indole-3-acetic acid
BASTA	a commercial herbicide containing ammonium-glufosinate
BLAST	basic local alignment search tool
bp	base pair

BSA	bovine serum albumin
C	cytosine
ca	carpel
CAL	CAULIFLOWER
CaMV	35S Cauliflower Mosaic virus 35S promoter
CAND1	CULLIN-ASSOCIATED NEDD8-DISSOCIATED 1
ChIP	chromatin immunoprecipitation
ChIP-Seq	chromatin immunoprecipitation combined with ultrahigh-throughput sequencing
CHX	Cycloheximide
Col-0	Columbia-0
CPIC	Complete Protease Inhibitor Cocktail
CRL	cullin-RING Ub ligases
csn	COP9 signalosome
CUL	cullin
Cys	cysteine
d	day
DAT	day after transfer the cold room to long-day growth conditions
dCAP	derived cleaved amplified polymorphism
DEX	dexamethasone
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide trisphosphatase
DOT	DOUBLE TOP
Dr	doctor
dsRNA	double-stranded DNA
DUB	deubiquitylating enzyme
<i>E. coli</i>	<i>Escherichia coli</i>
<i>e.g.</i>	<i>exempli gratia</i>
E6-AP	E6-associated protein

EAR-domain	ERF-associated amphiphilic repression domain
EBF	EIN3-BINDING F-BOX
EDTA	ethylenediaminetetraacetic acid
EIL1	EIN3-LIKE1
EIN3	ETHYLENE INSENSITIVE3
eq	equation
EtOH	ethanol
FIS	floral induction system
FM	floral meristem
FOXO4	Forkhead box O4
G	guanine
GA3	gibberellic acid
GFP	green fluorescent protein
Glu	glutamic acid
Gly	glycine
GO	gene ontology
GR	glucocorticoid
GRA	GRANNY
GUS	β -glucoronidase
hr	hour
HAT3	HOMEBOX-LEUCINE ZIPPER PROTEIN 3
HDAC	histone deacetylase repressor complex
HECT	homologous to E6-AP carboxy terminus
His	histidine
HRP	horseradish peroxidase
<i>ie</i>	<i>id est</i>
IM	inflorescence meristem
IPTG	isopropyl β -D-1-thiogalactopyranoside
IQD27	IQ-DOMAIN 27

JA	jasmonic acid
kb	kilobase
kDa	kilodalton
L-er	Landsberg <i>erecta</i>
LB	Luria Bertrani
LhG4	a chimeric transcriptional activator based on the lac repressor mutant lacI ^{his}
Leu	leucine
LFY	LEAFY
ln	neperian logarithm
LOV	light-oxygen-voltage
LUC	firefly luciferase
LUG	LEUNIG
M	molar
mAmp	miliampere
MBP	Maltose-Binding Protein
MCM8	MINICHROMOSOME MAINTENANCE 8
Met	methionine
mg, mL, mM	milligram, millilitre, millimolar
mg, mL, mM	microgram, microlitre, microgram
mRNA	messenger RNA
MS	Murashige and Skoog medium
mSRDX	mutated and inactive version of SRDX
mWUSB	mutated and inactive version of WUSB
n.d.	not determined
NAN	NANNY
NEB	New England Biolabs
ng, nm, nM	nanogram, nanometer, nanomolar
norm.	normalized
NOS	nopaline synthetase

°C	celcius
OD ₆₀₀	optical density measured at wavelength of 600 nm
Op	optimized lac operator
PAN	PERIANTHIA
PCR	polymerase chain reaction
Pe	petal
PhD	Doctor of Philosophy
Phe	phenylalanine
PI	PISTILLATA
PMI15	PLASTID MOVEMENT IMPAIRED 15
PMSF	phenylmethanesulfonylfluoride
primer	oligonucleotide primer
Pro	proline
PPR	pentatricopeptide repeat-containing
PVDF	polyvinylidene fluoride
RB	right border
RBE	RABBIT EARS
RGA	REPRESSOR OF <i>gal-3</i>
RING	Really Interesting New Gene
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
RT-qPCR	quantitative real-time polymerase chain reaction
SAM	shoot apical meristem
SCF	Skp1/Cullin/F-box
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrilimide gel electrophoresis
Se	sepal
siRNA	small interfering RNA

SOB	super optimal broth
SRDX	EAR repression domain
St	stamen
SUP	SUPERMAN
T	thymine
t1/2	half-life
T-DNA	transferred DNA of the tumour-inducing plasmid
TE	tris-EDTA buffer
TF	transcription factor
Thr	threonine
TIR1	Transport Inhibitor Response 1
Tm	annealing temperature of primers in PCR reactions
TRP6	PHOSPHORIBOSYLANTHRANILATE ISOMERASE 1
Ub	ubiquitin
UBC	Ub-conjugating catalytic domain
UFO	UNUSUAL FLORAL ORGANS
USP7/HAUSP	deubiquitylating enzyme herpesvirus-associated ubiquitin-specific protease
UTR	untranslated region
v/v	volume/volume
w/v	weight/volume
WUSB	WUSCHEL BOX
YEB	yeast extract broth
λ	decay constant

Table of Contents

1. Introduction.....	9
1.1. Protein degradation in eukaryotes	9
1.1.1. Ub-dependent proteolysis	10
1.1.2. Components of the Ub system.....	12
1.1.2.1. E1 enzymes	12
1.1.2.2. E2 enzymes	12
1.1.2.3. E3 enzymes	13
1.1.3. SCF complexes play essential roles in plants.....	16
1.1.3.1. Structure of SCF complexes.....	16
1.1.3.2. Functions of SCF complexes in <i>Arabidopsis</i>	17
1.2. Role of the Ub system in the regulation of transcription.....	19
1.2.1. Non-proteolytic roles of the Ub system in the regulation of transcription.....	20
1.2.1.1. Spatial distribution of transcriptional regulators	20
1.2.1.2. Regulation of transcriptional activator-promoter interaction	20
1.2.2. Proteolytic roles of the Ub system in the regulation of transcription	21
1.2.2.1. Regulation of the abundance of transcription factors	22
1.2.2.2. The Ub system as a co-regulator of transcription	22
1.3. Role of the F-box protein UFO during flower development in <i>Arabidopsis</i>	24
1.3.1. Initiation of flower development.....	24
1.3.2. The ABC model of flower development	24
1.3.3. Functions of LFY and UFO in flower development.....	26
1.4. Aims of the project	29
1.4.1. Investigating the role of UFO as a transcriptional co-activator during flower development.....	29
1.4.2. Genome-wide characterization of processes regulated by SCF complexes acting as transcriptional co-activators.....	31
1.4.3. Functional characterization of the closely related F-box proteins <i>NANNY</i> and <i>GRANNY</i> during <i>Arabidopsis</i> development.....	33
2. Materials and methods.....	35
2.1. Materials.....	35
2.1.1. Strains and plasmids.....	35
2.1.2. Plant material.....	35
2.1.3. Oligonucleotides.....	36

2.2. Methods	39
2.2.1. Microbiology methods	39
2.2.1.1. Preparation of chemically competent <i>E. coli</i> stbl2 and JM109 cells	39
2.2.1.2. Preparation of chemically competent <i>E. coli</i> TP611	40
2.2.1.3. Preparation of competent <i>Agrobacterium tumefaciens</i> cells	40
2.2.1.4. <i>E. coli</i> transformation	41
2.2.1.5. <i>A. tumefaciens</i> transformation	41
2.2.2. Plant-related methods	41
2.2.2.1. Growth conditions	41
2.2.2.2. Flower-dip plant transformation	41
2.2.2.3. Transient expression in <i>Arabidopsis</i> seedlings	42
2.2.2.4. Seed sterilization	42
2.2.2.5. Selection of transformants	42
2.2.2.6. Crosses and isolation of higher order mutants	43
2.2.2.7. Genotyping assays	43
2.2.3. Molecular biology methods	45
2.2.3.1. Plasmid DNA extraction from <i>E. coli</i>	45
2.2.3.2. Plant genomic DNA extraction	45
2.2.3.3. Genomic DNA extraction using the CTAB method	45
2.2.3.4. cDNA synthesis	46
2.2.3.5. PCR-based methods	46
2.2.4. Biochemical methods	47
2.2.4.1. Protein extraction from plants	47
2.2.4.2. Protein expression in <i>E. coli</i>	47
2.2.4.3. Purification of recombinant proteins fused to the maltose-binding protein (MBP)	48
2.2.4.4. Determination of protein concentration using Bradford	48
2.2.4.5. Preparation of protein extracts from nuclei for Western blot analysis	49
2.2.4.6. Protein separation by SDS-PAGE electrophoresis	49
2.2.4.7. Western blot analysis	50
2.3. Epifluorescence microscopy	50
2.4. Data analysis	51
2.4.1. Low-level microarray data processing and basic data handling	51
2.4.2. Higher level microarray analysis	51
2.5. Generation of transgenic plants	52
2.5.1. Isolation of plants expressing UFO fused to dominant repressor	

domains or their mutated versions	52
2.5.1.1. Creation of lines carrying the <i>35S_{pro}:UFO-SRDX</i> or <i>35S_{pro}:UFO-mSRDX</i> constructs	52
2.5.1.2. Generation of transgenic lines expressing the <i>35S_{pro}:UFO-WUSB</i> or <i>35S_{pro}:UFO-mWUSB</i> transgene	53
2.5.2. Isolation of plants with altered ASK1 function	53
2.5.2.1. Creation of lines carrying the <i>ASK1_{pro}:SRDX-ASK1</i> transgene	54
2.5.2.2. Generation of lines carrying the <i>35S_{pro}:SRDX-ASK1</i> or <i>35S_{pro}:mSRDX-ASK1</i> construct.....	54
2.5.2.3. Generation of <i>35S_{pro}:ASK1_{RNAi}</i> to disrupt ASK1 function.....	55
2.5.3. Isolation of complementation lines for ChIP	55
2.5.3.1. Creation of lines carrying the <i>UFO_{pro}:UFO-3xFLAG</i> rescuing construct	55
2.5.3.2. Generation of lines encoding the <i>UFO_{pro}:UFO-GFP</i> transgene.....	56
2.5.3.3. Isolation of lines carrying the <i>ASK_{pro}:GFP-ASK1</i> fusion.....	56
2.5.3.4. Generation of lines encoding the <i>35S_{pro}:GFP-ASK1</i> rescuing construct	57
2.5.3.5. Creation of lines carrying the <i>ASK1_{pro}:6xmyc-ASK1</i> rescue construct	58
2.5.4. Isolation of transgenic lines with the altered <i>NAN</i> and <i>GRA</i> mRNA levels	58
2.5.4.1. Generation of the <i>35S_{pro}:NAN&GRA_{amiRNA}</i> single and <i>nan-1 gra-1 35S_{pro}:NAN&GRA_{amiRNA}</i> triple mutants.....	58
2.5.4.2. Generation of lines carrying the <i>35S_{pro}:GRA</i> transgene	59
3. Role of UFO as a transcriptional co-activator during flower development	61
3.1. Introduction	61
3.2. Results.....	62
3.2.1. Characterization of lines ectopically expressing UFO fused to dominant repressor domains and their mutated versions	62
3.2.2. Isolation of <i>ufo-2</i> rescue lines expressing epitope-tagged versions of <i>UFO</i> for ChIP-Seq analysis	75
3.3. Discussion and future work.....	81
3.3.1. Comparison and suitability of the SRDX and WUSB domains.....	81
3.3.2. New insights into the functions of UFO.....	82
4. Transcriptional co-activation by SCF complexes is a widespread mechanism to control gene expression.....	84
4.1. Introduction	84
4.2. Results	84
4.2.1. Identification of physiological processes that are transcriptionally co-	

regulated by SCF complexes	84
4.2.1.1. Perturbation of ASK1 function using a dominant repressor domain	84
4.2.1.2. Effects of SRDX-ASK1 expression on SCF complex activity.....	94
4.2.1.3. Conclusion.....	103
4.2.2. Identification of genes responding to an inducible expression of SRDX-ASK1	104
4.2.2.1. Generation of plants allowing an inducible expression of SRDX-ASK1	104
4.2.2.2. Identification of genes regulated by SRDX-ASK1.....	109
4.2.3. Identification of a functional RNAi construct to target ASK1 mRNA	117
4.2.4. Genome-wide identification of genomic regions bound by ASK1	120
4.2.4.1. Isolation of lines expressing a functional epitope-tagged version of ASK1	120
4.2.4.2. Identification of genomic regions bound by ASK1 in seedlings.....	124
4.3. Discussion and future work.....	124
4.3.1. Transcriptional co-activation by SCF complexes	124
4.3.2. Future experiments.....	126
5. Characterization of closely related F-box proteins <i>NAN</i> and <i>GRA</i>	130
5.1. Introduction	130
5.2. Results	134
5.2.1. Characterization of plants mutant for <i>NAN</i> and <i>GRA</i>	134
5.2.1.1. Isolation of lines with reduced levels of <i>NAN</i> and <i>GRA</i> mRNAs.....	134
5.2.1.2. Detailed phenotypic characterization of plants with reduced levels of <i>NAN</i> and <i>GRA</i> mRNAs.....	138
5.2.1.3. <i>NAN</i> and <i>GRA</i> act antagonistically to <i>UFO</i>	139
5.2.2. Characterization of lines ectopically expressing <i>GRA</i>	146
5.2.3. Dissection of the genetic interactions between <i>NAN</i> and <i>GRA</i> mutants, and mutant plants for B function genes	148
5.3. Discussion and future work.....	150
6. References	156

Figure and Table List

Page Number is given in brackets.

Chapter 1 – Figures

Figure 1.1 – The Ub-proteasome system. (Page 11)

Figure 1.2 - Regulatory cycles of CRLs by CAND1 and RUBylation/de-RUBylation cycles of the cullin subunit. (Page 15)

Figure 1.3 - Quaternary structure of the human SCF^{Skp2} complex. (Page 17)

Figure 1.4 - Involvement of SCF complexes in auxin response. (Page 19)

Figure 1.5 - Non-proteolytic functions of ubiquitylation in controlling transcription factors. (Page 21)

Figure 1.6 - Two regulatory modes of transcriptional activators by Ub-mediated proteolysis. (Page 23)

Figure 1.7 - The ABC model. (Page 25)

Figure 1.8 - Floral phenotypes of *ufo-2* and *ufo-6* mutant flowers. (Page 27)

Figure 1.9 - Synchronized induction of flower development in the floral induction system. (Page 31)

Figure 1.10 - Experimental design to identify processes transcriptionnally co-regulated by components of SCF complexes. (Page 32)

Chapter 3 – Figures

Figure 3.1 - The sequences of the active and mutated versions of dominant repressor domains used in this study. (Page 62)

Figure 3.2 - Phenotypic comparison of lines expressing the *35S_{pro}:UFO-SRDX/WUSB* transgene or of *ufo* mutants. (Page 64)

Figure 3.3 - Phenotypic comparison of lines encoding *35S_{pro}:UFO-mSRDX/mWUSB* or *35S_{pro}:UFO*. (Page 69)

Figure 3.4 - Results of RT-qPCR experiments for 2 lines expressing the *UFO-mSRDX* fusion from the 35S promoter. (Page 73)

Figure 3.5 - Partial rescue of a *ufo-2* mutant by the introduction of a *UFO_{pro}:UFO-3xFLAG* construct. (Page 76)

Figure 3.6 - Characterization of *ap1-1 cal-1 ufo-2 API_{pro}:API-GR UFO_{pro}:UFO-GFP*. (Page 80)

Chapter 4 - Figures

Figure 4.1 - Results of RT-qPCR experiments for lines expressing the *SRDX-ASK1* fusion. (Page 86)

Figure 4.2 - Mutant phenotypes observed in plants expressing the *SRDX-ASK1* fusion from the 35S promoter. (Page 89)

Figure 4.3 - Expression levels of endogenous *ASK1* and *ASK2* in selected *35S_{pro}:SRDX-ASK1* lines. (Page 91)

Figure 4.4 - Phenotypic alterations observed in line J25 expressing the *mSRDX-ASK1* fusion from the 35S promoter. (Page 92)

Figure 4.5 - Results of RT-qPCR experiments for lines expressing the *mSRDX-ASK1* fusion from the 35S promoter. (Page 93)

Figure 4.6 - Cell-free degradation assays to test the functionality of SCF complexes. (Page 97)

Figure 4.7 - Determination of IAA17-LUC half-life in the presence of (m)*SRDX-ASK1* fusion proteins. (Page 101)

Figure 4.8 - The DEX-inducible system used in this study. (Page 105)

Figure 4.9 - Phenotypes observed in line 523-1 carrying the *35S_{pro}:LhG4-GR/pOp(6x)_{pro}:SRDX-ASK1* transgene. (Page 107)

Figure 4.10 - Kinetics of *SRDX-ASK1* induction following DEX treatment. (Page 109)

Figure 4.11 - Results of RT-qPCR experiments on selected genes to validate the microarray data. (Page 114)

Figure 4.12 - GO terms enriched among differently expressed genes. (Page 116)

Figure 4.13 - Characterization of an RNAi construct specific for *ASK1*. (Page 119)

Figure 4.14 - Configuration of the *GFP-ASK1* rescue constructs. (Page 122)

Figure 4.15 - Characterization of a 6xmyc-*ASK1* rescue line. (Page 123)

Figure 4.16 - Strategy to identify high-confidence *ASK1* direct targets. (Page 128)

Chapter 5 - Figures

Figure 5.1 - Identification of candidate genes that act redundantly with *UFO*. (Page 132)

Figure 5.2 - Expression levels of endogenous *NAN* and *GRA* in wild-type plants. (Page 134)

Figure 5.3 - Characterization of lines mutant for *NAN* and *GRA*. (Page 136)

Figure 5.4 - The floral phenotypes of weak and intermediate mutant alleles of *UFO*. (Page 141)

Figure 5.5 - Characterization of the *ufo-1 nan-1 gra-1* double mutant. (Page 142)

Figure 5.6 - Phenotypic characterization of *ufo-2 35S_{pro}:NAN&GRA_{amiRNA-1}* double mutants. (Page 145)

Figure 5.7 - Characterization of lines ectopically expressing *GRA*. (Page 147)

Figure 5.8 - Phenotypic comparison of the *nan-1 gra-1*, *pi-1*, *ap3-3* single mutants and the *nan-1 gra-1 ap3-3* and *nan-1 gra-1 pi-1* double mutants. (Page 149)

Figure 5.9 - Model of the role of *NAN/GRA* during flower development. (Page 155)

Chapter 2 - Tables

Table 2.1 - List of lines used in this work. (Page 35)

Table 2.2 - List of oligonucleotides used in this study. (Page 36)

Chapter 3 - Tables

Table 3.1 - Example of the frequency of different floral organ types in plants expressing *UFO-WUSB* from the 35S promoter. (Page 66)

Table 3.2 - Number of plants in each of the different phenotypic categories for lines carrying the *35S_{pro}:UFO-SRDX* transgene in the T2 generation. (Page 67)

Table 3.3 - Number of plants in each of the different phenotypic categories for lines carrying the *35S_{pro}:UFO-WUSB* transgene in the T1 generation. (Page 67)

Table 3.4 - Example of the frequency of different organ types in lines expressing the *35S_{pro}:UFO-mSRDX* or *35S_{pro}:UFO-mWUSB*. (Page 71)

Table 3.5 - Number of plants in each of the different phenotypic categories for lines carrying the *35S_{pro}:UFO-mSRDX* transgene in the T2 generation. (Page 72)

Chapter 4 - Tables

Table 4.1 - Assignment of different lines to phenotypic categories. (Page 87)

Table 4.2 - Summary of the different half-lives obtained for MBP-RGA. (Page 98)

Table 4.3 - Summary of the hybridizations carried out for the gene expression profiling of inducible SRDX-ASK1 lines. (Page 110)

Table 4.4 - Number of differentially expressed genes. (Page 111)

Table 4.5 - Number of differentially expressed genes after correction. (Page 113)

Chapter 5 - Tables

Table 5.1 - Results obtained using Expression Angler with *UFO* as a query. (Page 131)

Table 5.2 - Summary of the double mutants generated to study genetic interactions between *NAN*, *GRA* and *UFO*. (Page 139)

Table 5.3 Summary of other mutants crossed into the *nan-1 gra-1* mutant. (Page 153)

1. Introduction

1.1. Protein degradation in eukaryotes

“Everything flows, nothing stands still” said Heraclitus of Ephesus. He claimed that ever-present change is central to the universe. This notion applies to everything, including biological systems such as a cell’s proteome. It is known that the proteome is in a dynamic state of synthesis and degradation, which was shown for the first time in the late 1930s by Schoenheimer (Hochstrasser, 1996)). Nowadays, it is well established that protein turnover is crucial for the regulation of about every biological process in all organisms (reviewed in (Hochstrasser, 1996)).

The first pathway shown to mediate protein degradation involved specific organelles, termed lysosomes, in eukaryotes. For example, it has been shown that extracellular proteins such as blood coagulation factors, immunoglobins, albumin, or peptide hormones are degraded in primary lysosomes after their internalization *via* a series of vesicles that fuse with these organelles (reviewed in (Glickman and Ciechanover, 2002)). However, lysosomal protein degradation is not sufficient to explain the turnover of many other proteins. Indeed, it has been observed that even when lysosome-dependent proteolysis is inhibited, a significant group of proteins, mostly intracellular ones, is still hydrolyzed (reviewed in (Glickman and Ciechanover, 2002)). Moreover, these proteins have different half-life times that may also change in response to exogenous stimuli, whereas degradation in lysosomes occurs approximately at the same rate. Finally, in the 1970s it was discovered that some proteins required ATP to undergo hydrolysis (Hershko et al., 1980), which is in contrast with the ATP-independent proteolysis of lysosomes. All together, these observations suggested that a lysosome-independent pathway for protein degradation must exist.

Biochemical studies in a cell-free system from rabbit reticulocyte extracts, that are known to lack lysosomes, provided evidence for the existence of the hypothetical non-lysosomal mechanism of protein degradation (Etlinger and Goldberg, 1977). Further molecular and genetic analyses showed that this novel pathway of protein degradation required the highly conserved 76-residue protein ubiquitin (Ub) (Ciechanover et al., 1978).

1.1.1. Ub-dependent proteolysis

Ub-dependent proteolysis is a conserved process among eukaryotes (Hershko et al., 1980). The action of 3 enzymes is necessary to covalently attach Ub to a substrate protein (Hershko et al., 1983). Biochemical studies in rabbit reticulocytes have revealed that a Ub-activating enzyme (also known as E1) activates a Ub molecule in an ATP-dependent reaction (Figure 1.1). Successively, Ub is transferred from the E1-Ub intermediate to a Ub-conjugating enzyme (also termed E2), which results in the formation of E2-Ub intermediate. In the next step, the E2 enzyme catalyzes the transfer of activated Ub to a Ub ligase (or E3), which can recognize specific degradation signals in a substrate protein. Formation of an E2-E3 complex can lead to the generation of a E3-Ub intermediate in some cases, or to the direct covalent attachment of Ub to the protein substrate bound by the E3 enzyme (Deshaies and Joazeiro, 2009). In most cases, the covalent Ub-substrate isopeptide bond is generated between the C-terminal glycine of Ub and the ϵ -NH₂ group of an internal lysine residue in the substrate protein. A poly-Ub chain is generated by adding successive activated Ub moieties to the substrate. It was shown that a poly-Ub chain comprising at least four molecules of isopeptide-linked Ub is necessary for efficient protein recognition and degradation by a multisubunit protease termed the 26S proteasome (Thrower et al., 2000).

The 26S proteasome is composed of 2 sub-complexes. The 20S particle constitutes the cylindrical-shaped core of the complex that exhibits different protease activities (Tanaka, 2009). There are 6 proteolytic sites hidden inside the barrel of the 20S particle with a very narrow channel leading to them. Therefore, prior to entering the degradation chamber, the substrate must be recognized, unfolded and translocated into the 20S particle. These steps are carried out by the 19S particle, which has an ATPase activity essential for protein unfolding and channeling into the 20S particle (Smith et al., 2005; Liu et al., 2006). At the same time, the poly-Ub chain is removed by deubiquitylating enzymes (Hershko et al., 1980). The tight control of protein degradation through specific degradation signals and Ub conjugation is essential, as the 26S proteasome is not sequestered in a specific organelle and could thus degrade most accessible proteins (Nussbaum et al., 1998).

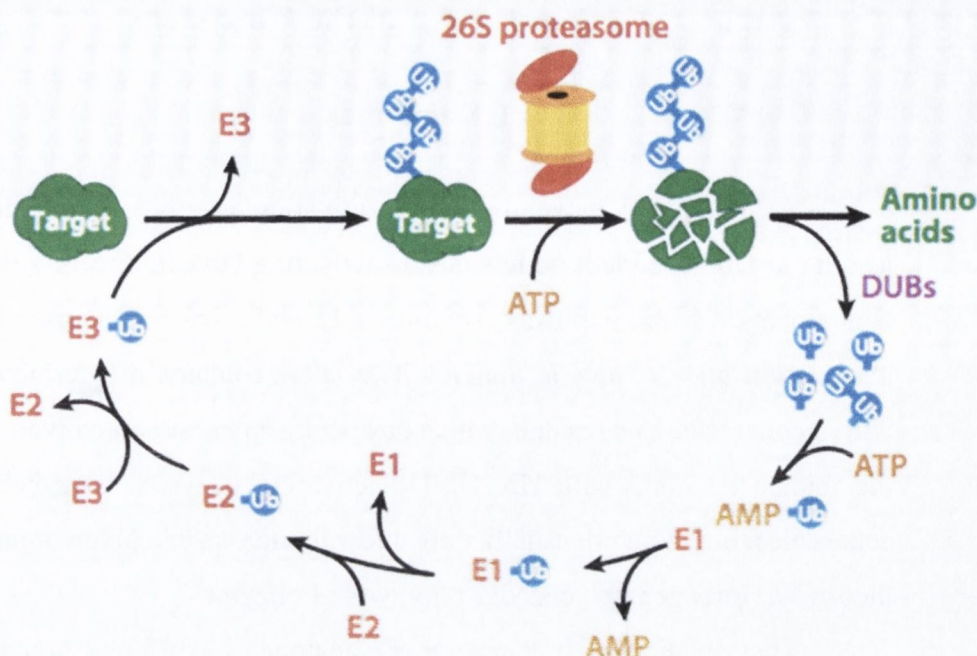


Figure 1.1 The Ub-proteasome system. The pathway begins with an ATP-dependent activation of Ub and the formation of a E1-Ub complex. This is followed by the transfer of Ub from the E1-Ub intermediate to a Ub-conjugating enzyme (E2). Next, the Ub ligase (E3) catalyzes the transfer of the Ub moiety to a substrate protein. After iterative conjugation of Ub, the poly-Ub chain attached to the substrate protein is recognized by the 26S proteasome, which leads to the ATP-dependent degradation of the ubiquitylated protein. The Ub molecules are released by deubiquitylating enzymes (DUBs) and can be reused in the next cycle. Figure adapted from (Hua and Vierstra, 2011).

The long list of substrates degraded *via* the Ub-proteasome system includes proteins involved in many cellular processes. For example, cell cycle regulators such as cyclins and cyclin-dependent kinase inhibitors are degraded in a Ub-dependent manner. It is also known that mutated, denaturated or misfolded proteins are eliminated from the cell by the Ub system. Finally, Ub-dependent proteolysis plays an important role in the regulation of transcription by being involved in the processing of transcription factors and co-factors and by regulating chromatin modifications, as well as post-translational modifications of histones (Section 1.2) (reviewed in (Glickman and Ciechanover, 2002)). To date, the molecular mechanisms underlying the role of Ub-dependent proteolysis in transcriptional regulation are understood in numerous cases. However, recently, novel models have been proposed to explain the role of the Ub system in the regulation of transcription. The aim of my Ph.D. project is to bring some insights into these new roles of the Ub system in the regulation of transcription, using the plant *Arabidopsis thaliana* as a model organism.

1.1.2. Components of the Ub system

1.1.2.1. E1 enzymes

As mentioned above, the E1 Ub-activating enzyme initiates the cascade of events leading to Ub-dependent proteolysis by activating Ub and directing it to the downstream components of the pathway (Figure 1.1). E1s have 2 conserved domains – an adenylation and a catalytic domain. The latter contains a C-terminal Ub-fold domain with a conserved Cys residue, which has been shown to be involved in the formation of the thiol ester bond with Ub. E1 Ub-activating enzymes have been identified in all eukaryotes, but there are usually only a few homologs in a given organism. For example, the *Arabidopsis* genome encodes only two E1 enzymes.

The isolation of mutants for E1 enzymes in different organisms has provided insights into the functions of these proteins. It has been shown that a mutation in the mammalian E1, *UBA1*, results in an arrest of the cell cycle at the G2/M phase transition (McGrath et al., 1991). Also, the yeast *UBA1* and *UBA2* genes have been found to be essential for growth, as double mutant yeast for these genes showed complete inhibition of the Ub-proteasome pathway, which resulted in the stabilization of multiple substrates (Ciechanover, 1994; Dohmen et al., 1995). Finally, it has been shown that the *Arabidopsis* E1 *AtUBA1* is involved in the response to pathogens (Goritschnig et al., 2007). Taken together, these observations show that Ub-activating enzymes are essential for a wide range of processes, as expected given their role in the Ub-proteasome system.

1.1.2.2. E2 enzymes

The next step in the conjugation of Ub to a substrate protein is the transfer of the activated Ub molecule from an E1 to an E2 Ub-conjugating enzyme (Figure 1.1). E2s share a highly conserved 150-200-residue Ub-conjugating catalytic (UBC) domain. The UBC domain contains a conserved Cys residue which accepts the Ub moiety via a thiol ester bond. Apart from the UBC fold, E2s may have additional extensions on their N- or/and C-termini. Ub-conjugating enzymes have been classified based on the existence of such domains: class I E2s do not have any extensions, while class II and III E2s contain an N- or C-terminal extension, respectively. Class IV E2s carry extensions on both their N- and C-termini. These extensions appear to be important for the role of an E2. For example, it has been shown that the C-terminal domain of the class III E2 Cdc34

is necessary for yeast viability and cell cycle progression, as its phosphorylation regulates its interaction with an E3 enzyme that regulates the G1-S phase transition in yeast (reviewed in (van Wijk and Timmers, 2010)).

1.1.2.3. E3 enzymes

An E3 ligase binds an E2-Ub conjugate and a protein substrate to catalyze the transfer of Ub to the substrate. After transfer of Ub, the E2 dissociates from the E3 ligase, so that a fresh E2-Ub conjugate may be bound by the E3 ligase.

The step catalyzed by E3 ligases is essential for the selection of protein substrates that should undergo degradation. Therefore, the tight regulation of E3 ligase activity is crucial. A first layer of regulation occurs at the substrate level. For example, in some cases substrates have to undergo phosphorylation prior to recognition by an E3 ligase and ubiquitylation (Lahav-Baratz et al., 1995). Conversely, a phosphorylated substrate may no longer be recognized by an E3, hence preventing its degradation by the Ub-proteasome system (Zachariae et al., 1998). The other type of regulation occurs at the E3 ligase level. Namely, it has been shown that some E3s undergo autoubiquitylation, followed by their proteasome-dependent degradation upon the absence of their substrate (Lai et al., 2002; Jones and Gellert, 2003). Moreover, E3s are known to undergo homo- or heterodimerization before binding the substrate (reviewed in (Hua and Vierstra, 2011)). Finally, the function of some E3s can be regulated by binding small molecules (reviewed in (Deshaies and Joazeiro, 2009)). For example, it has been shown that an interaction between the E3 ligase UBR1 and a dipeptide may enhance its Ub ligase activity towards a certain types of substrates (Turner et al., 2000). Other molecules known to be involved in the regulation of E3 ligase activity are hormones, which have been shown to stabilize the substrate-E3 interaction upon binding to an E3 ligase (reviewed in (Deshaies and Joazeiro, 2009)).

Genomes of eukaryotes usually encode significantly more E3 Ub ligases than other components of the Ub system. Moreover, E3 Ub ligases are the most diverse group among enzymes involved in the Ub-proteasome system, which is not surprising considering the role of E3s in substrate recognition. Despite the considerable structural and functional variety of E3s, they have been classified in only 2 main groups: E3s homologous to E6-AP carboxy terminus (also termed HECT-domain E3s) and “Really Interesting New Gene” E3s (also known as RING motif-containing E3s).

Ubiquitylation of a substrate by a HECT domain E3 ligase requires the formation

of a thioester bond between the E3 ligase and Ub before this molecule is transferred to the substrate protein. The conserved 350-residue HECT domain was initially identified in a human E3 ligase termed E6-associated protein (E6-AP), which is required for the degradation of the tumor suppressor p53. (Scheffner et al., 1993; Huibregtse et al., 1995). Although the *Arabidopsis* genome is thought to encode approximately 7 HECT-domain E3s, not much is known about their role in plants. To date, it has only been reported that these enzymes are involved in trichome development (Downes et al., 2003) and in leaf senescence (Petroski and Deshaies, 2005; Miao and Zentgraf, 2010).

Unlike HECT domain E3s, RING motif-containing E3 ligases do not require the formation of a thioester bond with Ub before transferring Ub to the protein substrate. The RING motif is defined by a pattern of conserved Cys and His residues that bind 2 Zn^{2+} ions and is required for the E2-E3 interaction (reviewed in (Deshaies and Joazeiro, 2009)). Although many RING-domain E3 ligases are single polypeptide proteins, it has been shown that a subset of these RING-domain proteins, such as RBX1/Roc1, RBX2/Roc2 and APC, act as part of multi-subunit E3 ligases. The best characterized and largest known class of such complexes are termed cullin-RING Ub ligases or CRLs (reviewed in (Bosu and Kipreos, 2008)). These complexes comprise different subunits, including a RING-domain protein, a cullin (noted CUL), as well as a substrate-recognition subunit and an adaptor that links the substrate-recognition domain to the rest of the complex.

The regulation of CRL activity is complex and involves covalent and non-covalent binding of regulatory proteins. The CULLIN-ASSOCIATED NEDD8-DISSOCIATED 1 (CAND1) protein is involved in one cycle of CRL regulation (Figure 1.2). CAND1 inhibits an assembly of CRL by transient and reversible binding to the CUL-RBX1 subcomplex. The U-shaped structure of CAND1 works as a clamp (Goldenberg et al., 2004), in that the C-terminal domain of CAND1 binds the CUL subunit, preventing it from interacting with the rest of the complex. Secondly, the N-terminal region of CAND1 prevents a modification of the CUL subunit, which is necessary to regulate CRL activity. This modification corresponds to the conjugation of the Ub-like protein Nedd8 (also known as RUB1 in plants) or its removal by the COP9 signalosome (CSN). Indeed, through the action of the RUB1-specific E1 and E2 enzymes, RUB1 is conjugated to the CUL subunit (del Pozo and Estelle, 1999; Gray et al., 2002; Lechner et al., 2002), leading to the activation of the CRL. Following substrate ubiquitylation, the CRL complex associates with the CSN, which removes RUB1 from the CUL subunit. De-RUBylation of CUL, leads to the dissociation of the

CUL/RBX sub-complex from the rest of the CRL and allows it to enter a new cycle of CAND1 binding or reassembly with other CRL components (Hua and Vierstra, 2011).

CRLs may form different types of complexes depending on the subunits involved. In fact, many alternative substrate-recognition subunits were identified, including those that contain an F-box, a SOCS box, a VHL box, a DWD box, or a BTB domain (reviewed in (Petroski and Deshaies, 2005)). A well-known example of a CRL is a Skp1/Cullin/F-box (SCF) complex, in which the substrate-recognition subunit is a protein with an F-box domain. SCF complexes are the focus of my work, and hence they will be described in more detail below.

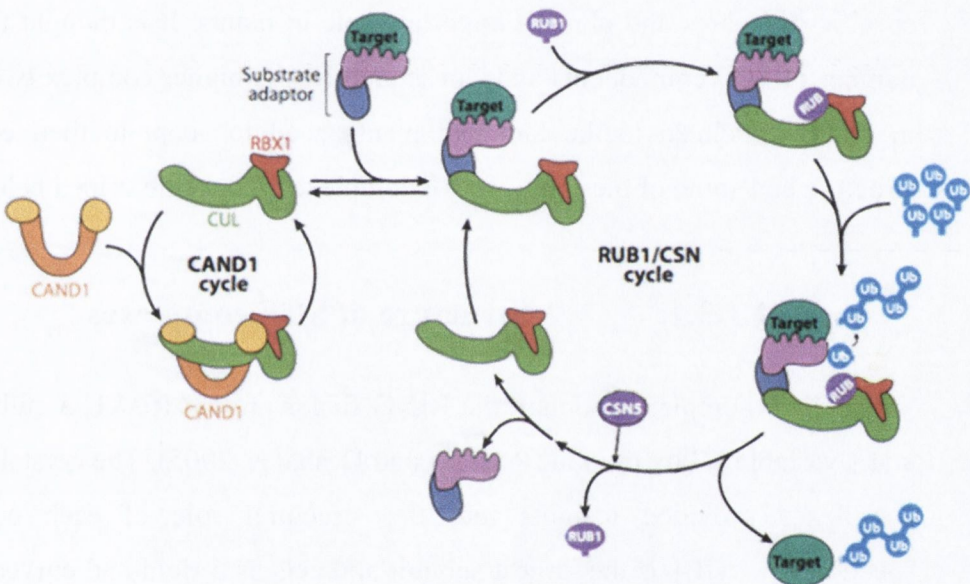


Figure 1.2 Regulatory cycles of CRLs by CAND1 and RUBylation/de-RUBylation cycles of the cullin subunit. CAND1 regulates the pool of CRLs by reversible and transient binding of a CUL-RBX1 sub-complex, which prevents the assembly of a functional CRL. The substrate-adaptor sub-complex recruits a target protein and binds to the CUL/RBX1 sub-complex that is not bound by CAND1, forming a CRL complex. This complex then enters the RUB1/CSN cycle, in which RUB1 is conjugated to the CUL subunit, leading to activation of the CRL complex and ubiquitylation of the substrate protein. Finally, the CRL complex associates with the COP9 signalosome (CSN) that has de-RUBylating activity. The CUL-RBX sub-complex and the substrate adaptor may be reused in a new cycle. Figure adapted from (Hua and Vierstra, 2011).

1.1.3. SCF complexes play essential roles in plants

Genome-wide analysis across different organisms have shown that the genomes of plants encode a much higher number of SCF components compared to the genomes of yeast and animals. For example, in the case of plants such as *Arabidopsis thaliana*, *Brachypodium distachyon*, *Medicago truncatula* or rice around 1,000 genes encoding F-box proteins have been identified in each of these plants (Hua et al., 2011). In contrast, the genomes of the yeast *Saccharomyces cerevisiae*, the fly *Drosophila melanogaster* or human appear to encode about 20, 27 and 69 F-box proteins, respectively (Skaar et al., 2009). Such a significant difference suggests that SCF complexes are involved in plant-specific processes and play an important role in plants. It is thought that the increased number of SCF components and, consequently, the higher complexity of the Ub system in plants is related to the fact that plants need to adapt to their environment. The structure and some of the roles of SCF complexes will be described below.

1.1.3.1. Structure of SCF complexes

SCF complexes contain the RING finger protein RBX1, a cullin (CUL1), Skp1 and a variable F-box protein (Petroski and Deshaies, 2005). The crystal structure of SCF complexes provided insights into the structural role of each component. More specifically, CUL1 is the largest subunit and acts as a rigid and curved scaffold for the complex (Figure 1.3) (Zheng et al., 2002). The N-terminal domain of CUL1 interacts with the adaptor protein Skp1, while the C-terminal domain of CUL1 forms a V-shaped cleft for RBX1 binding. The latter facilitates the recruitment of an E2 to the complex (Kawakami et al., 2001). The F-box protein comprises a tri-helical fold that forms an interlocked interface with a broad shallow pocket in Skp1 (Schulman et al., 2000). Moreover, the F-box protein confers its specificity to an SCF complex, as it is the F-box protein that recognizes specific degradation signals in a substrate protein. Substrate recognition is mediated by additional domains, which are usually involved in protein-protein interactions (*e.g.* LRR, Kelch repeats, Tubby, lectin-like and light-oxygen-voltage (LOV) domains) (Gagne et al., 2002; Yang et al., 2008; Xu et al., 2009; Hua et al., 2011). Therefore, it is not surprising that F-box proteins are the most diverse component of SCF complexes. For example, around 700-900 F-box proteins are predicted to be encoded in the *Arabidopsis* genome (Hua and Vierstra, 2011), whereas

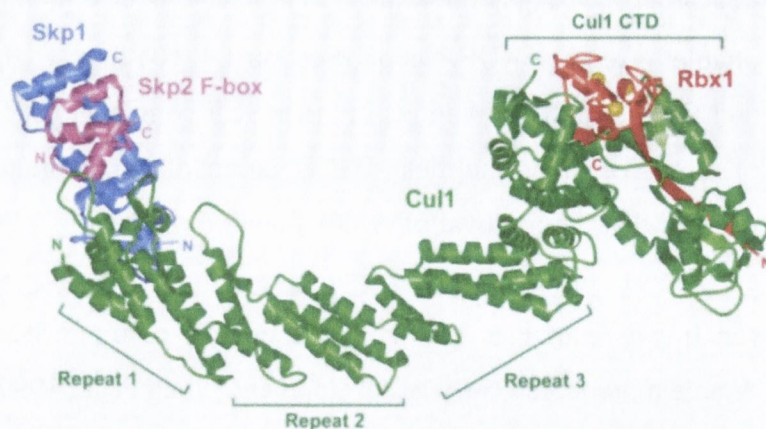


Figure 1.3 Quaternary structure of the human SCF^{Skp2} complex. The CUL1 subunit acts as a scaffold for the SCF complex with two binding sites: N-terminal for Skp1 and C-terminal for Rbx1. The adaptor protein Skp1 connects the F-box protein (here Skp2) that is responsible for substrate recognition with the rest of the complex. The function of Rbx1 is to facilitate binding of the E2 Ub-conjugating enzyme. Figure from (Zheng et al., 2002).

there are only 2 RBX subunits, 10 CUL proteins and 21 SKP1 homologs (named ASKs in *Arabidopsis*) (Gagne et al., 2002).

Theoretically, the number of possible SCF complexes is great as it is known that one particular ASK may interact with many different F-box proteins (Kuroda et al., 2012). However, there are a few factors limiting the potential number of SCF complexes. Firstly, it is likely that some proteins have to undergo some post-translational modifications (*e.g.* phosphorylation) (Lin et al., 2009) prior to assembly the functional SCF complex. Secondly, the presence of co-factors *in vivo* may be necessary for the assembly of functional SCF complex. Finally, ASKs and F-box proteins show a variety of expression patterns. Consequently, not every ASK is co-expressed with each F-box protein. Despite these restrictions, the number of reports on the multitude of biological functions of different SCF complexes in plants is still growing.

1.1.3.2. Functions of SCF complexes in *Arabidopsis*

There are many cellular and developmental processes that SCF components are involved in (reviewed in (Lechner et al., 2006)). For example, it has been shown that mutations in either proteins regulating SCF function or in SCF subunits such as RBX or CUL often result in pleiotropic and severe phenotypes, indicating that this class of E3

ligases is of special importance. Moreover, it was reported that *csn* mutants that are unable to remove RUB/Nedd8 (Section 1.1.2.3) from CUL1 are seedling lethal (Hotton and Callis, 2008). Mutations in other SCF component such as ASKs also lead to some phenotypical abnormalities. The isolation of a null mutant for *ASK1* (noted *ask1-1*) showed that the removal of ASK1 function resulted in a loss of pollen fertility (Zhao et al., 1999). Further genetic studies showed that this weak phenotype was due to functional redundancy with other members of the ASK family, as an *ask1-1 ask2-1* double mutant died early in development (Yang et al., 1999; Zhao et al., 1999).

A growing number of studies have provided insights into the role of another SCF component – the F-box protein. As mentioned above, the *Arabidopsis* genome is encodes around 900 different F-box proteins, indicating that they are likely to regulate a multitude of processes. This has been largely confirmed by the isolation of mutants for F-box proteins, which showed that they are involved in different phytohormone signaling pathways (*e.g.* auxin, jasmonic acid, strigolactone, ethylene and gibberellins), as well as in the regulation of cell division and developmental processes such as flower development. F-box protein and associated SCF complexes have also been shown to be involved in the response to environmental parameters such as light signaling, drought resistance, phosphate starvation, and the response of plants to pathogens (reviewed in (Hua and Vierstra, 2011)). Despite this multitude of functions, only about 50 F-box proteins have been functionally characterized so far. The identification of substrates that are recognized by them has been even more difficult, with only approximately 20 targets described to date (reviewed in (Hua and Vierstra, 2011)).

The function of SCF complexes is probably best known in plants in the case of the phytohormone auxin. Indeed, the role of F-box proteins in the response to phytohormones was first discovered by isolating mutant *Arabidopsis* plants that were deficient for their response to auxin (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Over the years, the molecular mechanism underlying auxin response and the role of SCF complexes has been unraveled. In the absence of auxin, the members of the family of auxin/indole-3-acetic acid (Aux/IAA) proteins bind and repress the activity of Auxin Response Factor (ARF) proteins, which are transcriptional activators of auxin-inducible genes (Figure 1.4) (Ulmasov et al., 1997; Tiwari et al., 2001; Tiwari et al., 2004). Upon the increase of auxin level, the interaction between the F-box protein Transport Inhibitor Response 1 (TIR1), or the related F-box proteins Auxin Receptor F-box Proteins (AFB1, AFB2 and AFB3), and their Aux/IAA targets is facilitated (Dharmasiri et al., 2005). The subsequent Ub-dependent degradation of Aux/IAA

proteins leads to the derepression of ARF transcription factors and to auxin-mediated transcriptional changes. The latter example also illustrates the involvement of the Ub system in the regulation of transcription, which is the main focus of my work. However, many different cases of Ub-dependent transcriptional regulation have been reported to date, which I will discuss more in detail below.

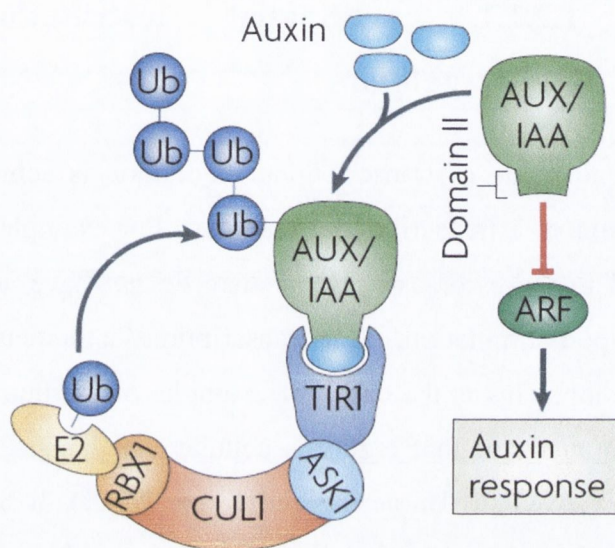


Figure 1.4 Involvement of SCF complexes in auxin response. Auxin signaling involves the ubiquitylation of Aux/IAA proteins by the SCF^{TIR1} E3 ligase. The F-box protein TIR1 binds the Aux/IAA proteins and also acts as an auxin receptor. Figure from (Vierstra, 2009).

1.2. Role of the Ub system in the regulation of transcription

It has been shown that gene expression is mainly controlled at the stage of transcription. The generation of proper RNA transcripts requires several steps, such as the formation of a pre-initiation complex, elongation, termination and polyadenylation. Additionally, processes such as the regulation of transcription factors or co-factors, and of chromatin modifications are also necessary for correct gene expression. It is therefore not surprising that the transcriptional machinery comprises many proteins and that the regulation of transcription is of high complexity. A growing number of studies have provided insights into the roles of the Ub system in the regulation of transcription.

However, in many cases the exact molecular mechanism awaits further investigation.

1.2.1. Non-proteolytic roles of the Ub system in the regulation of transcription

1.2.1.1. Spatial distribution of transcriptional regulators

One mode of transcriptional regulation is achieved by controlling the spatial localization of a transcriptional regulator. For example, some transcription factors are localized in the cytoplasm, where they do not have access to the promoter of target genes. Upon stimulation, these transcription factors can enter the nucleus and activate transcription. This is the case, for example, of the human Forkhead box O4 (FOXO4) transcription factor that regulates cellular metabolism, cell-cycle progression and cell death (reviewed in (Burgering and Kops, 2002)). It has been shown that FOXO4 is localized in the cytoplasm but upon increased cellular oxidative stress, FOXO4 undergoes mono-ubiquitylation followed by its translocation to the nucleus and subsequent transcriptional activation of FOXO4's target genes (Figure 1.5A). Moreover, the activity of the deubiquitylating enzyme herpesvirus-associated ubiquitin-specific protease (USP7/HAUSP) deubiquitylates FOXO4, which leads to its exclusion from the nucleus (van der Horst et al., 2006).

1.2.1.2. Regulation of transcriptional activator-promoter interaction

The Ub system may also affect the binding of a transcriptional activator to the promoter of its target genes in a non-proteolytic manner. For example, in yeast, the activation domain of the transactivator Gal4 is responsible for recruiting the ubiquitylation machinery to the promoter of target genes (Figure 1.5B). This leads to mono-ubiquitylation of the DNA binding domain of Gal4 and to the recruitment of the transcriptional machinery. It was also shown that mono-ubiquitylation and interaction of Gal4 with Rpt4 and Rpt6 (2 subunits of the 19S particle) protects Gal4 from degradation (Archer et al., 2008).

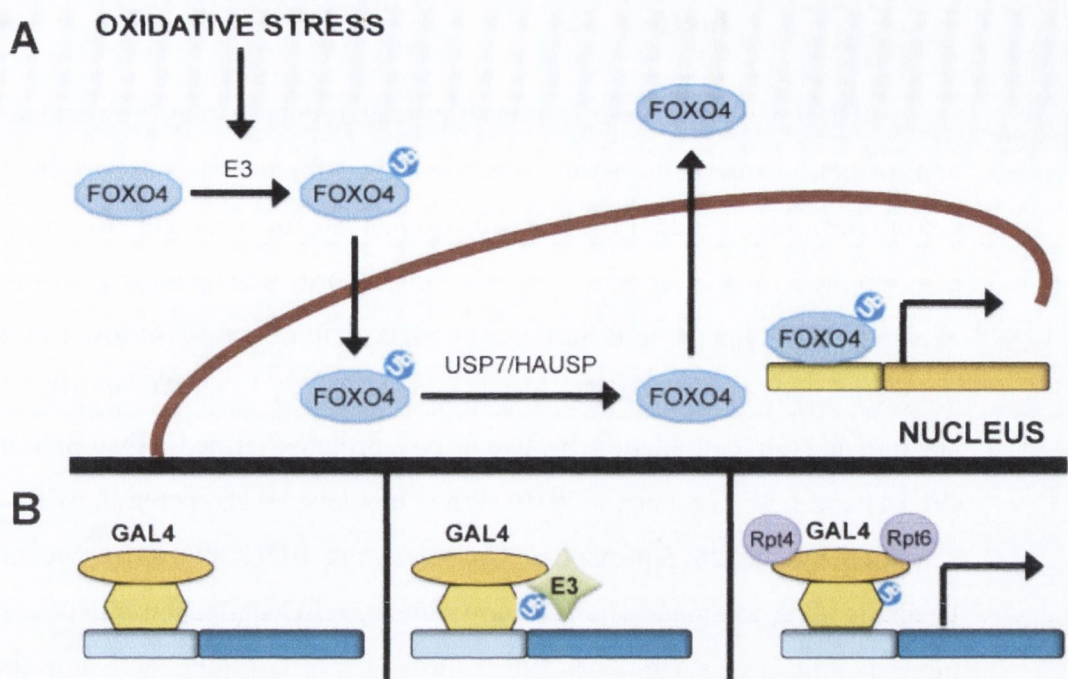


Figure 1.5 Non-proteolytic functions of ubiquitylation in controlling transcription factors. (A) Regulation of spatial distribution of a transcriptional regulator. In response to the cellular oxidative stress transcriptional regulator FOXO4 becomes monoubiquitylated. Next, the monoubiquitylated transcription factor is translocated to the nucleus where either activates transcription of its target genes or becomes deubiquitylated by USP7/HAUSP and excluded from the nucleus. **(B)** Activation by mono-ubiquitylation. Transcription factor Gal4 is mono-ubiquitylated on the promoter of target genes followed by activation of transcription. It has been shown that mono-ubiquitylation and interaction with the proteasomal subunits Rpt4 and Rpt6 is necessary to maintain transcription.

1.2.2. Proteolytic roles of the Ub system in the regulation of transcription

Besides the protein degradation-independent roles of the Ub system described above for the control of transcription, a growing number of studies have provided insights into proteolytic roles of the Ub system in transcriptional regulation as well. Two general mechanisms have been proposed (Geng et al., 2012), depending on whether transcriptional regulation requires binding of components of the Ub system to chromatin or not.

1.2.2.1. Regulation of the abundance of transcription factors

The level of a specific transcriptional regulator may be regulated in order to control gene expression, as has been described for the response to the phytohormone auxin (Section 1.1.3.2). In fact, such mechanism for the regulation of transcription is also required for the regulation of other phytohormone signaling pathways, such as for example that of the gaseous hormone ethylene (Figure 1.6A). At low ethylene levels, the transcriptional activators ETHYLENE INSENSITIVE3 (EIN3) and EIN3-LIKE1 (EIL1) are targeted for degradation by two F-box proteins termed EIN3-BINDING F-BOX1 (EBF1) and EBF2 (An et al., 2010). When ethylene levels increase, EBF1 and EBF2 are degraded by the 26 proteasome. Consequently, EIN3 and EIL1 accumulate to high levels, leading to an activation of downstream ethylene-response genes. To date, many more examples of a Ub-dependent control of transcriptional activator abundance have been reported (Maxwell et al., 1999; Zhu and Bunn, 2001; Baek et al., 2005).

1.2.2.2. The Ub system as a co-regulator of transcription

Intriguingly, it has also been reported that the degradation of some transcriptional activators is required for transcriptional activation. It has been proposed that a 'spent' transcriptional activator that remains bound to the promoter of target genes is no longer able to activate the transcription of its target genes and needs to be degraded and replaced by a 'fresh' transcriptional activator in order to maintain transcription. Only two examples of such a mode of regulation had been described in *Arabidopsis* until very recently (Chae et al., 2008; Inigo et al., 2012), but the number of cases is continuously growing. For example, in a recent report, it has been shown that the regulation of some jasmonic acid (JA) response genes occurs in this manner (Zhai et al., 2013) (Figure 1.6B), as a master regulator of the JA signaling pathway, MYC2, was shown to be phosphorylated, ubiquitylated and degraded by the 26S proteasome. Importantly, degradation of MYC2 occurs on the chromatin during the process of gene activation and MYC2 turnover appears to be necessary to maintain the transcription of target genes.

Another example of a component of the Ub system that plays a role as a transcriptional co-regulator in *Arabidopsis* is the F-box protein UNUSUAL FLORAL

ORGANS (UFO), which is involved in the regulation of flower development. Because part of the work I have carried out for my thesis focused on UFO, a more detailed introduction on flower development and the role of UFO in this process is described in the next section.

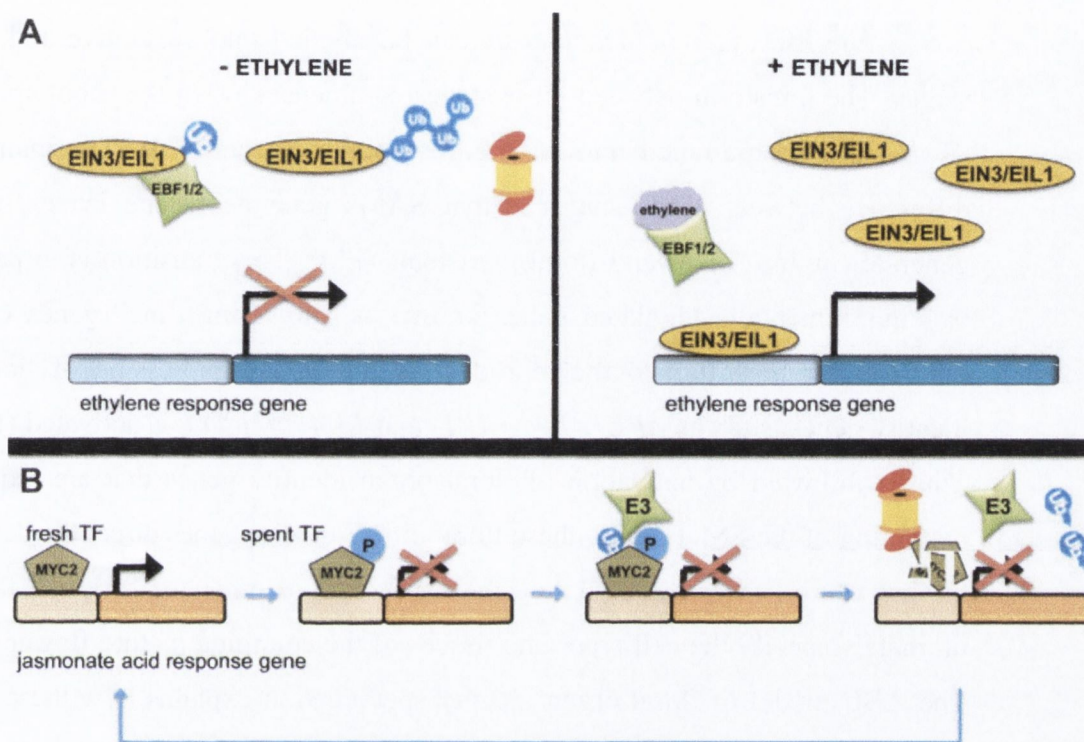


Figure 1.6 Two regulatory modes of transcriptional activators by Ub-mediated proteolysis. (A) Regulation of the abundance of a transcriptional activator. In the absence of the plant hormone ethylene, the transcription factors EIN3 and EIL1 are targeted by SCF^{EBF1/2} for degradation. In contrast, when EBF1/2 bind ethylene, EBF1/2 are degraded, leading to the accumulation of EIN3/EIL1. Consequently, ethylene-response genes are expressed. **(B)** Components of the Ub system as transcriptional co-activators. MYC2 is a master regulator of JA response. After activating transcription, MYC2 remains bound to the promoter elements of its target genes and becomes a “spent” transcription factor. It then undergoes phosphorylation, followed by poly-ubiquitylation and degradation, which enables a “fresh” MYC2 protein to bind to the free promoter of target genes and activate transcription again.

1.3. Role of the F-box protein UFO during flower development in *Arabidopsis*

1.3.1. Initiation of flower development

The life cycle of *Arabidopsis* can be divided into vegetative and reproductive stages. The transition between these stages commences when the shoot apical meristem (SAM) is reprogrammed into an inflorescence meristem (IM). The main phenotypic difference between these stages is that SAMs generate rosette leaves, whereas IMs generate cauline leaves and floral meristems (FM). This transition is in part controlled by environmentally-regulated genes known as “flowering time” genes (Ausin et al., 2005). After transition to the reproductive phase, the expression of floral meristem identity genes, such as *APETALA1* (*API*) and *LEAFY* (*LFY*), is activated (Parcy, 2005). This is followed by activation of floral organ identity genes that are required for the patterning of the FM. Finally, these floral organ identity genes direct the development of the immature flower by regulating the expression of other transcription factors, which ultimately specify the cell types and tissues of the emerging mature flower (Jack, 2004). The ABC model of floral organ identity specification explains how these transcription factors direct floral organ formation (Bowman et al., 1991; Coen and Meyerowitz, 1991).

1.3.2. The ABC model of flower development

An *Arabidopsis* flower consists of 4 whorls occupied (from the outer to the inner whorls) by 4 sepals, 4 petals, 6 stamens and 2 fused carpels. In the original ABC model that was proposed to explain the specification of these 4 types of organs, there are three functional categories of transcription factors (Figure 1.7) (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994; Ma and dePamphilis, 2000).

AP1 and APETALA2 (AP2), which belong to the group of A function proteins, specify sepal identity. AP1 and AP2 combined with the B class transcription factors APETALA3 (AP3) and PISTILLATA (PI) are required for normal petal development. AP3, PI and the C class homeotic transcription factor AGAMOUS (AG) specify stamen identity. Finally, AG alone is required to define carpel identity. Furthermore, the indeterminate phenotype of an *ag-1* flower (Figure 1.7E) indicates that the activity of

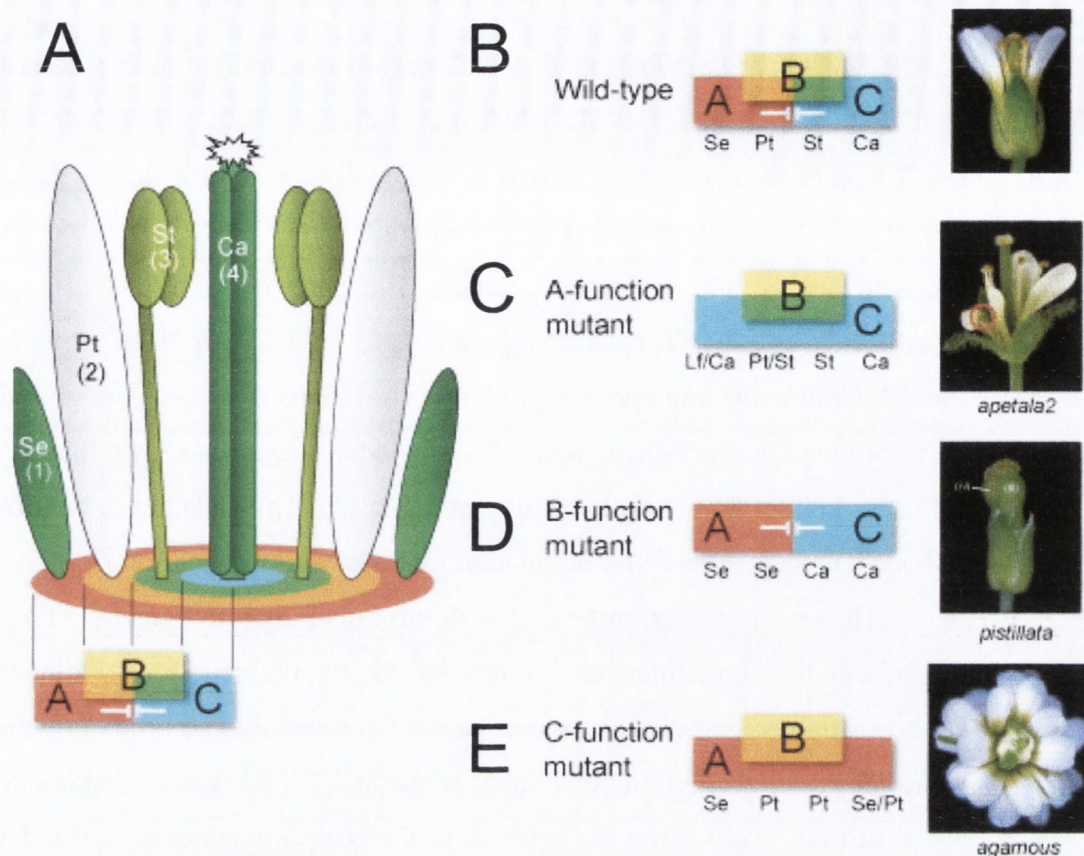


Figure 1.7 The ABC model. (A) Representation of an *Arabidopsis* flower showing sepals (Se), petals (Pt), stamens (St) and carpels (Ca) and the categories of transcription factors involved in the specification of each of these organs. (B) The interplay between A, B and C-function genes in a wild-type *Arabidopsis* flower. (C) An A-function mutant (*ap2*) shows a conversion of sepals into carpel-like structures and petals into stamen-like organs (an anther in whorl 2 is indicated with a circle), due to expansion of the C function into whorls 1 and 2. (D) A B-function mutant (*pi*). The second whorl has sepal identity and the third whorl presents carpels instead of stamens. This is in agreement with the presence of A function alone in whorls 1 and 2, while in whorls 3 and 4, only C function is active. (E) Flower from a C-function mutant (*ag*). In this mutant, A function has expanded into all whorls, resulting in the absence of reproductive organs. AG is also required for the termination of the floral meristem, as in an *ag* mutant, the floral meristem becomes indeterminate, leading to a reiteration of sepals and petals. Figure from (Causier et al., 2010).

AG is also required to generate a determinate flower. Moreover, A and C class genes are mutually antagonistic. Therefore, the expansion of A function is observed in C-function mutants (Figure 1.7D) and *vice versa* (Figure 1.7E).

1.3.3. Functions of LFY and UFO in flower development

The expression of the floral identity transcription factors described above is regulated by *LFY* (Parcy et al., 1998; Lenhard et al., 2001). Extensive studies over the last 20 years provided insights into the role of LFY during flower development. The flower-like structures produced in a mutant allele of LFY (*lfy-1*) shows a conversion of petals to sepals, and stamens to carpels, similarly to *ap3-1* and *pi-1* single mutants (Huala and Sussex, 1992). This suggested that LFY is required for the activation of *AP3* and *PI*. Chromatin immunoprecipitation experiments (ChIP) using an antibody against LFY followed by hybridization to whole genome arrays has shown that *AP3*, *PI* and *AG* are indeed direct LFY targets (Winter et al., 2011). This data hence confirmed that LFY activates directly these floral organ identity transcription factors.

However, in contrast to the floral organ identity genes, LFY is expressed throughout the floral meristem, implying the requirement for additional elements to define spatial expression of its target genes. For example, the F-box protein UFO, which is the substrate recognition component of the SCF^{UFO} E3 ligase complex in *Arabidopsis*, is one of these factors, that is required for the correct expression of the B-function gene *AP3* in whorls 2 and 3 only (Jack et al., 1992; Ingram et al., 1995; Levin and Meyerowitz, 1995; Lee et al., 1997). Indeed, flowers of the strong mutant allele of *UFO* (*ufo-2*) show morphological abnormalities that are reminiscent of those found in plants mutant for *AP3* or *PI*. Although the floral defects found in *ufo-2* flowers are quite variable, the most striking feature is the presence of sepals and carpels in whorls 2 and 3, respectively (Figure 1.8C). These mutant phenotypes strongly suggest that UFO plays a role in the specification of organs found in whorls 2 and 3. Furthermore, the characterization of flower development in plants expressing an inducible version of *UFO* to conditionally rescue the *ufo-2* mutant allowed to dissect more in detail the role of UFO during flower development (Laufs et al., 2003). This analysis suggests that at stage 2 of flower development (stages according to (Smyth et al., 1990)), UFO is essential for proper patterning of the petal, stamen and carpel primordia. After the patterning and identity of these primordia have been established, UFO is required for initiating petal primordia (Smyth et al., 1990), which is consistent with *UFO*'s expression pattern. Indeed, at stages 4/5, *UFO* expression pattern changes and becomes restricted to the second whorl, whereas at stage 2, it was expressed in the presumptive second and third whorls. Moreover, *in situ* hybridization experiments using *ufo-1* inflorescences to detect expression of *AP3* and *PI* showed that the presence of a

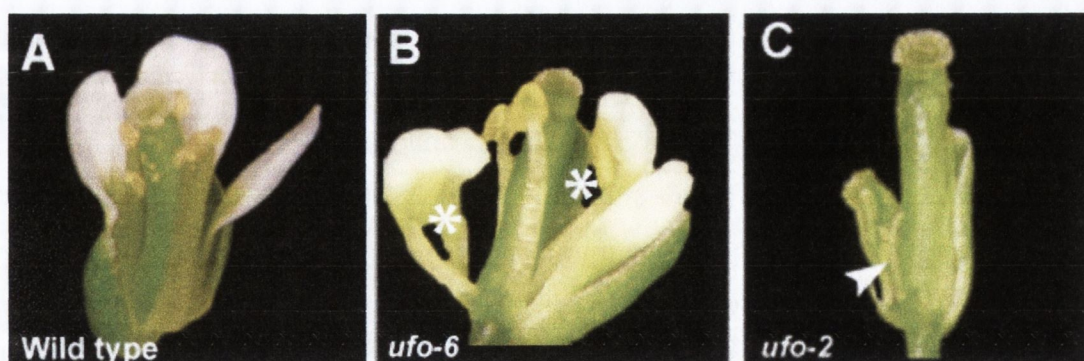


Figure 1.8 Floral phenotypes of *ufo-2* and *ufo-6* mutant flowers. (A) Wild-type flower, with four petals (only three visible, as one sepal was removed), six stamens (only five visible) and a gynoecium with two fused carpels. (B) Flower with a weak *ufo-6* mutant phenotype. One normal petal, two normal stamens and three chimeric petal-stamen organs (indicated by asterisks) are visible. The two outer sepals were removed to visualize the inner organs. The fourth whorl is not affected. (C) A *ufo-2* flower with a strong phenotype. Some stamens are replaced by filaments (indicated by an arrowhead). Petals and stamens are generally absent from the second and the third whorls. The phenotypes of flowers of the *ufo-2* mutants vary within the same plant and floral defects are most severe in early flowers (Lee et al., 1997).

functional UFO is required for expression of the B function genes in inflorescence meristems starting from the stage 4 (Samach et al., 1999).

Analysis of the genetic interactions between the *ufo-1* mutant and strong mutant alleles of *AP3* and *PI* (*ap3-1* and *pi-1*, respectively) confirmed the role of UFO in the regulation of B function. Indeed, unlike *pi-1* and *ap3-1* single mutant phenotypes, the flowers of the *ufo-1 ap3-1* and *ufo-1 pi-1* double mutants exhibited both sepal and carpel features characteristic of a strong *ufo* allele (Wilkinson and Haughn, 1995). It indicates that UFO acts upstream of AP3 and PI.

Because UFO is an F-box protein, it was hypothesized that its role in the regulation of B function would involve the formation of an SCF complex. The analysis of genetic interactions between the *ufo-6* weak mutant and the *ask1-1* mutant (Section 1.1.3.2) indicated that this was indeed the case. The *ufo-6* single mutant produces chimeric petal-stamen organs, and both the number and the size of petals are reduced (Figure 1.8B). However, an *ask1-1 ufo-6* double mutant has a stronger floral phenotype than each of the single mutants (Zhao et al., 1999). More specifically, the double mutant produced flowers similar to those found in the strong *ufo-2* mutant allele (Figure 1.8C) with sepals in the second whorl, carpelloid organs and filamentous structures in the third whorl. Moreover, it was shown that ASK1 physically interacts with UFO (Samach et al., 1999). All together, these data suggest that *UFO* and *ASK1* act together in the regulation

of B function during floral development in the context of SCF complexes. Hence, the initial hypothesis to explain the role of UFO in the regulation of B function during flower development was that it was required to degrade a negative regulator of B function to allow the expression of *AP3* and/or *PI*.

However, recent studies have suggested a different mechanism to explain the role of UFO in the regulation of B function. In both *Arabidopsis* and *Petunia*, it was reported that UFO (or DOUBLE TOP (DOT) in *Petunia*) interacts with LFY (or ABERRANT LEAF AND FLOWER (ALF) in *Petunia*) at the protein level (Chae et al., 2008; Souer et al., 2008). Experiments in which UFO was expressed as a fusion with a dominant repressor peptide further indicated that this F-box protein might act together with LFY on the promoter of its target genes. Indeed, plants that expressed this UFO fusion protein resembled either *ufo-2* mutants or showed a *lfy*-like mutant phenotype with a complete or partial transformation of flowers into inflorescence shoots in the most severe lines (Chae et al., 2008). The latter phenotype suggests that UFO is able to bind indirectly to DNA and that it is required for LFY's role as an activator of B function. This idea was further strengthened by the results of chromatin immunoprecipitation (ChIP) experiments, which showed that LFY and UFO bind to the same regions of the *AP3* promoter (Chae et al., 2008). Interestingly, experiments in the presence of proteasome inhibitors also suggested that degradation of LFY was required for its activity. Based on these observations, Chae *et al.* proposed a model in which SCF^{UFO} would be essential for the degradation of an inactive form of LFY that remains bound to the regulatory elements of the *AP3* promoter after activating transcription, so it can be replaced with a fresh active LFY molecule (see Section 1.2.2.2). From this point of view, UFO and possibly its associated SCF complex, act as *bona fide* transcriptional co-activators during flower development. Interestingly, similar results were found in *Petunia*, as DOT is required to promote ALF-mediated transcription (Souer et al., 2008).

As mentioned above, UFO is one of the only few examples in plants of transcriptional co-activation by a component of the Ub system. Therefore, it remains to be determined whether this type of transcriptional regulation by SCF complexes is widespread in plants. In fact, the same question remains unanswered in other model organisms, such as yeast or humans. My project therefore aims at determining which biological processes require transcriptional co-activation by SCF complexes using *Arabidopsis* as a model organism. In order to deepen our understanding of the molecular mechanisms underlying this type of transcriptional regulation, I also intend to identify, at the genome-wide level, genes that are regulated in the same manner as *AP3*.

1.4. Aims of the project

In the following project outline, experiments are described that aim at determining whether transcriptional co-activation by SCF-type E3 ligases (see Section 1.2) is a widespread mechanism to regulate gene expression. Another aim of my work is to characterize two F-box proteins encoded by At4g19865 (noted *NANNY*) and At4g19870 (termed *GRANNY*), which were hypothesized to be functionally redundant homologs of UFO, but whose function had never been examined.

1.4.1. Investigating the role of UFO as a transcriptional co-activator during flower development

As mentioned above, expression of UFO fused to a dominant transcriptional repressor domain, together with other experiments, led to the discovery that UFO acts as a transcriptional co-activator of the transcription factor LFY (Chae et al., 2008). One of the aims of my Ph.D. project is to determine which processes are transcriptionally co-regulated by SCF complexes in general. To this aim, I intend to express components of SCF complexes fused to dominant transcriptional repressor domains, as done with UFO. In order to validate the plasmids I generated for my Ph.D. project, I started by isolating and characterizing lines that ectopically express a UFO fused to 2 different types of repressor domains. The first one is the SRDX domain, which was used in conjunction with UFO (Chae et al., 2008). The SRDX domain is a short peptide that contains an ERF-associated amphiphilic repression (EAR) motif from the transcription factor SUPERMAN. It was shown that this short peptide was sufficient to recruit a histone deacetylase (HDAC) repressor complex, thus converting the DNA binding protein to which it is fused to into a dominant repressor (Hiratsu et al., 2004). The second repressor domain (noted WUS box or WUSB) was described more recently and corresponds to a short sequence of the WUSCHEL (WUS) transcription factor. It has been shown that fusion of a transcriptional activator (*e.g.* the *CUP-SHAPED COTYLEDON2* transcription factor) to the WUS box results in the conversion of this transcriptional activator into a dominant repressor (Ikeda et al., 2009), similarly to the role of the SRDX domain. In order to determine if the WUSB domain could be used in this study, I also generated *Arabidopsis* transgenic lines that ectopically express a UFO-WUSB fusion. As a control, and to verify that the observed phenotypes would be due to

the activity of the SRDX or WUSB domains, I generated transgenic plants expressing *UFO* fused to mutated versions of the SRDX and WUSB domains (noted mSRDX and mWUSB, respectively), which are known to be inactive (Hiratsu et al., 2004).

Another aim of my project is to gain further insights into the role of *UFO* as a transcriptional co-activator. To reach this goal, I aimed at identifying, at a genome-wide scale, the genes that are directly regulated by *UFO* using chromatin immunoprecipitation coupled to ultra high-throughput sequencing (ChIP-Seq). *UFO*'s role as a transcriptional co-activator of *AP3* expression mainly occurs at early stages of flower development, for which it is difficult to obtain sufficient amounts of tissue to apply genome-wide approaches such as ChIP-Seq. In order to overcome this problem, I will use the so-called floral induction system, which has been previously developed in the laboratory (Wellmer et al., 2006; O'Maoileidigh et al., 2013). This system is based on a double mutant for the two closely related genes *API* and *CAULIFLOWER (CAL)*, which act redundantly during the initiation of flower development. In the *apl cal* double mutant, flower development is temporarily blocked, which results in the massive over-proliferation of inflorescence-like meristems. In the floral induction system, the *apl cal* double mutant also carries a transgene encoding a fusion between *API* and part of the glucocorticoid receptor (GR) under control of the *API* promoter (this fusion is noted *API-GR*). Hence, *API-GR* is expressed in the same domain as endogenous *API*, but the fusion protein is excluded from the nucleus because of the interaction between the GR domain and heat-shock proteins in the cytoplasm. Upon dexamethasone (DEX) treatment, this interaction is disrupted, allowing the *API-GR* fusion to enter the nucleus. Once *API-GR* translocates into the nucleus, flower development is induced and hundreds of flowers develop in a synchronized manner (Figure 1.9), which allows the collection of large amounts of floral tissue at early stages of development for ChIP-Seq experiments.

In sum, this first part of my project will allow me to validate the tools that will be used to study the role of SCF complexes as transcriptional co-activators, while also leading to a better understanding of the roles of *UFO* as a transcriptional co-activator during flower development.

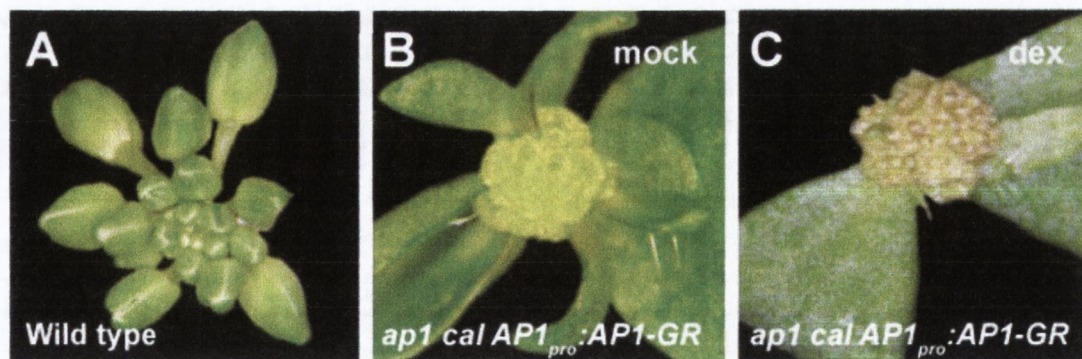


Figure 1.9 Synchronized induction of flower development in the floral induction system. (A) Picture of a wild-type inflorescence. Each of the flower buds is at a different developmental stage, with the youngest ones being hidden at the centre of the inflorescence. These characteristics of a wild-type inflorescence make it difficult to collect enough young floral buds of the same developmental stage for ChIP-Seq experiments. (B) Over-proliferation of inflorescence-like meristems in mock-treated plants of the floral induction system. (C) Induction of synchronized flower development after treatment of the floral induction system with a DEX-containing solution. Pictures in (B) and (C) were taken 4 d after a single treatment with either a mock or a DEX-containing solution.

1.4.2. Genome-wide characterization of processes regulated by SCF complexes acting as transcriptional co-activators

The second aim of my Ph.D. is to determine if transcriptional co-activation by components of the Ub system is a widespread mechanism to regulate gene expression. As mentioned in Section 1.1.2, the Ub system is composed of different types of enzymes (*e.g.* E1, E2 and E3), and the genome of *Arabidopsis* encodes over 1,000 different components of the Ub system (Smalle and Vierstra, 2004). It would hence be very difficult and time-consuming to test the role of each component as a transcriptional co-regulator. Therefore, in this work I will focus only on one type of component of the Ub system – SCF complexes (see Section 1.1.3).

The *Arabidopsis* genome encodes many different components of SCF complexes: 2 RBX subunits, 10 cullins, 21 ASKs and 700 to 900 different F-box proteins (reviewed in (Hua and Vierstra, 2011)). Given that the components can interact in any possible combination, there is a large number of possible SCF complexes. However, it is known that some SCF components, such as ASK1 and CUL1, are more often found in SCF Ub ligases, including the SCF^{UFO} complex (see Section 1.3.3), and consequently they are involved in the regulation of a wide range of physiological processes (see Section 1.1.3.2). Therefore, ASK1 appears as a good starting point for the analysis of the role of SCF-type Ub ligases as transcriptional co-regulators.

Because ASK1 is a common component of many SCF complexes, some of which will not act as transcriptional co-regulators, a manner of affecting solely the role of ASK1 as a transcriptional co-regulator needs to be used. In particular, this precludes the use of an *ask1* mutant, RNAi or amiRNAs, as these methods would affect all processes involving ASK1. To overcome this problem, I will express ASK1 as a fusion with the dominant repressor domains SRDX and WUSB (Figure 1.10). Plants expressing such fusions will be characterized phenotypically in order to identify physiological processes that might require ASK1-containing SCF complexes as transcriptional co-activators. Furthermore, I will generate plants ectopically expressing *ASK1* fused to mutated and inactive versions of the SRDX and the WUSB domains to verify that the observed phenotypes are indeed due to the activity of the dominant repressor domain used.

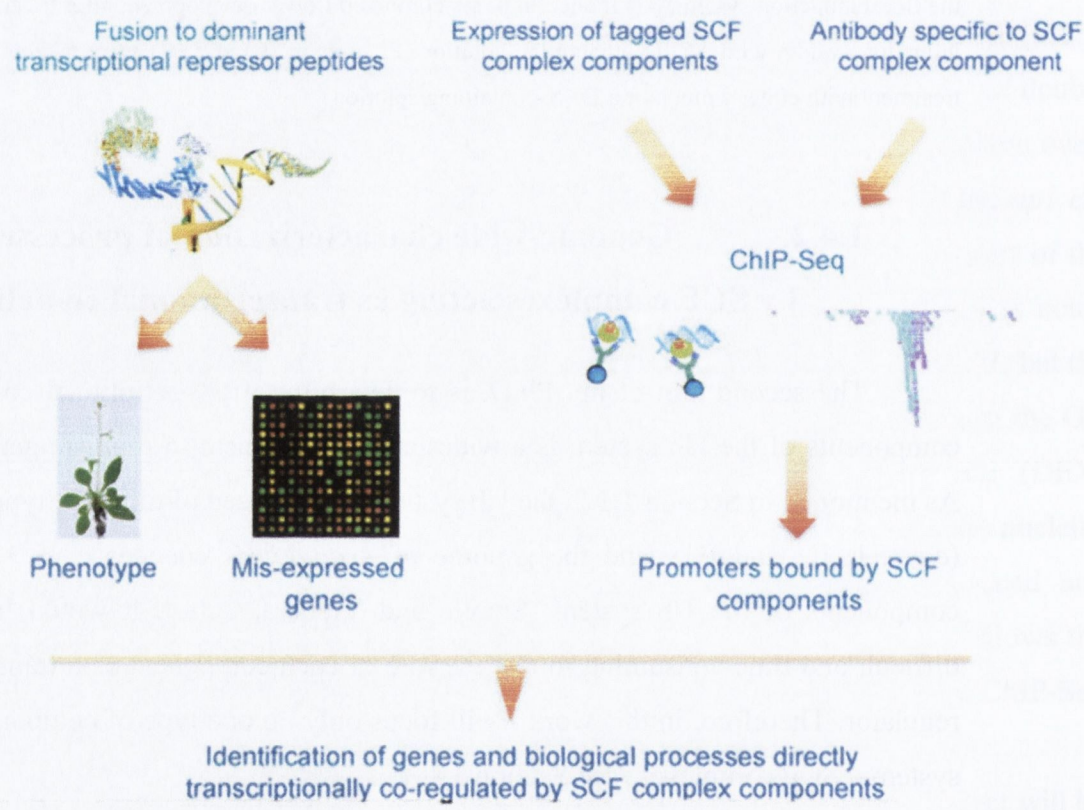


Figure 1.10 Experimental design to identify processes transcriptionally co-regulated by components of SCF complexes. One line of experimentation will aim at determining the effects that a perturbation of transcriptional activity of SCF components has on plant development. This will be assessed by expressing ASK1 fused to dominant transcriptional repressor peptides and by characterizing the resulting plants phenotypically. To gain further insights into the biological pathways that are affected, gene expression profiling experiments will also be carried out. In addition, using ChIP-Seq, genomic regions bound by ASK1 will be identified. Finally, the datasets from all these experiments can be combined allowing the discovery of genes and processes transcriptionally co-regulated by SCF complexes either directly or indirectly.

This phenotypic analysis will be completed by monitoring global gene expression changes in plants expressing the SRDX-ASK1 fusion protein. This will allow me to identify more specifically biological pathways that are likely to require transcriptional co-activation by SCF complexes for their regulation. To this end, I will use plants that express the SRDX-ASK1 fusion under the control of an inducible promoter, which will allow me to express the fusion protein at different developmental stages and follow the resulting gene expression changes using microarrays.

Finally, in order to identify promoters that are directly bound by ASK1-containing SCF complexes, I aim at carrying out ChIP-Seq experiments (Section 1.4.1). To this end, I will use two approaches. First, I will generate lines expressing an epitope-tagged version of ASK1 in the *ask1-1* mutant background to test the functionality of the fusion protein. Second, an antibody against ASK1 will be raised and will be tested for ChIP. These 2 approaches will be developed in parallel to increase the probability of obtaining a suitable reagent for these experiments. Moreover, they will serve as controls for each other.

The results of the ChIP-Seq experiment will be analyzed in parallel with those obtained from global gene expression profiling experiments upon inducible expression of SRDX-ASK1. This comparison will allow to generate a list of genes whose promoters are bound by ASK1 and whose transcriptional regulation requires this SCF component.

1.4.3. Functional characterization of the closely related F-box proteins *NANNY* and *GRANNY* during *Arabidopsis* development

The characterization of null mutants of *UFO* homologs in *Petunia hybrida* and in *Solanum lycopersicon* showed that, in the absence of a functional *UFO* homolog, almost all functions of the respective *LFY* homologs are abolished, including the promotion of floral meristem fate (Lippman et al., 2008; Souer et al., 2008). In contrast, *ufo* mutants in *Arabidopsis* show only defects during the floral meristem patterning (Section 1.3.3). Therefore, it has been proposed that at least one unknown gene acts in a redundant manner with *UFO* in *Arabidopsis* (Souer et al., 2008). Although genetic screens have been carried out on plants mutant for *UFO*, genes with redundant functions have not been identified using this method. This could be due to the fact that more than

one gene is involved in this process and/or that closely linked genes in the genome could fulfill this function. To identify genes that could act redundantly with *UFO*, I have therefore used a candidate approach.

Using co-expression analysis (Section 5.1), I have identified At4g19865 (called *NANNY* or *NAN*) and its close homolog At4g19870 (termed *GRANNY* or *GRA*) as possible candidates. These two F-box proteins share 77% sequence identity among each other and are therefore likely to be functionally redundant. To test whether these genes are indeed redundant with *UFO*, I intend to phenotypically analyze plants with reduced levels of *NAN* and *GRA* mRNA. I will also test possible genetic interactions between *NAN*, *GRA* and *UFO*. Furthermore, if *NAN* and *GRA* indeed play a role during floral development, overexpression of one of these genes in plants may lead to phenotypic defects as well. To test this, lines that ectopically express *GRA* will be generated and characterized.

In summary, these experiments will yield new insights into the functions of *NAN* and *GRA*, and possibly *UFO*.

2. Materials and methods

2.1. Materials

2.1.1. Strains and plasmids

The following bacterial strains have been used in the experiments and assays:

- *E.coli* JM109: *e14*–(*McrA*–) *recA1 endA1 gyrA96 thi-1 hsdR17 (rK– mK+)* *supE44 relA1 Δ(lac-proAB)* [*F'* *traD36 proAB lacIqZΔM15*]
- *E. coli* stbl2TM: *F*–, *mcrA*, *Δ(mcrBC-hsdRMS-mrr)*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *supE44*, *relA1*, *λ*–, *Δ(lac-proAB)*
- *E. coli* TP611: *thi thr leuB6 lacY1 tonA21 supE44 hsdR hsdM recBC lop-11 cya-610 pcnB80 zad:Tn10* (Glaser, et al. 1993)
- *E. coli* XL1 blue: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F'* *proAB lacIqZΔM15 Tn10 (Tetr)*]
- *A. tumefaciens* C58 pGV2260 (McBride and Summerfelt, 1990)

2.1.2. Plant material

For all experiments, *A. thaliana* accessions Landsberg *erecta* (L-*er*) or Columbia-0 (Col-0) were used. Lines used for this work are presented in Table 2.1.

Table 2.1 List of lines used in this work.

Line	Accession	Reference
<i>ap1-1 ag-1 cal-1 AG_{pro}:AG-GFP</i> <i>AP1_{pro}:AP1-GR</i>	L- <i>er</i>	(O'Maoileidigh et al., 2013)
<i>ap1-1 cal-1 AP1_{pro}:AP1-GR</i>	L- <i>er</i>	(O'Maoileidigh et al., 2013)
<i>ap3-3</i>	L- <i>er</i>	(Jack et al., 1992)
<i>ask1-1</i> (or ET2441)	L- <i>er</i>	(Yang et al., 1999)
<i>pi-1</i>	L- <i>er</i>	(Bowman et al., 1989)
<i>ufo-1</i>	Col-0	(Wilkinson and Haughn, 1995)
<i>ufo-2</i>	L- <i>er</i>	(Levin and Meyerowitz, 1995)
<i>ufo-6</i>	L- <i>er</i>	(Levin and Meyerowitz, 1995)
<i>ufo-13</i>	Col-0	(Durfee et al., 2003)

GT_5_111847	Col-0	This work
SAIL_272_A02	Col-0	This work
SALK_076200.55.75	Col-0	This work

2.1.3. Oligonucleotides

Table 2.2 List of oligonucleotides used in this study.

NAME	SEQUENCE (5'-3')
3'OCS	GGTAAGGATCTGAGCTACACATGCTC
35S	GAAAAAGAAGACGTTCCAACCAC
At259	ATACTTATCCTACGGTGATTTTGG
At261	ATGTCTGCGAAGAAGATTGTG
At262	AACATAAGGAAGAAGAACTGG
At278	CTCCTTACGCTGTGAAAAACCT
At281	CGATGAATTCTCCAAACTTTTAAATATATGCATTATGCTTTA
At303	GGATCTTGATGCGGAAGCTAGAGCTGGATTTGCT
At304	AGCAAATCCAGCTCTAGCTTCCGCATCAAGATCC
At350	CACCTCCTCTTCTTGACCGC
At351	CCAAGGGTTATGTTTTTGAGTTTGATGG
At376	GACCGCAAGAGAACGATGA
At377	GCACACGCGCAATTACCA
At420	CCTTTCTTCAAAGCCAAGATTGC
At421	AATATAGAATAGTTAGTACATGTTGTGGG
BART3	GCAGAGATCCGAATTATCAGCCTTC
DM242	AAGCGGTTGTGGAGAACATGATACG
DM243	TGGAGAGCTTGATTTGCGAAATACCG
DM355	CCGAATCCGATCCAAGAAGAATGAGC
DM356	TGCTCGTAGTTAGATCCTCCTGGC
DM400	TCCGACAAGATCCTTTGAGCAC
DM401	ATAAATATGGATTGCACGACGAGTC
FLAG1	CAGTTGGTCTGGTGTCAAAAATC
FLAG2	CCCGGGTCTAGATTACTTGTC
GFP1	ATGAGTAAAGGAGAAGAACTTTTCACT

GFP2	TTATTTGTATAGTTCATCCATGCCATG
KH1	GAAAATGCATAAGTTGAGGTTGTGGGTTGAA
KH2	GGTTATGGAAACGAAGAGAGAAGA
KH3	TCTTCTCTCTTCGTTTCCATAACCATGAGTAAAGGAGAAGA ACTTTTCACT
KH4	TTTTACGCGTCGCAGCTGCAGCTTTGTATAGTTCATCCATG CCATG
KH5	AAAAGGTACCATGTCTGCGAAGAAGATTGTGT
KH6	TTACTCTAGAAACATAAGGAAGAAGAACTGGTTG
KH10	TTTTCCCGGGACAGACTCCAGGAAATGGAAG
KH15	AAAAGAATTCTAAAATGGATTCAACTGTGTTCAT
KH16	TTTTGAATTCCACAGACTCCAGGAAATGGAAG
KH17	TCTTGTGTAACCCTCTTGTCGG
KH20	AAAAGGATCCGCGGCAGCCGCTATGTCTGCGAAGAAGATT GTGT
KH21	TTT TTC TAG ATC ATT CAA AAG CCC ATT GGT TC
KH26	TTTTACGCGTTTATGGAAACGAAGAGAGAAGAGAAA
KH42	GATCTATTATTGGGGTTATGCATTCTCTCTTTTGTATTCC
KH43	GAATGCATAACCCCAATAATAGATCAAAGAGAATCAATGA
KH44	GAATACATAACCCCATTAATAGTTCACAGGTCGTGATATG
KH45	GAACTATTAATGGGGTTATGTATTCTACATATATATTCT
KH46	TAATAACACTTGAAAACGGCAATAAACATA
KH49	AATTGAATTGAATCGATTGCG
KH50	AAACTAGTTGCATGTGGTGGG
KH51	TGATCCAGGTTTATGCAGTCC
KH52	TCTTCTCAACCATGGAGTTGG
KH53	AAATTTTCCCATCGTGGAAC
KH54	AATGGATTGTTGACCAGTTGG
KH67	TTTAAAGCTTCATAACCATGTCTGCGAAGAAGATT
KH70	ATTTTCTAGACTAGCAGGAATTCAAGTCCTCTTCA
KH72	GAGGTTGCCAAGGATTAAAGGACGAA
KH73	CTTTCCTTCAATCACTCCGCTTCTGT
KH74	GAGGTCCCAATGTGTGATGGAGAG
KH76	GGGCATCGACGTACGCTTCCTCTCTTCCCTATGCACGGTGA

	AGATTAAT
KH77	CTAGATTAATCTTCACCGTGCATAGGGAAGAGAGGAAGCG TACGTCGATGCCC
KH79	GCTGCTACAAGAATAAGCCTCCCTTTGCTCTCTAACAAGTC T
KH81	CGAGATGTCCCTGGATCTTGATTTGG
KH82	CCTCGAAAGATTCACCATCGGAACTC
KH87	TAAACTGCAGATGAAACGCCAAGCAAAACC
KH88	AAATTCTAGATCAAACCGAGACAACGAGAC
KH95	GACTCCAGAAGAGATCCGCACAACGT
KH125	GAAGAAGAACTGGTTGCTTGCTGC
KH134	TTTACTGCAGATGAGTAAAGGAGAAGAAGCTTTTCACT
KH135	ATTTCTCGAGCTTTGTATAGTTCATCCATGCCATGTG
KH137	TCATTCAAACGCCCACTGATTCTCAC
KH138	CAGAGGAAGAAGAAGAGGTTCGCC
KH192	TTTATCTAGAGCTGATTTTATGAAGATCGATCAAGC
KH217	GCTTGGAGATCTGAGAAATCTCCTCATCGGAGCTTGAATCA ATAT
KH218	AATGCAACCTCGCCTTCACAGC
KH219	GACTCAGACTTGGGAATGCCTTTCTG
KH261	GGGCATCGACGTACGGCGCCTGCCTTCGCCATGCACGGTG AAGATTAAT
KH262	CTAGATTAATCTTCACCGTGCATGGCGAAGGCAGGCGCCG TACGTCGATGCCC
KH271	TGACGGGAACAGAGACAAGATACTCC
KH272	ATAGTCGAGGATGCTCTTCGTCAACG
KH273	ATGTTGTTAGGGAATCGACCACGG
KH274	GTG ATC CTC GTT ATG CTT GCT GTT CT
KH275	CATCGTCGTCTCATGATGTTTCCTCC
KH276	TGATCCTCTTGGAAGATGGAGTGTGC
KH277	GAT ACG TTG GCA GTG TGG TCT CAA
KH278	AAGTAGGCATCGTCGGGTAATCTTCC
KH294	AACTGCAACGAGGTTCTCTTAACAC

KH295	CGAGATCGAACGGATGCCTCAG
KH298	TCAGACGGAGAGGTCTTGCAGG
KH299	TCCGCATCAAGATCCAGGGATC
KH300	GCCTACAAGAATCTTGCGTTGGCTAA
KH301	CATATGGTCTGGAGCACCGTCTAGT
LB2	CCAAACTGGAACAACACTCAACCCTATCTC
LB_CSHL	CCGGTATATCCCGTTTTTCGT
LB_SAIL	GCTTCCTATTATATCTTCCCAAATTACCAATACA
SW11	ACAAAGTCCGAGACCACCAGATG
SW12	CCTCTTGCGTTGCTTGCTATAGC
SW28	AGAGGATAGAGAACCAGACAAATCGA
SW29	GTTTAGAGAGATGGTGTACGTGG
SW30	GATTACTGTTGTCCTTCCATGG
SW31	ATCTAGGGTTAAAGATTCAAGGG
SW181	TCCGGACTCAGATCAAGCA
SW182	TTCCATTTCATCCTCAAGACG
SW2483	GTATCGATTAAAGCTATGGAGC

2.2. Methods

2.2.1. Microbiology methods

2.2.1.1. Preparation of chemically competent *E. coli* stbl2 and JM109 cells

Highly competent *E. coli* cells were prepared as described in (Inoue, et al. 1990). stbl2 cells were streaked on a LB plate (Appendix 1) supplemented with 100 µg/mL tetracycline and incubated overnight at 37°C. Then a 2 mL starter culture in LB supplemented with 100 µg/mL tetracycline (Appendix 1) was inoculated and grown overnight at 37°C with shaking. The following day, 200 µL of the starter culture were used to inoculate 250 mL SOB medium (Appendix 1) supplemented with 100 µg/mL tetracycline. The culture was grown with shaking (200-250 rpm) at 18-25°C until an OD₆₀₀ of 0.6 was reached. Then the cell culture was cooled on ice for 10 min. After

cooling, the cells were spun at 4,000xg for 10 min at 4°C and the supernatant was discarded. The cell pellet was resuspended in 80 mL ice-cold TB buffer (Appendix 1). The cells were centrifuged again at 4,000xg for 10 min at 4°C and the supernatant was discarded. The bacterial pellet was resuspended in 20 mL ice-cold TB. Then DMSO was added slowly until a final concentration of 7% (v/v) was reached. Cells were incubated on ice for 10 min and aliquoted. Aliquots were frozen in liquid nitrogen and stored at -80°C.

2.2.1.2. Preparation of chemically competent *E. coli* TP611

The strain was streaked on a LB plate and incubated at 37°C. The following day a single colony was grown in a 3 mL LB culture at 37°C. Then the pre-culture was used to inoculate 40 mL of LB and incubated at 37°C until an OD₆₀₀ of 0.36 was reached. The culture was then chilled on ice for 10 min, and spun at 4,000 rpm for 6 min at 4°C. The pellet was then resuspended in 20 mL of ice-cold 100 mM CaCl₂, 25% (v/v) glycerol and incubated on ice for 20 min. Then cell suspensions were spun at 4,000 rpm for 6 min at 4°C and resuspended in 1 mL ice-cold 100 mM CaCl₂, 25% glycerol and aliquots of 200 µL were frozen in liquid nitrogen. Competent cells were stored at -80°C.

2.2.1.3. Preparation of competent *Agrobacterium tumefaciens* cells

Agrobacterium tumefaciens cells from the C58 pGV2260 strain were streaked on a LB plate supplemented with 100 µg/mL rifampicin and 100 µg/mL carbenicillin and incubated for 2 d at 28°C.

A single colony was then used to inoculate a 5 mL LB pre-culture with appropriate antibiotics and grown overnight at 28°C. Then 250 mL of LB with appropriate antibiotics and supplemented with 0.2 g/L MgSO₄ was inoculated with 0.5 mL of the pre-culture and grown at 250 rpm at 28°C until an OD₆₀₀ of about 1.0 was reached. The culture was then chilled on ice and spun at 4°C for 10 min at 4,500xg. The pellet was resuspended in 20 mL ice-cold sterile 10 mM CaCl₂, spun again at 4°C for 10 min at 4,500xg. The pellet was resuspended in 4 mL ice-cold sterile 10 mM CaCl₂. Aliquots of 200 µL were frozen in liquid nitrogen and stored at -80°C.

2.2.1.4. *E. coli* transformation

For each transformation reaction, 100 μ L of competent cells were thawed on ice. Plasmid DNA or an aliquot of a ligation reaction was added while keeping the cells on ice. The cells were then transferred to 42°C for 90 seconds and then back to ice for 1 min. After the heat shock, 1 mL LB was added to each transformation and incubated at 37°C for 1 hr. An aliquot of each reaction was plated on LB plates with appropriate selection.

2.2.1.5. *A. tumefaciens* transformation

Competent cells were thawed at room temperature in aliquots of 100 μ L for each transformation and 2-5 μ L of purified plasmid was added. The cells were frozen and kept in liquid nitrogen for 5 min, then thawed at room temperature. Subsequently 1 mL of LB was added and incubated at 28°C for 4 h. Cultures were spun for 5 min at 5,000 rpm at room temperature, 500 μ L of supernatant was discarded and cells were resuspended. 100 μ L of this cell suspension was plated on LB plates with the appropriate antibiotics.

2.2.2. Plant-related methods

2.2.2.1. Growth conditions

Plants were grown on a medium consisting of compost, perlite and vermiculite in a ratio of 5:2:3. The plants were grown under constant illumination at 20°C after being incubated at 4°C in the dark for 3 d.

2.2.2.2. Flower-dip plant transformation

Agrobacterium-mediated *Arabidopsis* transformation was carried out using the floral-dip method (Clough and Bent, 1998).

2.2.2.3. Transient expression in *Arabidopsis* seedlings

In order to carry out transient gene expression analysis in *Arabidopsis* seedlings the protocol described in (Li et al., 2009) was applied. Briefly, seeds of lines carrying the *ASK1_{pro}:6xmyc-ASK1* transgene (Section 2.5.3.5) were surface sterilized (Section 2.2.2.4) followed by sowing on 0.5xMS agar plates supplemented with 20 µg/mL phosphinotricin. Plates were incubated in the cold room at 4°C for 3 d and transferred to the plant room for growth at 20°C. On the fifth day, *A. tumefaciens* carrying the pML-BART and pML-BART 35S_{pro}:ASK1_{RNAi} vectors were streaked onto YEB agar (Appendix 1) supplemented with 100 µg/mL carbenicillin, 100 µg/mL spectinomycin and 100 µg/mL rifampicin and incubated for 48 h at 28°C. *A. tumefaciens* colonies were scraped from the plates and resuspended in 1 mL of washing solution (Appendix 1). The OD₆₀₀ was measured and the cell suspensions were diluted in the co-cultivation medium (Appendix 1) to reach a final OD₆₀₀ of 0.25.

Next, approximately 40 seedlings were taken from the Petri dishes and soaked in 20 mL co-cultivation medium (Appendix 1) containing *A. tumefaciens* carrying either the pML-BART or pML-BART 35S_{pro}:ASK1_{RNAi} transgenes. Seeds were left to co-cultivate in the dark for 36 hr. After this, 3-4 washes with sterile H₂O were carried out. Seedlings were then transferred onto 0.5xMS agar plates, incubated at 23°C for 3 d. Finally, seedlings were collected in liquid nitrogen and stored at -80°C before further analysis.

2.2.2.4. Seed sterilization

Seeds were sterilized using hypochlorous acid generated by mixing 100 mL bleach (Domestos) with 3 mL concentrated hydrochloric acid. Sterilization was performed for 3-4 h in a sealed container.

2.2.2.5. Selection of transformants

In order to select for gene cassettes carrying a kanamycin resistance gene, sterilized seeds were plated on 0.5xMS agar plates containing 50 µg/mL kanamycin. The plates were placed in the dark at 4°C for 3 d and allowed to germinate under long-day

conditions. Plants that formed true leaves were transferred onto soil and genotyped for the presence of the correct transgene.

In order to select for transformants carrying an ammonium-glufosinate (BASTA) resistance gene, seedlings were sprayed three times in 3-day intervals with 0.15% (v/v) BASTA (Bayer). Resistant seedlings were transferred on fresh soil and genotyped for the presence of the transgene.

2.2.2.6. 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 2.2.2.7) 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.

2.2.2.7. Genotyping assays

To genotype the *ap3-3* the dCAPS method was used. The PCR was performed with primers SW28 and SW29, which resulted in a product of 182 bp. After digestion of the PCR product with *Clal* a fragment of 182 bp indicated a wild-type and bands at 160 bp and 22 bp indicated the presence of the *ap3-3* allele.

ask1-1 mutants were genotyped by PCR using the following combinations of oligonucleotides: At261 and At262 (for the wild-type allele) and At262 and LBCSHL (for the T-DNA). To distinguish between the tagged version of ASK1 and endogenous *ASK1* in lines carrying rescue constructs, plants were genotyped using primers At261 and KH46 (to check for the absence of T-DNA). The latter oligonucleotide is downstream of the sequence used in the rescue constructs.

The presence of the *pi-1* mutant allele was confirmed by using the dCAPS method. The PCR reaction was performed with primers SW30 and SW31. After digestion of the PCR product with *FokI* fragments of 130 bp and 90 bp indicated a wild-type and a band at 220 bp indicated the presence of the *pi-1* allele.

ufo-1 mutants were genotyped by PCR using oligonucleotides At278 and KH217 followed by the digestion of the resulting PCR product with the restriction enzyme *SspI*.

Samples from wild-type plants showed the presence of a band at 248 bp, whereas DNA from *ufo-1* mutant plants resulted in 2 bands at 201 bp and 47 bp.

ufo-2 mutants were genotyped by PCR using oligonucleotides At350 and At351. The resulting PCR product was digested with the restriction enzyme *Afl*III. In the case of wild-type plants a band at 1274 bp was detected, whereas DNA from *ufo-2* mutant plants resulted in 2 bands at 703 bp and 571 bp.

In the case of *ufo-6* mutants, the presence of the mutation was checked by PCR using oligonucleotides At278 and KH79. Subsequently, the resulting PCR product was digested using the restriction enzyme *Xba*I. Samples from wild-type plants showed the presence of a band at 202 bp, whereas *ufo-6* mutant plants resulted in 2 bands at 158 bp and 44 bp.

GT line was genotyped by PCR using the following combination of primers: KH49 and KH50 (for the wild-type allele), and KH50 and LBCSHL (for the T-DNA).

SALK T-DNA insertion line was genotyped by PCR using the following oligonucleotides: KH51 and KH52 (for the wild type) and KH52 and LB2 (for the T-DNA).

nan-1 gra-1 mutant line was genotyped by PCR using two sets of primers: KH53 and KH54 (for the wild-type allele), as well as KH54 and LB_SAIL (for the presence of the insertion).

In rescue lines encoding the *ASK1_{pro}:GFP-ASK1* and *35S_{pro}:GFP-ASK1* rescue constructs, the presence of the rescuing transgenes were verified by PCR using GFP1 and GFP2.

The position of the *UFO_{pro}:UFO-GFP* transgene in the genome of the *ufo-2* rescue line carrying this transgene was determined by Dr Emmanuelle Graciet using the Thermal Asymmetric Interlaced PCR. This line was genotyped by PCR using the following oligonucleotides: At376 and At377 (for the wild type allele), as well as At376 and BART3 (for the presence of the insertion).

In the case of rescue lines with *ASK1_{pro}:6xmyc-ASK1*, the presence of the tagged version of ASK1 was checked using SW2483 and KH70. The position of the *ASK1_{pro}:6xmyc-ASK1* transgene in the genome of the *ask1-1* rescue line carrying this transgene was determined by Dr Emmanuelle Graciet using the Thermal Asymmetric Interlaced PCR. This line was genotyped by PCR using the following oligonucleotides: At420 and At421 (for the wild type allele), as well as At420 and BART3 (for the presence of the insertion).

Plants carrying the *API_{pro}:API-GR* transgene were genotyped by PCR using the

following sets of primers: DM400 and DM401 (for the wild-type allele), as well as DM400 and BART3 (for the T-DNA).

2.2.3. Molecular biology methods

2.2.3.1. Plasmid DNA extraction from *E. coli*

Bacterial DNA was obtained after inoculation of 5 mL LB medium with the appropriate selection. Cultures were grown overnight at 37°C at 220 rpm. They were subsequently spun at 3,500 rpm for 5 min. After discarding the supernatant, the plasmids were purified using the commercial kit for plasmid extraction from Fermentas.

2.2.3.2. Plant genomic DNA extraction

In order to isolate genomic DNA suitable for PCR amplification, small quantities of plant tissue (*e.g.* a leaf) were treated following the protocol described in (Edwards et al., 1991). Briefly, the plant tissue was ground in 1.5 mL tubes using a pestle after addition of 400 µL Edward's extraction buffer (Appendix 1) and spun at full speed in a centrifuge to pellet the cell debris. Then, 300 µL of the supernatant was transferred into fresh 1.5 mL tubes, and 300 µL isopropanol were added. The solution was centrifuged at full speed for 5 min to pellet the DNA. After discarding the supernatant, the DNA pellets were rinsed with 500 µL 70% ethanol and spun at maximum speed for 3 min. The supernatants were discarded and the DNA pellets were air-dried. Finally, the genomic DNA was resuspended in 75 µL H₂O.

2.2.3.3. Genomic DNA extraction using the CTAB method

This procedure was applied to generate contaminant-free genomic DNA preparations from plant tissue, which were used as template in PCR reactions for cloning purposes. Briefly, plant tissue was ground in CTAB buffer (Appendix 1) and purified by phenol-chloroform extraction with phase-lock tubes (5-prime) twice. The DNA was then precipitated using isopropanol and resuspended in molecular biology grade water once the pellet had dried.

2.2.3.4. cDNA synthesis

Total RNA was isolated from plant tissue using the Spectrum Plant Total RNA kit (Sigma-Aldrich) according to the manufacturer's instructions. RNA was reverse transcribed using oligo(dT)18 primers and the RevertAid reverse transcriptase (Fermentas) according to manufacturer's instructions. cDNA obtained from this reaction was used for molecular cloning purposes.

2.2.3.5. PCR-based methods

♦ *Quantitative PCR*

Primers were designed to have a melting temperature (T_m) of $60 \pm 1^\circ\text{C}$. These parameters along with primer dimerization 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 cDNA, 1 μL of 10 μM primers and 3 μL of molecular biology grade water. An equivalent time of 60 sec per 1 kb of DNA was given to generate the amplicons. Primer sequences are detailed in Table 2.1.

In order to detect mRNA of endogenous *UFO* in the *35S_{pro}:UFO-mSRDX* lines the oligonucleotides KH300 and KH301 were used (both complementary to *UFO* 3'UTR region). To detect mRNA of *UFO-mSRDX* in plants carrying the *35S_{pro}:UFO-mSRDX* transgene KH298 (complementary to the exon of *UFO*) and KH299 (complementary to the *mSRDX* sequence) were used.

To detect the mRNA of *(m)SRDX-ASK1*, oligonucleotides KH81 (complementary to the junction between *SRDX* and *ASK1* exon 1) and KH82 (in *ASK1* exon 1) were used. The mRNA of endogenous *ASK1* was detected using primers KH95 and KH125 (complementary to *ASK1* 3'UTR region). Finally, to detect mRNA of endogenous *ASK2*, primers KH137 and KH138 were used.

To validate the microarray data, the lines carrying either *35S_{pro}:LhG4-GR/pOp(6x)_{pro}:SRDX-ASK1* or *35S_{pro}:LhG4-GR/pOp(6x)_{pro}:mSRDX-ASK1* transgenes were tested for 4 selected genes using the following sets of oligonucleotides: primers KH277 and KH278, KH271 and KH272, KH275 and KH276 and KH273 and KH274 to detect mRNA of At1g31710, At1g52820, At1g74890 and At4g39795, respectively (Table 2.1).

NAN and *GRA* mRNAs were detected using oligonucleotides KH72 and KH73, or KH218 and KH219, respectively. In order to determine the expression level of genes involved in flower development, primers DM355 and DM356, SW181 and SW182, SW11 and SW12, and KH294 and KH295 were used for *AG*, *AP3*, *PI* and *UFO*, respectively.

To normalize the data, oligonucleotides DM242 and DM243, complementary to the reference gene *At1g13320* were used.

♦ *Colony PCR*

In order to check the presence of relevant inserts, after *E. coli* transformation with a ligation reaction, colonies on plate were tested by PCR. Single colonies were picked from the plate and resuspended in 10 μ L LB. Then 1.5 μ L of the cell suspension was used as PCR template in a 15 μ L reaction mix.

2.2.4. Biochemical methods

2.2.4.1. Protein extraction from plants

For Western blot analysis, 10-day-old seedlings were collected and frozen in liquid nitrogen. Samples were then ground in liquid nitrogen and the powder was resuspended in 1x CCLR buffer (Promega) supplemented with 1 mM PMSF and plant protease inhibitor (Sigma-Aldrich, diluted 100-fold). After centrifugation for 5 min at 14,000g at 4°C, the supernatants were removed and used for the subsequent analysis.

2.2.4.2. Protein expression in *E. coli*

E. coli JM109 was transformed with pMALc5x RGA (kindly provided by Dr. Xing-Wang Deng). After overnight growth at 37°C, the cells were scraped from the LB plates supplemented with 100 μ g/mL carbenicillin and 35 μ g/mL chloramphenicol by adding 2 mL LB medium to the surface. The cell suspension was then transferred to a 50 mL LB culture supplemented with 100 μ g/mL carbenicillin and 35 μ g/mL chloramphenicol and incubated at 37°C until an $OD_{600} \sim 0.5$ was reached. The cell cultures were then grown at 42°C for 30 min to induce expression of *E. coli* chaperones, followed by chilling on ice for 15 min. Expression of the recombinant proteins was

induced by adding isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. The cells were then grown for 4 h at 25°C. Cultures were spun at 3,500 rpm at 4°C for 10 min.

2.2.4.3. Purification of recombinant proteins fused to the maltose-binding protein (MBP)

Cell pellets were resuspended in the resuspension solution (Appendix 1). The cell extract was then submitted to sonication 4 times for 30 sec, followed by spinning down at 4°C at 14,000 rpm for 10 min. The supernatant, which contained soluble proteins, was collected. 0.5 mL amylose resin (NEB) was equilibrated by spinning down for 1 min at 4°C at 1000 rpm, followed by washing 5 times with 1 mL column buffer.

To allow binding of the MBP fusion proteins, the protein extracts were mixed with the resin and incubated for 2 h at 4°C with rotation. All downstream steps were carried out at 4°C. In order to eliminate proteins that bound aspecifically to the amylose resin, the latter was washed using 6 mL of column buffer, until no proteins could be detected in the flow through using the Bradford reagent. MBP-RGA fusion proteins were eluted from the amylose resin by applying elution buffer (Appendix 1). 500 μ L fractions were collected and protein concentration was measured to identify fractions that contained the highest amount of the MBP fusion proteins. The buffer was then changed by dialysis of the MBP-RGA fusion protein against dialysis buffer (Appendix 1). This step was carried out overnight at 4°C. The purified proteins were aliquoted, frozen in liquid nitrogen and stored at - 70°C.

2.2.4.4. Determination of protein concentration using Bradford

Protein concentration was determined using the Bradford reagent (Sigma-Aldrich). Different concentrations, ranging from 1 mg/mL to 10 mg/mL of a bovine serum albumine (BSA) solution were used for calibration.

2.2.4.5. Preparation of protein extracts from nuclei for Western blot analysis

500 μ L of inflorescence tissue from the *apl-1 cal-1 API_{pro}:API-GR* was collected, frozen in liquid nitrogen and stored at -70°C . The tissue was ground in liquid nitrogen and 250 μ L M1 buffer (Appendix 1) was added followed by grinding on ice. Then 650 μ L of M1 buffer was added. The ground tissue was filtered twice using a pre-wet circular piece of Miracloth (Calbiochem®) with M1 buffer. The collected filtrate was spun down at 5°C at 9,000 rpm for 1 min to pellet crude nuclei. To wash the pellet, it was resuspended in 0.9 mL M2 buffer (Appendix 1) and spun down at 5°C at 9,000 rpm for 1 min. This washing step was repeated twice. Then the pellet was washed with 0.9 mL M3 buffer (Appendix 1) followed by spinning down at 5°C at 2,000 rpm for 5 min. After centrifugation the pellet was resuspended in 20 μ L ChIP Lysis Buffer (Appendix 1). Chromatin was sonicated using a Diagenode Bioruptor at 4°C . “High” intensity sonication cycles were applied, which consisted of 30 sec sonication, followed by 30 sec without sonication. To open nuclear envelope and shear the chromatin into small fragments, 7 cycles of sonication were applied. To pellet insoluble material, the sheared chromatin sample was spun down at 5°C at 12,000 rpm for 10 min. After centrifugation the supernatant was collected. An aliquot of the nuclear extract was diluted 1:10 in H_2O and DNA concentration was determined using the Qubit HS kit (Invitrogen). To load equal amounts of DNA on SDS gel, the nuclear extract samples were normalized using DNA concentrations.

2.2.4.6. Protein separation by SDS-PAGE electrophoresis

Proteins were denatured by adding 2X SDS loading buffer (Appendix 1), followed by incubation at 95°C for 5 min. After centrifugation at maximum speed for 1 min at room temperature, the supernatants were loaded on SDS-PAGE gels with the appropriate acrylamide concentration. The stacking and separating gels were prepared using respectively stacking buffer (Appendix 1) and separating buffer (Appendix 1).

2.2.4.7. Western blot analysis

Protein extracts were separated on a SDS-PAGE acrylamide gels, which were run in Tris-glycine buffer (Appendix 1) at 50 mA, followed by transfer to a PVDF membrane in transfer buffer (Appendix 1) at 50 V.

After transfer, equal protein loading was checked by Ponceau staining. To this aim, the PVDF membrane was soaked in 7.5% (v/v) acetic acid for 5 min, followed by incubation in Ponceau solution (Appendix 1) for 5 min with shaking. The excess of dye was washed with 7.5% acetic acid. The protein-bound dye was rinsed away with several washes in 1x PBS-T (prepared from 10x PBS (Appendix 1) with 0.05% Tween 20 (v/v) and 5% (w/v) non-fat milk. When the staining was no longer visible, the antibody solution was prepared in PBS-T with 5% milk and was added to the membrane.

The detection of Myc-tagged proteins was carried out using a 1:5,000 dilution of a mouse monoclonal antibody (Sigma), GFP fusions were detected using a rabbit α -GFP antibody (AbCam) diluted 8,000 fold, while anti-MBP antiserum (NEB) was used to detect MBP-tagged proteins diluted 1:10,000.

Incubation with the primary antibody was carried out overnight at 4°C. The next day, the membrane was washed 3 times 5 min with 1x PBS-T. The secondary antibody (α -rabbit or α -mouse conjugated to horse radish peroxidase (HRP)) was then added and incubated for 4 h at room temperature with mild shaking. The excess of secondary antibody was rinsed 3 times for 5 min with 1x PBS-T and proteins were detected using the Amersham ECL Plus Western Blotting Detection Reagent (GE Healthcare) by chemiluminescence reading carried out with the LAS-3000 Intelligent Dark Box Image Reader (Fujifilm).

2.3. 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.

2.4. Data analysis

2.4.1. Low-level microarray data processing and basic data handling

The analyses of data obtained from microarray experiments were performed by Patrick Ryan using the R statistical software Version 2.14.1 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).

Agilent median signals and background were read into R and background correction was performed using the *backgroundCorrect*-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.

2.4.2. Higher level microarray analysis

Base-level annotations were retrieved from the Bioconductor homepage, (package *arabidopsis.db0* Version 2.5.0) and an annotation package for the custom-made 44K Agilent array was generated using *AnnotationDBI* package as described in the SQL Forge vignette.

Gene set enrichment analysis were performed using the Cytoscape freeware (<http://www.cytoscape.org/>) and the BINGO 2.44 plugin (<http://www.psb.ugent.be/cbd/papers/BiNGO/Home.html>) (Maere et al., 2005) with default settings. GO terms that had an adjusted *P*-value < 0.05 were judged as being statistically over-represented in the microarray datasets. Heatmaps were generated using the BAR HeatMapper Plus Tool freeware (http://bar.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi).

2.5. Generation of transgenic plants

2.5.1. Isolation of plants expressing UFO fused to dominant repressor domains or their mutated versions

2.5.1.1. Creation of lines carrying the *35S_{pro}:UFO-SRDX* or *35S_{pro}:UFO-mSRDX* constructs

In order to generate a dominant repressor version of UFO, the SRDX domain (SLDLDLRLGFA) from the plant transcription factor SUPERMAN was translationally fused to the coding region of *UFO* under the control of the 35S promoter. The primers KH15 and KH16 (Table 2.2), which incorporated *EcoRI* sites into the amplified product, were used to amplify the *UFO* coding region from the wild type (accession: Col-0) flower cDNA. This PCR product was then ligated between the 35S promoter and the SRDX domain using *EcoRI* sites to create an in frame translational fusion. The orientation of the insert was checked by restriction analysis.

In order to generate the mutated and inactive version of *35S_{pro}:UFO-SRDX*, noted *35S_{pro}:UFO-mSRDX*, two complementary primers, At303 and At304 (Table 2.2), were designed. These primers encode point mutations in the SRDX domain in order to mutagenize it from the LDLELRL sequence (SRDX) into LDAEARA (mSRDX). Two PCRs were carried out using pBJ36 *35S_{pro}:UFO-SRDX* as a template using oligonucleotides KH17 and At304, as well as At303 and 3'OCS (Table 2.2). Both products were mixed together in a 1:1 ratio and they annealed to each other through the newly generated mSRDX sequence, due to the fact that At303 and At304 are complementary. It served as a template for the third PCR reaction using primers KH17 and 3'OCS. The final PCR product and pBJ36 *35S_{pro}:UFO-SRDX* were digested using *XbaI* and *SalI*, followed by ligation.

The generated constructs, pBJ36 *35S_{pro}:UFO-SRDX* and pBJ36 *35S_{pro}:UFO-mSRDX* were checked by sequencing. *NotI* digestion was then used to transfer the DNA regions encoding for the different fusion proteins into the binary vector pML-BART. Plants were transformed as described in Section 2.2.2.2. Colony PCRs and genotyping were performed using primers KH17 and At304 (Table 2.2).

2.5.1.2. Generation of transgenic lines expressing the $35S_{pro}$:UFO-WUSB or $35S_{pro}$:UFO-mWUSB transgene

In order to generate an alternative dominant repressor version of UFO, the WUSB domain (TLPLFPMH) from the plant transcription factor WUSCHEL was used. The oligonucleotides KH76 and KH77 (Table 2.2) were mixed in the molar proportion of 1:1. The resulting DNA fragment encoded the WUSB domain and also contained *SmaI* and *XbaI* restriction sites for ligation. The vector pBJ36 $35S_{pro}$ was digested using *SmaI* and *XbaI*, and the WUSB-containing fragment was ligated. The resulting vector (noted pBJ36 $35S_{pro}$:WUSB) was verified by sequencing. The *UFO* coding region was digested out from the pBJ36 $35S_{pro}$:UFO-SRDX vector (Section 2.5.1.1) using *EcoRI*, the fragment encoding the *UFO* coding regions was then ligated between the $35S$ promoter and the WUSB domain of the pBJ36 $35S_{pro}$:WUSB plasmid. The orientation of the insert was checked by restriction analysis. The resulting vector was noted pBJ36 $35S_{pro}$:UFO-WUSB.

In order to generate a mutated and inactive version of WUSB domain – mWUSB (TAPAFAMH), the oligonucleotides KH261 and KH262 (Table 2.2) were mixed in the molar proportion of 1:1. The resulting DNA fragment encoded the mWUSB domain and also contained *SmaI* and *XbaI* restriction sites for ligation. The vector pBJ36 $35S_{pro}$:UFO-WUSB was digested using *SmaI* and *XbaI*, and the mWUSB-containing fragment was ligated.

The resulting vectors pBJ36 $35S_{pro}$:UFO-WUSB and pBJ36 $35S_{pro}$:UFO-mWUSB were verified by sequencing. *NotI* sites were then used to transfer these constructs into the binary vector pML-BART. Plants were transformed as described in Section 2.2.2.2. Colony PCRs and genotyping were performed using primers KH17 and KH77 (Table 2.2).

2.5.2. Isolation of plants with altered ASK1 function

It has been shown that the expression of an epitope-tagged version of *ASK1* with the tag at the C-terminus results in dominant-negative effects (Wang and Yang, 2006). Therefore, I generated *ASK1* N-terminally fused to SRDX.

2.5.2.1. Creation of lines carrying the *ASK1_{pro}:SRDX-ASK1* transgene

The SRDX domain was used in order to generate a version of ASK1 that would act as a dominant repressor under the control of its own promoter. Firstly, complementary primers (KH20 and KH21 (Table 2.2)) encoding the SRDX domain were annealed. The DNA fragment including a start codon, but no stop codon was ready to be ligated into pBJ36 digested using *Bam*HI and *Xba*I. The resulting plasmid, noted pBJ36 SRDX^N, was checked by sequencing and could be used to generate N-terminal fusions with the SRDX domain under the control of endogenous promoters. In order to amplify ASK1 promoter region, primers KH1 and KH26 (Table 2.2) were used. They contained a *Nsi*I or a *Mlu*I site, respectively. The resulting PCR product was digested with *Nsi*I and *Mlu*I and ligated into pBJ36 SRDX^N which was cut with the same enzymes. This resulted in a vector noted pBJ36 ASK1_{pro}:SRDX^N. Finally, the genomic region coding for *ASK1* was amplified using primers KH20 and KH6 (Table 2.2), which incorporated *Bam*HI and *Xba*I sites into the amplified product. The PCR product was ligated using *Bam*HI and *Xba*I sites into pBJ36 ASK1_{pro}:SRDX^N, resulting in the pBJ36 ASK1_{pro}:SRDX-ASK1 vector.

The sequence of pBJ36 ASK1_{pro}:SRDX-ASK1 was verified by sequencing. Subsequently, the two *Not*I sites were used to transfer this construct into the binary vector pML-BART. Plants were transformed as described in Section 2.2.2.2. Colony PCR and genotyping were performed using primers At303 and KH6 (Table 2.2).

2.5.2.2. Generation of lines carrying the *35S_{pro}:SRDX-ASK1* or *35S_{pro}:mSRDX-ASK1* construct

To generate the dominant repressor version of ASK1 under the control of the constitutive promoter, the sequences of the SRDX domain and the 35S promoter were used.

Initially, the SRDX domain was excised from the pBJ36 SRDX^N vector (see Section 2.5.2.1) using *Xho*I and *Age*I digestion, followed ligation into the pBJ36 35S_{pro} vector that had been digested with the same enzymes. The resulting vector, noted pBJ36 35S_{pro}:SRDX^N contains an SRDX domain with a start codon, but no stop codon. Thus, it may be used for N-terminal fusions.

Primers KH20 and KH21 (Table 2.2), which incorporated *Bam*HI and *Xho*I sites

into the PCR product, were used to amplify the ASK1 coding region from wild-type (accession: Col-0) flower cDNA. This PCR product was then ligated between the promoter and SRDX domain of pBJ36 35S_{pro}:SRDX^N using *Bam*HI and *Xho*I digestion.

In order to generate pBJ36 35S_{pro}:mSRDX-ASK1, two complementary primers (At303 and At304 (Table 2.2)) were used. Two PCRs were carried out using pBJ36 35S_{pro}:SRDX-ASK1 as a template: using either oligonucleotides 35S and At304 or At303 and KH21 (Table 2.2). Both PCR products were mixed together in 1:1 ratio and annealed to each other. The resulting DNA fragment was used as a template for the third PCR reaction using primers 35S and KH21 (Table 2.2). The PCR product and pBJ36 35S_{pro}:SRDX-ASK1 were digested using *Pst*I and *Afl*III restriction enzymes, followed by the ligation.

The sequences of both vectors pBJ36 35S_{pro}:SRDX-ASK1 and pBJ36 35S_{pro}:mSRDX-ASK1 were verified by sequencing. Subsequently, *Not*I sites were used to transfer these constructs into the binary vector pML-BART. Plants were transformed as described in Section 2.2.2.2. Colony PCRs and genotyping were performed using primers At303 and KH6 (Table 2.2).

2.5.2.3. Generation of 35S_{pro}:ASK1_{RNAi} to disrupt ASK1 function

The pBJ36 and pML-BART 35S_{pro}:ASK1_{RNAi} constructs were generated by Dearbhaile McEvoy (unpublished). Plants were transformed as described in Section 2.2.2.2. Genotyping was performed using primers KH192 and 3'OCS (Table 2.2).

2.5.3. Isolation of complementation lines for ChIP

2.5.3.1. Creation of lines carrying the UFO_{pro}:UFO-3xFLAG rescuing construct

To generate an alternative epitope-tagged version of UFO, genomic sequence including the promoter region was excised from pBJ36 UFO_{pro}:UFO-GFP (see Section 2.5.3.2) using *Eco*RI and *Sma*I digestion. The resulting fragment encoding UFO_{pro}:UFO was ligated into pBJ36 3xFLAG digested using *Eco*RI and *Sma*I. This resulted in a plasmid noted pBJ36 UFO_{pro}:UFO-3xFLAG. *Not*I sites were then used to transfer

UFO_{pro}:UFO-3xFLAG into the binary vector pML-BART. The population segregating for *ufo-2* (noted *ufo-2/+*) was transformed as described in Section 2.2.2.2. Colony PCRs and genotyping for the presence of the 3xFLAG tag were performed using primers FLAG1 and FLAG2 (Table 2.2). Plants were also genotyped for the *ufo-2* mutation as described in Section 2.2.2.7.

2.5.3.2. Generation of lines encoding the UFO_{pro}:UFO-GFP transgene

In order to generate a tagged version of *UFO*, the primers At281 and KH10 (Table 2.2), which incorporated *Eco*RI and *Sma*I sites to the PCR product, were used to amplify the *UFO* genomic sequence including the promoter region from wild-type (accession: Col-0) genomic DNA. The chosen sequence was shown to be sufficient for the *UFO* expression in the correct domain (Hobe, 2004). The resulting PCR fragment and the pBJ36 GFP vector were digested using *Eco*RI and *Sma*I, followed by ligation. *Not*I sites were then used to transfer the UFO_{pro}:UFO-GFP fusion into the binary vector pML-BART. A population segregating for *ufo-2* (noted *ufo-2/+*) was transformed as described in Section 2.2.2.2. Colony PCRs and genotyping for the presence of GFP were performed using primers GFP1 and GFP2 (Table 2.2). Plants were also genotyped for the *ufo-2* as described in Section 2.2.2.7. In order to isolate the *ufo-2* rescue line homozygous for the UFO_{pro}:UFO-GFP transgene I genotyped plants using At376 and At377 (for the wild type allele), as well as At376 and BART3 (for the presence of the insertion) (Table 2.2).

2.5.3.3. Isolation of lines carrying the ASK_{pro}:GFP-ASK1 fusion

To generate a GFP-tagged version of ASK1, the promoter region that had been already described (Wang and Yang, 2006) was amplified from the wild type (accession: Col-0) using primers KH1, which incorporated a *Nsi*I restriction site and KH2. The GFP sequence was amplified from the pBJ36 GFP vector using oligonucleotides KH3 (whose sequence partially overlapped with KH2) and KH4 (Table 2.2), that incorporated a *Mlu*I restriction site and lacked a stop codon. The two resulting PCR products were mixed in a 1:1 ratio and the overlapping PCR with oligonucleotides KH1 and KH4 (Table 2.2)

was used to generate a DNA fragment encoding *ASK1*'s promoter and a GFP tag for N-terminal fusion. The PCR fragment and the pBJ36 vector were digested using *NsiI* and *MluI* and ligated. This resulted in a vector noted pBJ36 *ASK1_{pro}:GFP^N*. The *ASK1* genomic region (Wang and Yang, 2006) was amplified from the wild type (accession: Col-0) using primers KH5 and KH6 which incorporated *Acc65I* and *XbaI* restriction sites. The resulting fragment and pBJ36 *ASK1_{pro}:GFP^N* vector were digested using these two enzymes and ligated. *NotI* sites were then used to transfer the *ASK1_{pro}:GFP^N-ASK1* fragment into the binary vector pML-BART. A population segregating for *ask1-1* (noted *ask1-1/+*) was transformed as described in Section 2.2.2.2. Colony PCRs and genotyping for the presence of the GFP tag were performed using primers GFP1 and GFP2 (Table 2.2). Plants were also genotyped for the *ask1-1* as described in Section 2.2.2.7.

2.5.3.4. Generation of lines encoding the *35S_{pro}:GFP-ASK1* rescuing construct

pBJ36 *ASK1_{pro}:GFP^N-ASK1* (Section 2.5.3.3) was used as a template to amplify the GFP sequence using oligonucleotides KH134 and KH135 (Table 2.2), which also incorporated *PstI* and *XhoI* restriction sites, as well as a start codon. The resulting PCR fragment and pBJ36 *35S_{pro}* were digested using *PstI* and *XhoI* and ligated. This resulted in pBJ36 *35S_{pro}:GFP^N*.

In a second step, pBJ36 *35S_{pro}:SRDX-ASK1* (see Section 2.5.2.2) and pBJ36 *35S_{pro}:GFP^N* were digested using *Acc65I* and *AgeI* restriction enzymes and the fragment encoding *ASK1* cDNA was ligated into pBJ36 *35S_{pro}:GFP^N* to generate pBJ36 *35S_{pro}:GFP-ASK1*. *NotI* sites were then used to transfer the *35S_{pro}:GFP-ASK1* fragment into the binary vector pML-BART. A population segregating for *ask1-1* (noted *ask1-1/+*) was transformed as described in Section 2.2.2.2. Colony PCRs and genotyping for the presence of the GFP^N tag were performed using primers GFP1 and GFP2 (Table 2.2). Plants were also genotyped for the *ask1-1* as described in Section 2.2.2.7.

2.5.3.5. Creation of lines carrying the *ASK1_{pro}:6xmyc-ASK1* rescue construct

In order to generate an alternative epitope-tagged version of *ASK1*, the 6xmyc tag was used. To this end, the promoter region of *ASK1* was cut from pBJ36 *ASK1_{pro}:SRDX-ASK1* (Section 2.5.2.1) using *SacI* and *MluI*. The resulting fragment was cloned into pBJ36 6xmyc (the tag included a start codon but no stop codon, so it could be used for a translational N-terminal fusion, see Section 2.5.2) after digestion with *SacI* and *MluI*. This resulted in a vector noted pBJ36 *ASK1_{pro}:6xmyc*.

In a second step, the genomic region of *ASK1* was amplified from genomic DNA of wild-type plants (accession: Col-0) using oligonucleotides KH67 and KH6 (Table 2.2) that incorporated *HindIII* and *XbaI* restriction sites. The vector pBJ36 *ASK1_{pro}:6xmyc* and the resulting PCR fragment were cut using *HindIII* and *XbaI* and ligated. The resulting vector is noted pBJ36 *ASK1_{pro}:6xmyc-ASK1*. *NotI* sites were then used to transfer *ASK1_{pro}:6xmyc-ASK1* into the binary vector pML-BART. A population segregating for *ask1-1* (noted *ask1-1/+*) was transformed as described in Section 2.2.2.2. Colony PCRs and genotyping for the presence of the transgene were performed using primers At259 and KH70 (Table 2.2). Plants were also genotyped for the *ask1-1* as described in Section 2.2.2.7. In order to isolate the *ask1-1* rescue line homozygous for the *ASK1_{pro}:6xmyc-ASK1* transgene in the 5.3 line I genotyped plants using At420 and At421 (for the wild type allele), as well as At420 and BART3 (for the presence of the insertion) (Table 2.2).

2.5.4. Isolation of transgenic lines with the altered *NAN* and *GRA* mRNA levels

2.5.4.1. Generation of the *35S_{pro}:NAN&GRA_{amiRNA}* single and *nan-1 gra-1 35S_{pro}:NAN&GRA_{amiRNA}* triple mutants

To disrupt functions of *NAN* and *GRA*, an amiRNA was designed using the Web microRNA designer (<http://wmd3.weigelworld.org>). Briefly, the *Arabidopsis* Genome Initiative (AGI) numbers of the genes of interest were submitted to the database and a list of microRNAs were presented which have the potential of perturbing the function of

the genes 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 five or less mismatches to the amiRNA, and hybridization energies of other genes with five 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 is chosen, an Oligo Designer application (<http://wmd3.weigelworld.org>) generates oligonucleotides that allows site-directed mutagenesis to be performed on the pRS300 plasmid, which contains a copy of the endogenous *Arabidopsis* miR319a (At4g23713) (Palatnik et al., 2003). Four rounds of PCR were performed in order to generate the amiRNA precursor (Table 2.2 for the oligos used in these reactions). The final product was cut with *Bam*HI and *Eco*RI and ligated into a vector (pBJ36) containing the 35S promoter. After verifying the sequence of the amiRNAs by sequencing, the fragment containing the promoter and the amiRNA was sub-cloned into the binary vector using *Not*I sites into pML-BART or pART-27. The latter vector confers the resistance to kanamycin which its use to transform BASTA-resistant plants. The pML-BART 35S_{pro}:NAN&GRA_{amiRNA} vector was used to transform *L-er* whereas the pART-27 35S_{pro}:NAN&GRA_{amiRNA} vector to super-transform the population homozygous for the *nan-1 gra-1* mutant allele as described in Section 2.2.2.2. The resulting *nan-1 gra-1* 35S_{pro}:NAN&GRA_{amiRNA} plants were genotyped for the presence of the *nan-1 gra-1* mutant allele as described in Section 2.2.2.7. Colony PCRs and genotyping assays for the presence of the 35S_{pro}:NAN&GRA_{amiRNA} transgene in all obtained lines were performed using the primers 35S and 3'OCS (Table 2.2).

2.5.4.2. Generation of lines carrying the 35S_{pro}:GRA transgene

In order to generate the pBJ36 35S_{pro}:GRA plasmid, cDNA from wild-type (accession: *L-er*) flower was used as a template to amplify the coding region of *GRA* using oligonucleotides KH87 and KH88 (Table 2.2), which incorporated *Pst*II and *Xba*I restriction sites, as well as start and stop codons. The resulting PCR fragment and pBJ36 35S_{pro} were digested using *Pst*II and *Xba*I and ligated. This resulted in the pBJ36

35S_{pro}:GRA vector. *NotI* sites were then used to transfer 35S_{pro}:GRA into the binary vector pML-BART. Wild-type plants (accession: Col-0) were transformed as described in Section 2.2.2.2. Colony PCRs and genotyping for the presence of the transgene were performed using primers KH74 and KH88 (Table 2.2).

3. Role of UFO as a transcriptional co-activator during flower development

3.1. Introduction

The aim of my Ph.D. is to determine if transcriptional co-activation by components of the Ub system is a widespread mechanism to control gene expression. As mentioned above (Section 1.3.3), UFO is an F-box protein, which is a component of SCF-type E3 ligases. Importantly, expression of UFO fused to the SRDX dominant repressor domain indicated that UFO might act as a transcriptional co-activator of LFY during flower development (Chae et al., 2008). I intend to use the SRDX domain fused to other SCF components to determine if transcriptional co-regulation by components of the Ub system is a widespread mechanism used to control gene expression. In a first step I validated the use of such fusion proteins by generating transgenic lines expressing the UFO-SRDX fusion and by testing if I could obtain similar phenotypes to those described by Chae *et al.* (2008). Moreover, shortly after starting my Ph.D., a novel dominant repressor domain (noted WUSB) was described (Ikeda et al., 2009) (Section 1.4.1). I therefore also aimed at comparing the efficiency of the WUSB and SRDX domains by generating plants expressing a fusion of UFO with the WUSB domain. Furthermore, in order to control for the use of these dominant repressor domains, I also generated plants that expressed UFO fused to mutated and inactive versions of these domains (noted mSRDX and mWUSB) (Figure 3.1). Finally, to obtain further insights into the role of UFO as a transcriptional co-activator, I aimed at identifying the genomic regions bound by UFO at a genome wide-scale. To this end, I generated plants that expressed a functional epitope-tagged version of UFO under the control of the *UFO* promoter in order to carry out ChIP-Seq experiments.

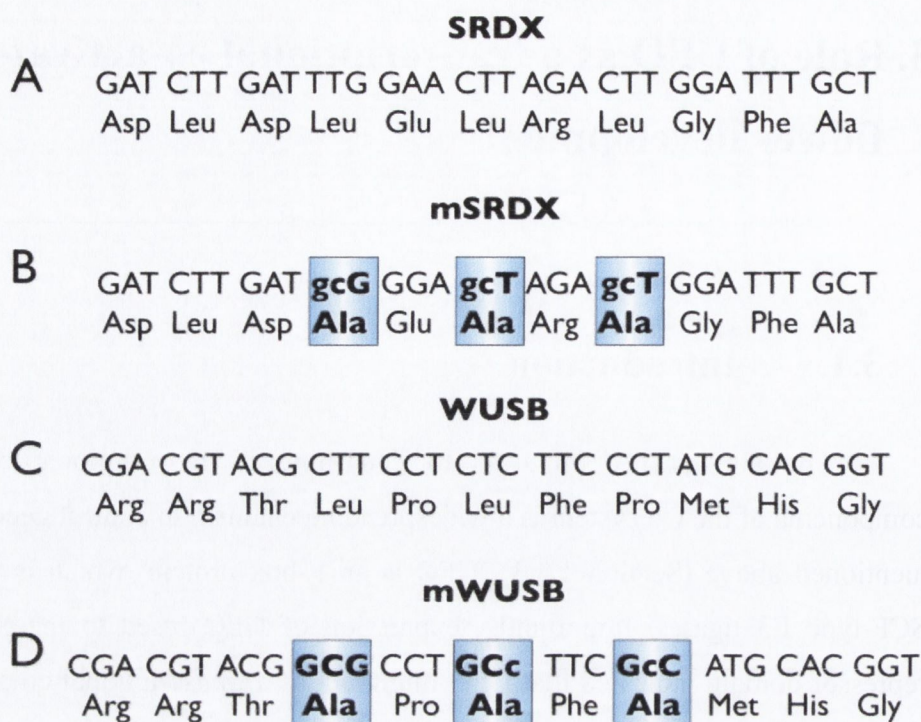


Figure 3.1 Sequences of the active and mutated versions of the dominant repressor domains used in this study. (A) Sequence of the SRDX domain. (B) Sequence of the mSRDX domain. (C) Sequence of the WUSB dominant repressor domain. (D) Sequence of the mWUSB domain. Upper lines: nucleotide sequences encoding the different domains. Lower lines: corresponding amino acid sequences. Mutated nucleotides to obtain the mSRDX and mWUSB domains are indicated by lowercase. Blue boxes indicate the amino acid changes introduced in order to generate mSRDX and mWUSB domains.

3.2. Results

3.2.1. Characterization of lines ectopically expressing UFO fused to dominant repressor domains and their mutated versions

In order to test the use of the SRDX and WUSB domains, the *UFO* coding sequence was translationally fused to either the SRDX or the WUSB domains and placed under the control of the 35S promoter (Sections 2.5.1.1 and 2.5.1.2, respectively). This promoter was chosen, because plants expressing UFO-SRDX from the 35S promoter had been previously described and shown to exhibit phenotypic alterations that resembled those found in *lfy* mutant plants (Chae et al., 2008), which would serve as a reference to test the functionality of my constructs and to compare the efficiency of the

SRDX and WUSB domains. The constructs described above were then introduced into wild-type plants. Fourteen and 32 primary transformants were obtained that carried the *35S_{pro}:UFO-SRDX* and the *35S_{pro}:UFO-WUSB* transgenes, respectively.

Overall, the transgenic lines that expressed either the *UFO-SRDX* or the *UFO-WUSB* fusions from the 35S promoter showed similar phenotypic alterations as those described by Chae *et al.* (2008). I classified the lines obtained into 4 categories, based on the strength of the floral defects that were observed (Figure 3.2 and Table 3.1). Plants showing ‘no phenotype’, formed flowers that were indistinguishable from wild-type ones. Plants showing a ‘weak phenotype’ formed flowers in which whorls 1 and 4 were unaffected compared to the wild type. In contrast, the second and the third whorls showed defects: the number of normal petals and stamens was reduced and chimeric petal-stamen organs were observed. Moreover, filamentous structures were also produced instead of stamens. These ‘weak’ lines phenocopied weak *ufo* mutant alleles, such as *ufo-6* or *ufo-13* (Figure 3.2C and F). Plants exhibiting an ‘intermediate phenotype’ showed similar defects to ‘weak’ lines in whorls 2 and 3, but their fourth whorl was also affected (Figure 3.2G and H). More specifically, carpels did not fuse properly, aerial ovules and ectopic carpelloid tissue was also observed in the third and/or fourth whorls. Importantly, similar floral defects are found in the strong *ufo-2* mutant allele (Figure 3.2I). In later-arising flowers of these plants, structures characteristic of *lfy* mutants were found, in that flowers terminated with fused carpeloid leafy organs (Figure 3.2J) and intermediate structures between inflorescence shoots and flowers subtended by bracts were also observed (Figure 3.2K). These phenotypic defects suggest that at late stages of development, LFY functions are disrupted in the intermediate UFO-SRDX/WUSB lines.

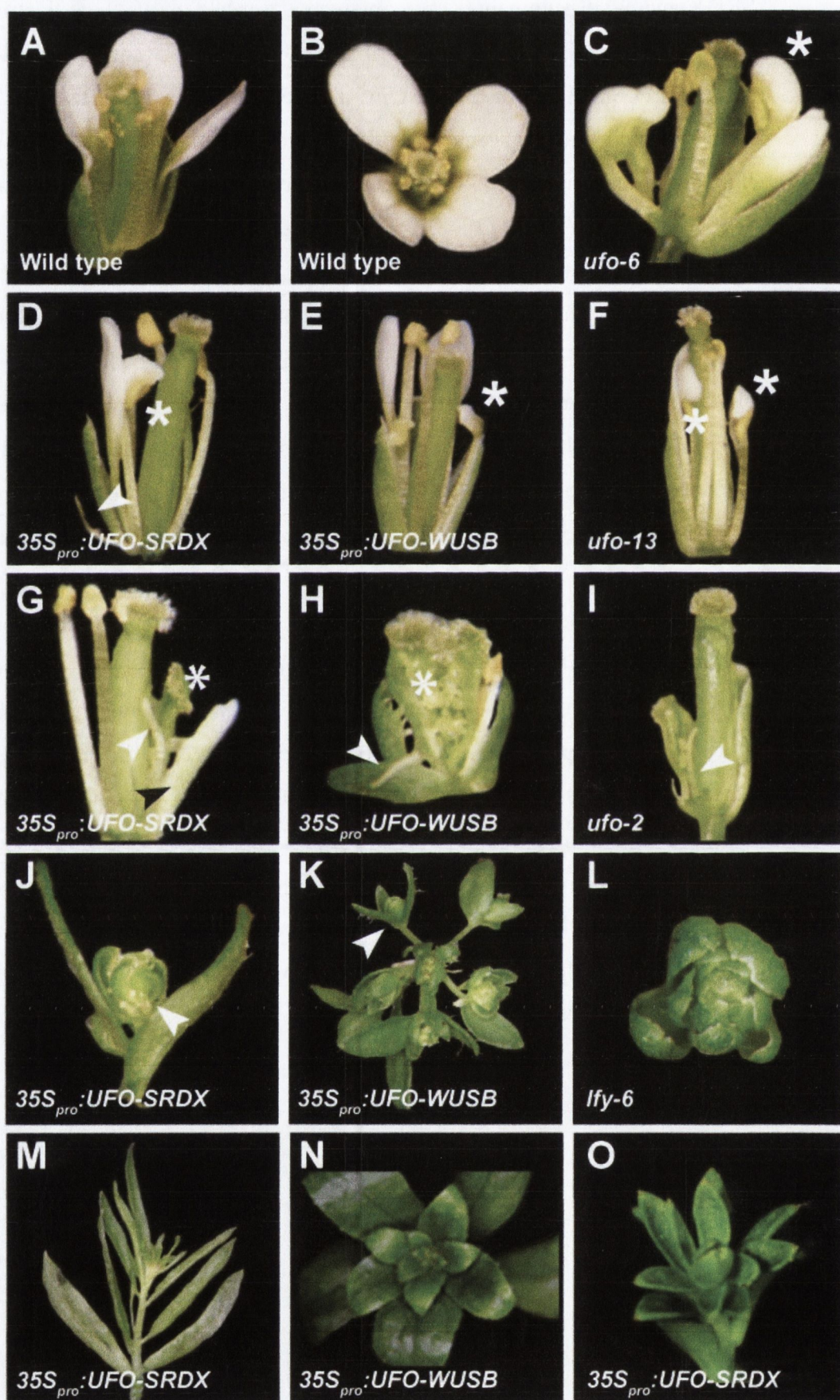


Figure 3.2 Phenotypic comparison of lines expressing the $35S_{pro}:UFO-SRDX/WUSB$ transgene or of *ufo* mutants. (A) Wild-type flower (side view, one sepal and one petal were removed). (B) Wild-type flower viewed from the top. (C) Characteristic flower of a weak mutant allele of *UFO* (*ufo-6*), with a reduced number of normal stamens and the presence of chimeric organs with both stamen and petal identity (indicated by asterisk) (D) Floral abnormalities observed in a $35S_{pro}:UFO-SRDX$ line with a weak phenotype. Chimeric petal-stamen organs are found (indicated with an asterisk), as well as filaments instead of stamens (arrowhead). (E) Floral defects observed in a $35S_{pro}:UFO-WUSB$ line with a weak phenotype. Chimeric petal-stamen organs (asterisk) are found. (F) Floral defects found in the weak *ufo-13* mutant allele. Flowers produce chimeric petal-stamen organs (asterisks). (G) Flower of a plant encoding $35S_{pro}:UFO-SRDX$ and that showed an intermediate phenotype. Only one small petal (black arrowhead) was formed. Filaments are observed instead of stamens (white arrowhead) and carpeloid organs presenting stigmatic tissue are also present (asterisk). (H) Floral defects observed in a $35S_{pro}:UFO-WUSB$ line with an intermediate phenotype. No wild-type petals are formed. One stamen and filamentous structures were visible in the third whorl. Stigmatic tissue and aerial ovules (asterisk) are formed. (I) Floral abnormalities of the strong *ufo-2* mutant. Wild-type petals and stamens are absent. Filamentous structures (arrowhead) often replace stamens; aerial ovules and ectopic carpeloid tissue are also visible. (J) A flower terminated with fused carpeloid leafy organs (arrowhead). (K) Flowers transformed into inflorescence shoots. The flowers resemble those found in the strong *lfy* alleles (arrowhead). The internodes are elongated. (L) Flower of a strong mutant allele of *LFY* (*lfy-6*) with reiterated sepals. (M-O) The lateral structures of plants expressing the $35S_{pro}:UFO-SRDX$ or $35S_{pro}:UFO-WUSB$ fusions in lines classified as strong. No floral organs are produced but leaf-like organs arise in a spiral phyllotaxy which resembles phenotypes observed in *ap1 lfy* double mutants (Huala and Sussex, 1992; Weigel et al., 1992). (M) Structures formed in plants encoding $35S_{pro}:UFO-SRDX$ with a strong phenotype. (N) Lateral structure observed in a $35S_{pro}:UFO-WUSB$ line with a strong phenotype. (O) Figure from Chae *et al.*, 2008. In all pictures, outer organs were removed to visualize the inner whorls.

Finally, in flowers of plants classified as having a ‘strong phenotype’, no floral organs are produced (Figure 3.2M and N). Instead, leaf-like organs arose with a spiral arrangement (in contrast to whorls in wild-type flowers). The flowers of these plants resemble those found in the most severe lines obtained by Chae *et al.* (2008) (Figure 3.2O) and show defects observed in the strong *ap1 lfy* double mutant (Huala and Sussex, 1992; Weigel et al., 1992). This result therefore suggests that both *LFY* and *AP1* functions are affected in these lines, as previously suggested (Chae et al., 2008).

Table 3.1 Example of the frequency of different floral organ types in plants expressing *UFO-WUSB* from the 35S promoter. The organ count of 3 representative lines of each phenotypic category is presented (see full details in the main text). Organ counts were carried out in plants of the T1 generation and numbers correspond to the average number of each type of organ among 25 flowers scored per line.

LINE	SEPAL	SEPALOID PETALS	PETALS	PETAL- STAMEN CHIMERIC ORGANS	FILAMENTS	STAMENS	CARPELOID STAMENS	CARPELS	TOTAL
WT	4.00	0	4.00	0.10	0	5.90	0	2.00	16.00
No phenotype									
C1.9	4.00	0	3.95	0.05	0.00	6.00	0	2.00	16.00
C1.14	4.00	0	4.00	0.10	0.00	5.90	0	2.00	16.00
C2.40	4.00	0	4.00	0.00	0.00	6.00	0	2.00	16.00
Weak phenotype									
C1.8	4.00	0	0.88	2.02	1.08	0.80	0.08	1.92	10.96
C2.39	4.00	0	0.76	2.60	1.04	2.44	0.08	1.92	12.84
C2.51	4.00	0.08	1.96	3.16	0.20	3.20	0	2.00	14.60
Intermediate phenotype									
C1.6	4.36	0	0.56	1.88	1.64	0.44	0.64	1.36	10.88
C2.45	4.00	0	1.04	2.28	0.72	2.28	0.24	2.00	12.56
C1.19	4.00	0	3.50	1.00	0.00	4.50	0.40	1.60	15.00
Strong phenotype									
C1.12	0	0	0	0	0	0	0	0	0
C1.15	0	0	0	0	0	0	0	0	0
C1.18	0	0	0	0	0	0	0	0	0

♦ *Comparison of lines expressing UFO-SRDX and UFO-WUSB*

Lines expressing UFO-SRDX and UFO-WUSB have similar phenotypic abnormalities. In order to assess if one of the dominant repressor domains was more efficient than the other, I tried to estimate if the expression of one of the fusion proteins led to a higher proportion of plants with a strong phenotype. In the case of the 14 independent transformants carrying the *35S_{pro}:UFO-SRDX* transgene, 4 showed a strong phenotype in the T1 generation, which represents 28% of the total number of transformants obtained. Although I was not able to characterize the remaining transformants in the T1 generation in detail, they were fertile, and hence did not fall into

the ‘strong phenotype’ category. The characterization of the T2 generation for these remaining lines indicated that they showed a wide range of phenotypes (Table 3.2), which makes it difficult to infer the phenotype of the parent in the T1 generation.

Table 3.2 Number of plants in each of the different phenotypic categories for lines carrying the *35S_{pro}:UFO-SRDX* transgene in the T2 generation. Floral defects were assessed in 20 plants of the T2 generation per independent T1 parental line. Phenotypic categories are as detailed in the main text.

Line	No phenotype	Mild	Intermediate	Strong
C2.7	20	0	0	0
C2.3	19	1	0	0
C2.8	11	7	0	2
C2.2	8	10	0	2
C2.6	1	10	7	2
C2.5	1	7	10	2
B5.15	3	12	0	5
B5.14	2	8	2	8
C2.14	2	7	2	9
B5.13	0	5	1	14

In the case of the 32 independent transformants carrying the *35S_{pro}:UFO-WUSB* transgene, I quantified the number of different floral organs in the T1 generation (25 flowers per transformant were scored) and assigned them to one of the phenotypic categories described above (Table 3.3). Based on these counts, 9 plants (corresponding to 28% of the total number of T1 plants obtained) showed a strong phenotype.

Table 3.3 Number of plants in each of the different phenotypic categories for lines carrying the *35S_{pro}:UFO-WUSB* transgene in the T1 generation. Floral defects were assessed in 25 flowers per line. Phenotypic categories are as detailed in the main text.

Phenotypic Category	Number of lines
No phenotype	3
Mild	10
Intermediate	10
Strong	9

In sum, the phenotypic alterations observed in plants expressing the UFO-SRDX and UFO-WUSB fusion proteins indicate that the plasmids I generated are functional, and that both domains are suitable. The comparison of the number of transformants with a strong phenotype further suggests that these domains have comparable efficiencies, at

least when fused to UFO.

♦ *Characterization of lines expressing UFO-mWUSB and UFO-mSRDX*

To test whether the observed phenotypes are indeed related to the presence of a functional dominant repressor domain fused to UFO and not to dominant-negative effects related to the addition of a peptide sequence at the C-terminus of UFO, control lines were generated that expressed UFO fused to the mSRDX or the mWUSB domains (Figure 3.1). I expected that the resulting plants would either look like the wild type (*e.g.* if the fusion is not expressed at very high levels), or would show defects similar to those found in plants that overexpress UFO alone (Lee et al., 1997). To test that this was indeed the case, the *UFO* coding sequence was translationally fused to either the mSRDX or the mWUSB domains and placed under the control of the 35S promoter (Sections 2.5.1.1 and 2.5.1.2, respectively). These constructs were then used to transform wild-type plants. Thirteen and 66 independent T1 lines carrying the *35S_{pro}:UFO-mSRDX* and the *35S_{pro}:UFO-mWUSB* transgenes, respectively, were obtained and characterized.

The transgenic lines expressing either the *35S_{pro}:UFO-mSRDX* or *35S_{pro}:UFO-mWUSB* transgene fell into 3 phenotypic categories (Figure 3.3 and Table 3.4). The first category corresponds to plants that appeared phenotypically wild type. This was the case for 24 lines encoding the *35S_{pro}:UFO-mWUSB* (none of the *35S_{pro}:UFO-mSRDX* transformants showed such phenotype). The second category includes plants that showed developmental defects similar to those described upon ectopic expression of *UFO* from the 35S promoter (Lee et al., 1997). Although, the phenotype of these plants is difficult to assess, as it varies depending on whether flowers are formed at early or late stages of development, one clear characteristic of *35S_{pro}:UFO* lines is the formation of leaves with serrated margins. Hence I used predominantly this trait to assign some of the transformants I obtained to this category (Figures 3.3H and I). In addition to serrated leaves, the *35S_{pro}:UFO-mSRDX/mWUSB* I obtained also showed floral defects similar to those described for *35S_{pro}:UFO* lines (Lee et al., 1997). The most severely affected flowers had supernumerary petals (Figure 3.3F), extra stamens formed at the apparent expense of carpels (Figure 3.3E) and often reproductive organs with both stamen and carpel identity were formed. Furthermore, unlike what has been reported by Lee *et al.* (1997), plants carrying the *35S_{pro}:UFO-mSRDX/mWUSB* transgene occasionally produced chimeric petal-stamen organs. Five and 25 lines encoding *35S_{pro}:UFO-mSRDX* and *35S_{pro}:UFO-mWUSB*, respectively, fell into this phenotypic class.

Contrary to my expectations, some transformants had a different phenotype and belong to a third category. These plants formed flowers with a reduced number of petals and stamens, as well as filaments and chimeric petal-stamen organs. Furthermore, unlike plants that ectopically express UFO, these plants did not show any leaf morphology defects. These transformants therefore resembled weak mutant alleles of *UFO*, such as *ufo-6*, or plants that express UFO fused to a functional SRDX domain and that have a weak phenotype (see above). For the $35S_{pro}:UFO-mSRDX$ construct, 8 independent transformants showed such phenotypes (Figure 3.3B), while 17 transformants had such defects for the $35S_{pro}:UFO-mWUSB$ transgene (Figure 3.3C).

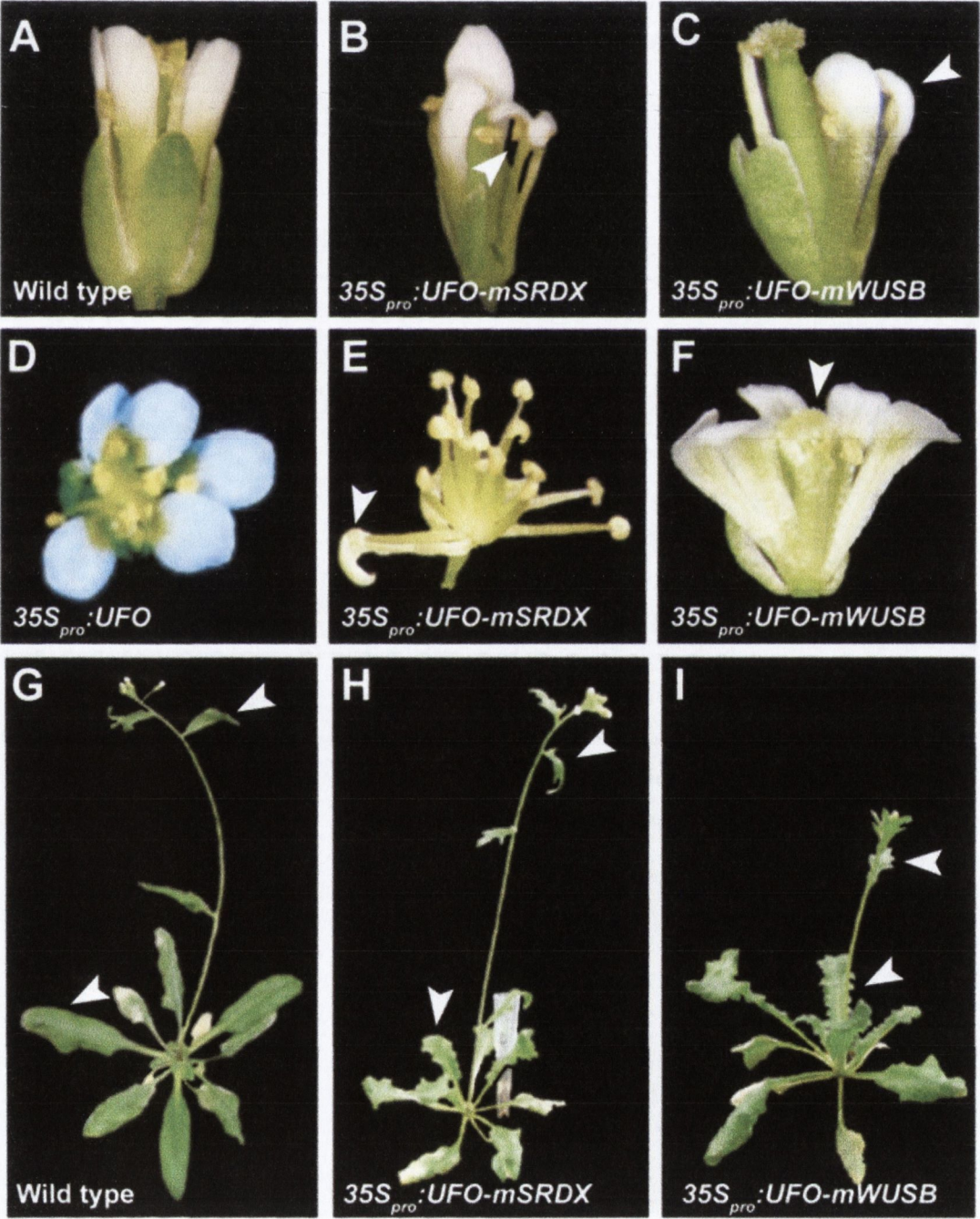


Figure 3.3 Phenotypic comparison of lines encoding $35S_{pro}:UFO-mSRDX/mWUSB$ and $35S_{pro}:UFO$. (A) Side view of a wild-type flower. (B-C) Floral defects observed in $35S_{pro}:UFO-mSRDX/mWUSB$ lines showing flowers with chimeric petal-stamen organs (arrowheads), as well as a reduction in the number of organs in the second and third whorls. (D) Phenotypic abnormalities observed in plants ectopically expressing *UFO*. Supernumerary petals and stamens are produced. Figure from Lee *et al.* (1997). (E-F) Floral defects observed in a $35S_{pro}:UFO-mSRDX/mWUSB$ lines with the expected *UFO* overexpression phenotype. (E) Floral defects observed in a *UFO-mSRDX* line. No sepals, petals and carpels are produced. Supernumerary stamens are produced in all whorls and chimeric petal-stamen organs are also found (arrowhead). (F) Phenotypic abnormalities observed in plants carrying the $35S_{pro}:UFO-mWUSB$ transgene. The number of stamens is reduced and supernumerary petals are produced. The gynoecium is not formed properly due to the presence of an extra carpel (arrowhead). (G) Five-week-old wild-type plant. Note the absence of serrations on rosette and cauline leaves (arrowheads). (H-I) Five-week-old $35S_{pro}:UFO-mSRDX$ (H) and $35S_{pro}:UFO-mWUSB$ (I) plants with the characteristic leaf phenotype found in plants ectopically expressing *UFO* (*i.e.* the presence of serrated rosette and cauline leaves, as indicated by arrowheads).

Table 3.4 Example of the frequency of different organ types in lines expressing the *35S_{pro}:UFO-mSRDX* or *35S_{pro}:UFO-mWUSB*. Organ counts were carried out in plants of the T1 generation. None of the transformants carrying the *35S_{pro}:UFO-mSRDX* transgene had a wild-type-like phenotype. The numbers correspond to the average number of each type of organ among 20 flowers scored per line. The organ count of 3 representative lines of 3 categories is presented (full details in main text). Petal/stamen correspond to chimeric organs with both petal and stamen identity; Stamen/carpel correspond to organs intermediate between stamen and carpel identity.

LINE	SEPAL	SEPALOID PETAL	PETAL	PETAL/STAMEN	FILAMENT	STAMEN	STAMEN/CARPEL	CARPEL	TOTAL
WT	4.00	0	4.00	0	0	5.90	0	2.00	16.00
No phenotypic defects									
UFO-mWUSB									
G6.6	4.00	0	4.00	0	0	6.00	0	2.00	16.00
G3.21	4.05	0	4.00	0	0	6.00	0	2.00	16.05
G3.3	4.00	0	3.95	0	0	6.10	0	2.00	16.05
UFO overexpression phenotype									
UFO-mSRDX									
D1.36	3.35	0.10	4.35	0.15	0.10	5.65	2.80	1.30	17.80
D1.37	4.00	0	4.00	0	0.05	5.30	6.00	0	19.35
D1.35	4.05	0	4.10	0.25	0	5.70	6.30	0	20.40
UFO-mWUSB									
G4.20	4.00	0	4.00	0	0	5.50	4.00	0	17.50
G4.21	4.00	0	4.00	0	0	5.65	4.00	0	18.15
G3.19	4.10	0	3.80	0	0	6.30	6.00	0	20.20
Mild UFO-SRDX/WUSB phenotype									
UFO-mSRDX									
D1.40	3.95	0.05	1.75	1.50	0.40	2.70	0	2.00	12.35
D1.39	3.90	0.05	2.15	1.35	0.10	4.10	0	2.00	13.65
D1.42	4.20	0.05	0.25	5.35	0.25	2.15	0	2.00	14.25
UFO-mWUSB									
G3.17	4.00	0	0.70	1.55	0.15	4.70	0	2.00	13.10
G6.5	4.15	0	1.10	1.95	0.60	4.10	0	2.05	13.95
G3.24	4.00	0	3.00	0.25	0.05	5.45	0	2.00	14.75

Although most plants had the expected phenotypes (*i.e.* appeared either wild type or similar to *35S_{pro}:UFO* lines), the observation that a large proportion of the transformants expressing the UFO-mSRDX/mWUSB proteins exhibited floral defects found in *ufo-6* or in UFO-SRDX/WUSB lines suggests that the phenotype observed in the latter lines might not be solely due to the activity of the repressor domain when fused to UFO. In order to further understand the origin of these mutant phenotypes in UFO-mSRDX/mWUSB lines, I tried to determine if they correlated with the level of expression of the fusion. To this end, I analyzed the T2 generation of some of the transformants that fell into the third phenotypic class and I carried out RT-qPCR analyses to determine the expression level of *UFO-mSRDX* in plants with different phenotypes. When I propagated the T2 generation of 8 independent lines encoding *35S_{pro}:UFO-mSRDX*, I observed that the resulting populations had plants that exhibited all 3 categories of phenotypes (Table 3.5). Although the number of plants observed is too small to do a proper segregation analysis, this results suggest that the different phenotypic classes might originate from different expression levels of the fusion. In order to assess this possibility, I carried out RT-qPCR analyses and tested the expression level of the *UFO-mSRDX* fusion in plants from all 3 phenotypic categories (Figure 3.4).

Table 3.5 Number of plants in each of the different phenotypic categories for lines carrying the *35S_{pro}:UFO-mSRDX* transgene in the T2 generation. Floral defects were assessed in 15 plants of the T2 generation per independent T1 parental line. Phenotypic categories are as detailed in the main text.

Line	No phenotypic defects	UFO Over-expression	Mild UFO-SRDX
D1.31	4	1	10
D1.39	0	6	9
D1.40	5	1	9
D1.32	6	4	5
D1.42	7	5	3
D1.43	6	6	3
D1.33	5	8	2
D1.38	4	9	2

In these experiments, young floral buds from plants representing each phenotypic category (derived from lines D1.31 and D1.42) were collected, followed by RNA extraction and cDNA synthesis. Next, I carried out qPCR experiments using a set of oligonucleotides designed to amplify specifically the *UFO-mSRDX* mRNA (Figure 3.4A). Because of time constraints, I was not able to test additional lines and repeat the

experiment, but the preliminary data obtained suggest that plants showing the typical UFO overexpression phenotype had the highest level of expression for UFO-mSRDX, while plants showing either no phenotype or a weak UFO-SRDX phenotype expressed the UFO-mSRDX fusion at low levels. Because the weak UFO-SRDX phenotype is also reminiscent of that found in weak *ufo* mutant alleles, I also checked if the levels of endogenous *UFO* mRNA were affected in plants of the 3 categories and found that this was not the case (Figure 3.4B).

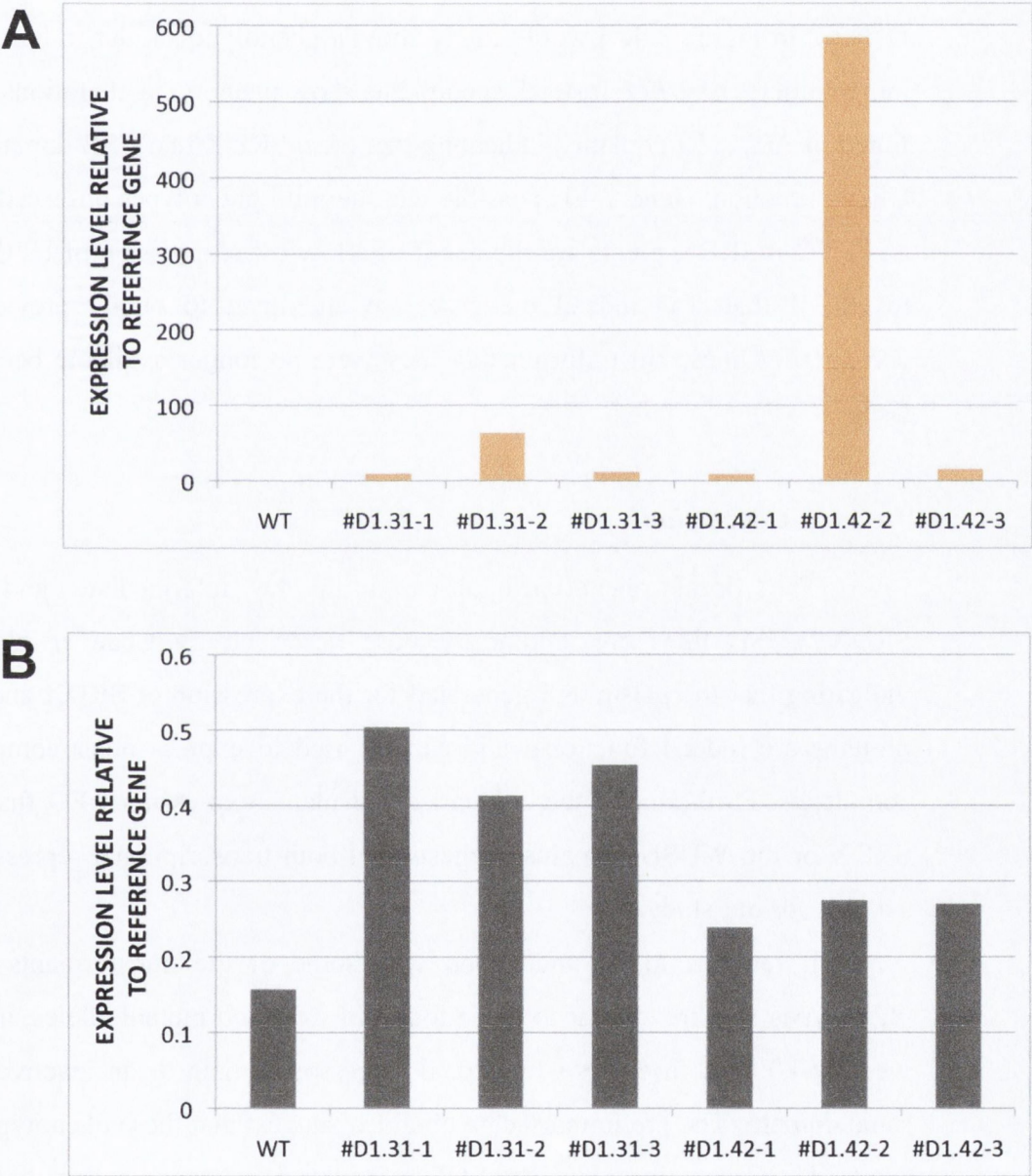


Figure 3.4 Results of RT-qPCR experiments for 2 lines expressing the *UFO-mSRDX* fusion from the 35S promoter. Expression levels of **(A)** the *UFO-mSRDX* fusion (orange bars) and **(B)** endogenous *UFO* (brown bars) were determined for 2 independent lines (D1.31 and D1.42) carrying the *35S_{pro}:UFO-mSRDX* transgene in the T2 generation. ‘1’, ‘2’ and ‘3’ after the name of the line correspond to the phenotypic category: wild-type, UFO gain-of-function and mild SRDX, respectively (details in the main

text). The results are presented relative to the reference gene. RNA was extracted from young floral buds. No error bars are presented for the expression assays because these values represent technical duplicates.

Although I did not have sufficient time to carry out additional biological replicates for these RT-qPCR experiments, these preliminary results suggest that the *ufo-6*-like phenotype observed in some of the lines expressing *UFO-mSRDX/mWUSB* is unlikely due to a partial loss of function of UFO because of the mSRDX/mWUSB peptides. Indeed, if the presence of the mSRDX or mWUSB domains affected UFO's function, plants that express these fusions at higher levels (*e.g.* plants D1.31-2 and D1.42-2 in Figure 3.4A) would likely show a phenotype similar to that found in strong mutant alleles of *UFO*. Instead, such plants show phenotypic alterations similar to those found in *35S_{pro}:UFO* plants, indicating that the mSRDX/mWUSB domains do not affect UFO's function. Hence, it is possible that the mild phenotypes observed in *UFO-SRDX* and *UFO-mSRDX* plants are the result of a low overexpression of UFO itself. In order to test if that was indeed the case, we attempted to obtain previously published *35S_{pro}:UFO* lines, but unfortunately they were no longer available because of sterility issues.

◆ Conclusion

The floral phenotypes observed in the intermediate and strong *UFO-SRDX/WUSB* lines are similar to those described by Chae *et al.* (2008), hence indicating that the plasmids I generated for the expression of SRDX and WUSB fusion proteins are indeed functional and can be used to express other components of SCF complexes. Furthermore, the comparison of plants expressing UFO fused to either the SRDX or the WUSB domains suggests that both transcriptional repressor domains are suitable for our study.

It remains to be understood why some of the transformants obtained show phenotypes that are similar to those found in weak *ufo* mutant alleles, independently of whether UFO is fused to a functional repressor domain or an inactive version of the same domain. The preliminary data obtained suggest that these phenotypes might be the result of a weak overexpression of UFO, but this conclusion needs to be confirmed after additional biological replicates have been carried out and after plants encoding a *35S_{pro}:UFO* construct have been isolated and characterized.

3.2.2. Isolation of *ufo-2* rescue lines expressing epitope-tagged versions of *UFO* for ChIP-Seq analysis

To obtain further insights into the molecular mechanisms underlying the role of *UFO* as a transcriptional co-activator during flower development, I aimed at identifying the genomic regions bound by *UFO* at early stages of flower formation using ChIP-Seq. In order to immunoprecipitate *UFO* and *UFO*-containing complexes, antibodies specific for this F-box protein are necessary. Because no antibodies against *UFO* are available, I generated constructs that would allow expression of different epitope-tagged versions of *UFO* under the control of the *UFO* promoter, which comprises the genomic region starting ~ 3.8 kb upstream of the start codon (this promoter region was shown to be sufficient to restore expression of *UFO* in a similar pattern as that found by *in situ* hybridizations (Hobe, 2004)). The different rescue constructs were then introduced into the *ufo-2* mutant background to test the functionality of the fusion proteins and avoid competition with endogenous *UFO* during the immunoprecipitation procedure. Because it is difficult to predict which tag will lead to a functional protein, I attempted to test the efficiency of two epitope tags in parallel.

♦ *Partial rescue of ufo-2 by a UFO-3xFLAG fusion protein*

First, I tried to obtain rescue lines that would express *UFO* fused to 3 repeats of the FLAG sequence (noted *UFO_{pro}:UFO-3xFLAG*) (Figure 3.5A) and transformed a *ufo-2/+* segregating population with the resulting construct (Section 2.5.3.1).

Two independent primary transformants were obtained and genotyped for the presence of both the transgene and the *ufo-2* mutation. One was homozygous for the *ufo-2* mutation, while the other transformant was wild type for *UFO*. The latter was not characterized further. A preliminary characterization of the transformant in the *ufo-2* background in the T1 generation indicated that the *ufo-2* phenotype was partially rescued as plants were fertile. The T2 population of this transformant was not phenotypically homogenous and presented plants with different phenotypes. The majority of plants expressing *UFO-3xFLAG* showed a partial rescue of the *ufo-2* phenotype. In particular, similarly to their parent, these plants were fertile and produced flowers with a reduced number of petals and stamens, ranging from 1 to 3 petals and 2 to 6 stamens with viable pollen (Figure 3.4F and G). Based on the absence of chimeric petal-stamen organs, these plants appeared to have a weaker mutant phenotype than the weak *ufo-6* mutant allele (Figure 3.4D). Moreover, despite the selection for plants that

carried the transgene, some plants were indistinguishable from the *ufo-2* mutant, with a lack of wild-type petals and stamens and the presence of filamentous structures (Figure 3.5E). Finally, no wild-type looking plants could be identified, indicating that this construct only led to partial rescue of the *ufo-2* mutant phenotype, at least in this one particular line that was isolated.

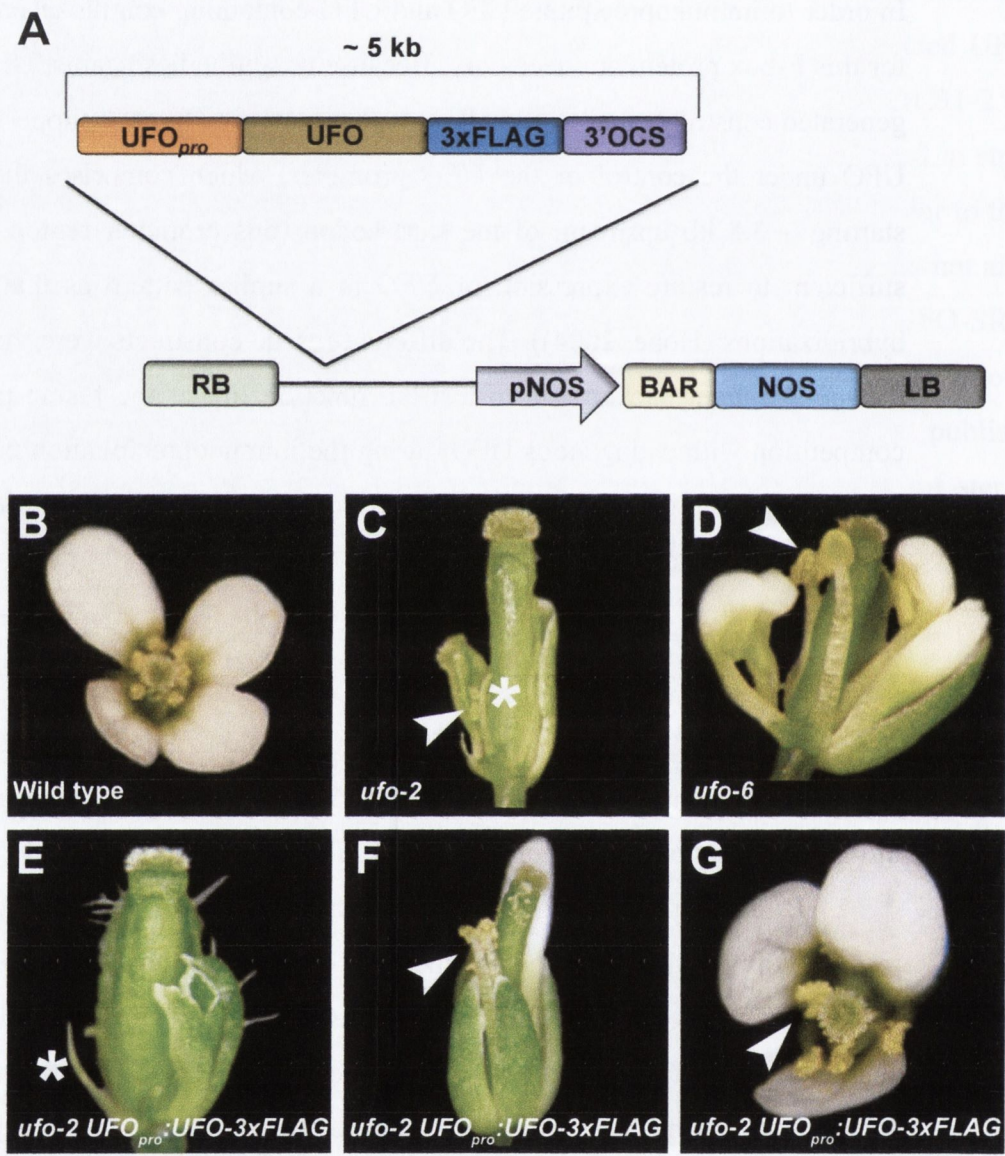


Figure 3.5 Partial rescue of a *ufo-2* mutant by the introduction of a *UFO_{pro}:UFO-3xFLAG* construct. (A) A scheme of the T-DNA mediating expression of an *UFO_{pro}:UFO-3xFLAG*. The binary vector pML-BART was used in conjunction with *Agrobacterium tumefaciens* to transform a *ufo-2*/+ population. (B) Top view of a wild-type flower. (C) Flower of a *ufo-2* mutant. Filaments (asterisk) and aerial ovules (arrowhead) were observed. (D) Floral phenotype of the weak *ufo-6* mutant. Two wild-type stamens (arrowhead) and three chimeric petal-stamen organs are visible. (E) Flower of *ufo-2* plants carrying *UFO_{pro}:UFO-3xFLAG* transgene that did not rescue the mutant phenotype. Filament is indicated by an asterisk. Flowers were indistinguishable from *ufo-2* mutant flowers. (F) Partial rescue of the *ufo-2* mutant

phenotype by UFO-3xFLAG. A reduced number of petals and stamens are formed. Stamens produce viable pollen (arrowhead). **(G)** Partial rescue of the *ufo-2* phenotype by the UFO-3xFLAG fusion. Three wild-type petals and 4 stamens (arrowhead) were produced. 3'OCS, octopine synthase terminator sequence; RB, right border of T-DNA; LB, left border of T-DNA; pNOS, nopaline synthase promoter sequence; BAR, BASTA® resistance gene; NOS, nopaline synthase terminator sequence.

Because I only isolated one transformant, it is difficult to conclude whether the partial rescue is due to the presence of the 3xFLAG tag at the C-terminus of UFO, or to potentially low expression levels of the fusion. I did not attempt to isolate additional transformants to distinguish between these 2 possibilities, as the use of a GFP tag in parallel yielded more promising results.

◆ *Rescue of ufo-2 by expression of a UFO-GFP fusion protein*

In the laboratory, ChIP-Seq experiments have been successfully carried out using transcription factors that were expressed from endogenous promoters and that had been fused to GFP. In order to take advantage of the expertise and protocols developed in the laboratory, I generated a plasmid carrying a C-terminal fusion of UFO to GFP under the control of the endogenous *UFO* promoter (noted *UFO_{pro}:UFO-GFP*) (Figure 3.6A). This vector was introduced into a *ufo-2/+* segregating population (Section 2.5.3.2).

Two independent primary transformants were obtained and genotyped for the presence of both the transgene and the *ufo-2* mutation. One was heterozygous for the *ufo-2* mutation and one was wild type for *UFO*. I first carried out a more detailed characterization of the progeny of the transformant obtained in the *ufo-2/+* background. In the T2 generation I selected for the *UFO_{pro}:UFO-GFP* transgene and all plants appeared phenotypically normal. Plants were therefore genotyped to determine which ones were homozygous for the *ufo-2* mutation, as well as to check for the presence of the rescue construct. The genotyping results confirmed that plants that were homozygous for the *ufo-2* mutation and contained the *UFO-GFP* rescue construct formed wild-type flowers (data not shown), indicating that the construct used resulted in the full rescue of the *ufo-2* mutant phenotype and that the line obtained could be characterized in more detail and possibly used for ChIP-Seq experiments.

In order to characterize more in detail this rescue line, I checked if the UFO-GFP fusion protein was present *in planta* using Western blot analysis (Section 2.2.4.7) of protein extracts (Section 2.2.4.1) from whole inflorescences of plants carrying the *UFO_{pro}:UFO-GFP* transgene. Although a GFP-specific antibody that had been

previously shown to recognize efficiently GFP fusion protein in plant extracts was used, I could not detect proteins corresponding to UFO-GFP (data not shown). As mentioned above (Section 1.3.3), *UFO* is expressed only in young flower buds. Because whole inflorescences were collected for this Western blot analysis, proteins from older flowers that do not express UFO are likely over-represented in the protein extract. I also attempted to check whether UFO-GFP was expressed in the same expression pattern as that described for endogenous UFO by *in situ* hybridization (Samach et al., 1999). To this end, I used fluorescence microscopy techniques, but did not observe any GFP signal (data not shown). Again, the lack of signal could be due to the fact that *UFO* is expressed only in young floral buds, which are difficult to visualize using whole inflorescences, even after careful dissection.

Because a large amount of synchronized floral tissue is required for ChIP-Seq analysis, I crossed the *ufo-2 UFO_{pro}:UFO-GFP* line described above into the floral induction system (noted *ap1-1 cal-1 API_{pro}:API-GR*) (O'Maoileidigh et al., 2013). The F1 population was allowed to self fertilize and in the F2 population I isolated a line that was homozygous for *ap1-1 cal-1 ufo-2* and also encoded the *UFO_{pro}:UFO-GFP API_{pro}:API-GR* T-DNAs. In the F3 generation, in order to isolate a line that was also homozygous for the *API_{pro}:API-GR* and the *UFO_{pro}:UFO-GFP*, I used information obtained from TAIL PCR reactions carried out by Dr. Diarmuid O'Maoileidigh and Dr. Emmanuelle Graciet, respectively. A line homozygous for all mutations or transgenes was then used for further characterization.

The use of the floral induction system should result in a significant enrichment for the tissue in which *UFO* is normally expressed, suggesting that the UFO-GFP fusion protein could likely be detected in this background. Hence, in order to check if *UFO-GFP* was indeed expressed in this line and to determine if it was expressed in the same pattern as endogenous *UFO*, I used Western blot analysis, as well as fluorescence microscopy. For the Western blot analysis, I collected inflorescences from *ap1-1 cal-1 ufo-2 API_{pro}:API-GR UFO_{pro}:UFO-GFP* plants 4 d after treatment with a DEX-containing solution (approximately stages 5-6 of floral development (Smyth et al., 1990; Wellmer et al., 2006)) and purified nuclei (Section 2.2.4.5). Moreover, as a positive control for the Western blot, I also collected floral tissue 4 d after treatment with a DEX-containing solution from an *ap1-1 cal-1 ag-1 API_{pro}:API-GR AG_{pro}:AG-GFP* line, which had been shown to express the AG-GFP fusion protein at a detectable level. Finally, to control for cross-reacting proteins I also collected floral tissue from *ap1-1 cal-1 API_{pro}:API-GR* plants (Wellmer et al., 2006; O'Maoileidigh et al., 2013). After

immunoblotting with a GFP-specific antibody, proteins migrating at ~ 80 kDa and ~ 50 kDa were identified which correspond to the expected molecular weights of UFO-GFP (78 kDa) and AG-GFP (56 kDa), respectively (Figure 3.6). These results suggest that the UFO-GFP fusion protein is indeed expressed in the *ap1-1 cal-1 ufo-2 API_{pro}:API-GR UFO_{pro}:UFO-GFP* line I generated for ChIP-Seq.

I next checked if the UFO-GFP signal was expressed in the same pattern as that described for endogenous UFO (Samach et al., 1999), using fluorescence microscopy (Figures 3.6C and D). It has been shown that *UFO* transcript is detected first in the central dome of the floral meristem at ~ stage 2 of flower development, and then becomes progressively restricted to the presumptive whorls 2 and 3 (stages ~ 3 to 6). Around stage 6, *UFO* is expressed at the base of petals (Lee et al., 1997; Samach et al., 1999). To characterize the expression pattern of UFO-GFP in the floral induction system, I took pictures before and 3 d after treatment with a DEX-containing solution. Before treatment, floral buds correspond to ~ stage 2 of flower development and, as expected, the signal coming from the UFO-GFP fusion was detected in the centre of each floral meristem. In contrast, when tissue was collected 3 d after treatment with a DEX-containing solution (~ stage 4), UFO-GFP appeared to be expressed in a ring-like pattern surrounding the central zone, but the fluorescence signal and the resolution were too low to observe clearly the expression pattern of UFO-GFP. In order to overcome these limitations and confirm that UFO-GFP is indeed expressed in the expected pattern, I intend to use confocal microscopy in the future.

In sum, the rescue construct leads to expression of UFO-GFP and is sufficient to fully rescue the *ufo-2* mutant phenotype, suggesting that this line should be suitable for ChIP-Seq experiments and the genome-wide identification of UFO binding sites. Unfortunately, because of time constraints I was not able to carry out the ChIP-Seq experiment at this stage.

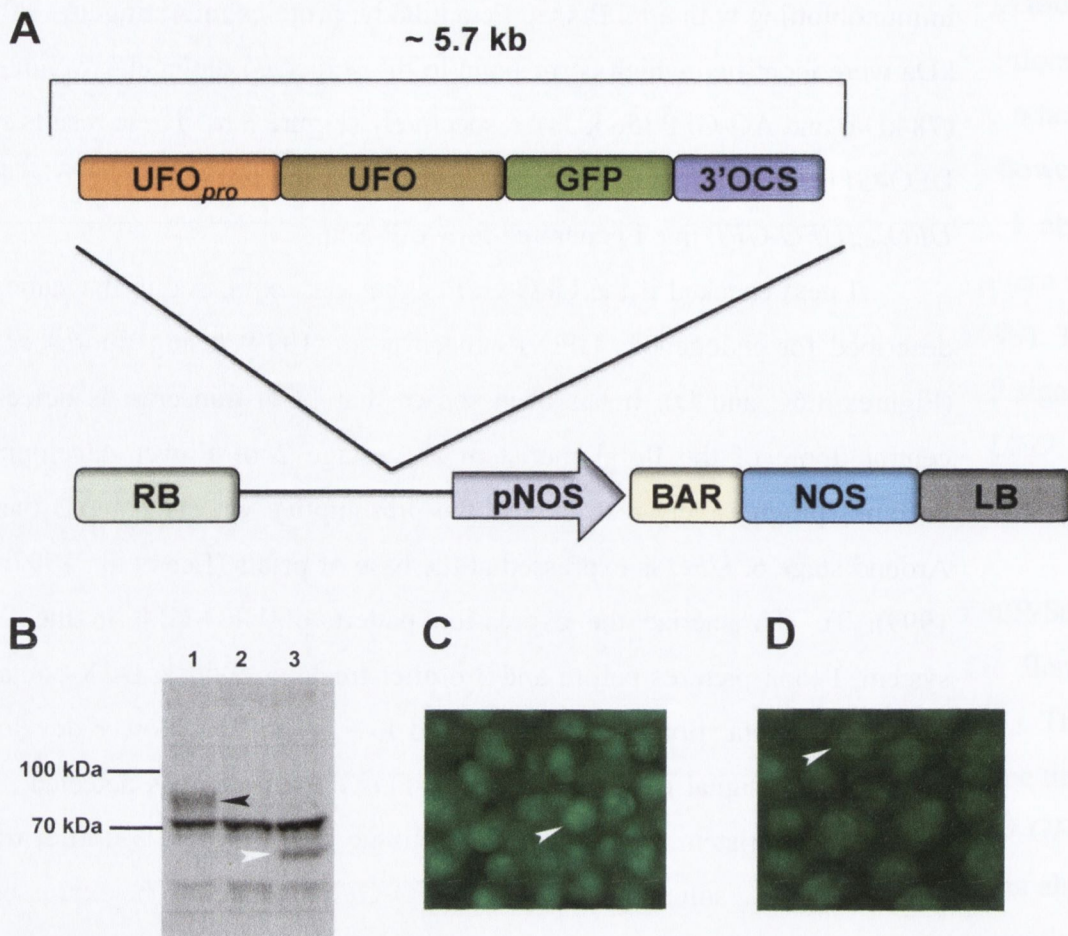


Figure 3.6 Characterization of *ap1-1 cal-1 ufo-2 API_{pro}:API-GR UFO_{pro}:UFO-GFP*. (A) A schematic of the T-DNA mediating expression of an *UFO_{pro}:UFO-GFP*. The binary vector pML-BART was used in conjunction with *Agrobacterium tumefaciens* to transform wild-type plants. For full details see Section 2.5.3.2. (B) Western blot analysis of *ap1-1 cal-1 ufo-2 API_{pro}:API-GR UFO_{pro}:UFO-GFP* and *ap1-1 ag-1 cal-1 AG_{pro}:AG-GFP API_{pro}:API-GR* resuce lines and *ap1-1 cal-1 API_{pro}:API-GR*. Proteins were extracted from nuclei from flowers collected 4 d after DEX treatment. Lane 1: *ap1-1 cal-1 ufo-2 API_{pro}:API-GR UFO_{pro}:UFO-GFP*. A band at approximately 80 kDa is observed (black arrowhead) which is the expected size of UFO-GFP fusion protein. Lane 2: *ap1-1 cal-1 API_{pro}:API-GR*; lane 3: *ap1-1 ag-1 cal-1 AG_{pro}:AG-GFP API_{pro}:API-GR*. A band at approximately 50 kDa is observed (white arrowhead) which is the expected size of AG-GFP fusion protein. The antibody against GFP was used. Three independent biological replicates were carried out. (C-D) Fluorescent microscopic images of inflorescences (C) before DEX treatment and (D) 3 d after DEX treatment. Arrowheads indicate fluorescence. 3'OCS, octopine synthase terminator sequence; RB, right border of T-DNA; LB, left border of T-DNA; pNOS, nopaline synthase promoter sequence; BAR, BASTA® resistance gene; NOS, nopaline synthase terminator sequence; UTR, untranslated region.

3.3. Discussion and future work

My work on the F-box protein UFO aimed at (i) determining if the SRDX and WUSB domains were indeed suitable for our study using other components of SCF complexes, by testing the plasmids I generated and comparing the efficiency of the 2 repressor domains; (ii) gain further insights into UFO functions in the regulation of flower development through the detailed characterization of lines expressing UFO-SRDX or UFO-WUSB fusion proteins and by identifying the regions of the genome that are bound by UFO using ChIP-Seq analysis.

3.3.1. Comparison and suitability of the SRDX and WUSB domains

In order to test the plasmids I generated to express SRDX and WUSB fusion proteins, I isolated plants that express *UFO-SRDX* and *UFO-WUSB* under the control of the 35S promoter. The lines I obtained overall recapitulated the phenotypic abnormalities that had been previously described for 35S_{pro}:UFO-SRDX plants (Chae et al., 2008). For example, some lines showed phenotypes similar to those found in strong *lfy* mutants: flowers terminated with fused carpeloid leafy organs and intermediate structures between inflorescence shoots and flowers subtended by bracts were also observed (Figures 3.2J and K), indicating that expression of UFO-SRDX/WUSB impairs *LFY* function. Furthermore, similarly to the results reported by Chae *et al.*, some plants that expressed the UFO-SRDX/WUSB fusion proteins did not produce floral organs (Figure 3.2M and N). Instead, leaf-like structures arose in a spiral arrangement, similarly to the phenotype found in *ap1 lfy* double mutants (Huala and Sussex, 1992; Weigel et al., 1992), suggesting that UFO could regulate not only *LFY*, but also *AP1* activity, as suggested previously (Chae et al., 2008).

Despite the similarities between the results I obtained using UFO-SRDX/WUSB lines and those published by Chae *et al.* 2008, I also found differences. More specifically, I identified UFO-SRDX/WUSB with phenotypic defects typical of weak mutant alleles of *UFO*, such as *ufo-6* (Durfee et al., 2003), in that they formed flowers with a reduced number of petals and stamens, as well as chimeric petal-stamen organs and filamentous structures (Figures 3.2D and E). More surprisingly, similar defects were also found in plants expressing the control *UFO-mSRDX/mWUSB* fusion. Indeed, I expected that these plants would present similar phenotypic defects as those reported for

plants expressing *UFO* alone from the 35S promoter (Lee et al., 1997). The results of RT-qPCR experiments on one of these *UFO-mSRDX* lines revealed that plants that showed a weak *ufo* mutant phenotype also expressed the *UFO-mSRDX* fusion to lower levels than plants showing the typical phenotype for ectopic expression of *UFO*. This result indicates that these unexpected phenotypic abnormalities are unlikely to be the result of impaired *UFO* function due to the addition of a short peptide to the C-terminus of *UFO* (if that was the case, then plants expressing *UFO-mSRDX* at high levels would resemble strong *ufo* mutant alleles such as *ufo-2*). These novel phenotypes hence rather suggest that mis-expression of *UFO* at low levels could affect the developmental programs of petals and stamens. I am currently investigating this possibility by generating 35S_{pro}:*UFO* lines that I will characterize in detail.

Finally, by comparing the strength of the phenotype found in plants expressing *UFO-SRDX* or *UFO-WUSB*, it appears that both repressor domains have similar efficiencies, at least when used in conjunction with *UFO*. This result indicates that when studying the role of *ASK1* as a transcriptional co-regulator, I can use both domains, which could serve as a control for the robustness of the mutant phenotypes that might be observed.

3.3.2. New insights into the functions of *UFO*

Even though *UFO* was identified almost 20 years ago (Wilkinson and Haughn, 1995), many ambiguities remain as to the molecular mechanisms underlying *UFO*'s functions in the regulation of flower development. The use of genetic approaches have shown that *UFO* is necessary for the proper patterning of the petal, stamen and carpel primordia, and that it acts upstream of the B class transcription factor *AP3* and *PI* (Wilkinson and Haughn, 1995). Furthermore, genetic interactions and biochemical studies have shown that the role of *UFO* is dependent on the formation of a functional SCF complex, hence indicating that *UFO* is likely to act through the degradation of target proteins. It was initially hypothesized that *UFO* targets for degradation a negative regulator of B function, thus allowing the expression of *AP3* and/or *PI*. More recently, it was proposed that *AP2* could be this negative regulator (Krogan et al., 2012), but no experimental data has been obtained so far to test this possibility. Furthermore, 2 recent papers focusing on the functions of *UFO* in *Arabidopsis* or of its ortholog (*DOT*) in *Petunia*, have revealed that *UFO* is likely to regulate flower development by acting as a transcriptional co-activator of *LFY*. Hence, it is expected that *UFO* will bind, together with *LFY*, to the promoter of some of this transcription factor's target genes. Such

binding has been shown in the case of the *AP3* promoter (Winter et al., 2011). Indeed, Chae *et al.* (2008) used whole inflorescences from a line expressing a UFO-MYC fusion under the control of the 35S promoter lines for ChIP followed by qPCR and showed that UFO could bind to the same promoter elements as LFY in the *AP3* promoter. However, it remains unknown whether UFO binds to the promoter of other genes that are also regulated by LFY, or to the promoter of other genes, that are not LFY targets.

In order to answer these questions, I have generated lines that express UFO-GFP under the control of *UFO*'s endogenous promoter in the floral induction system. These lines will allow me to collect large amounts of young floral buds, at stages at which UFO acts to activate the expression of B function genes, likely in conjunction with LFY. This was not the case in the study by Chae *et al.* (2008), as these authors used whole inflorescences, *i.e.* tissue that is enriched for late stages of flower development. Furthermore, these authors expressed UFO-MYC from the 35S promoter, which can lead to artefacts. Hence, the reagents I have generated will likely reveal new physiological functions of UFO and more importantly could contribute to a better understanding of the molecular mechanisms underlying its functions.

One difficulty when analyzing data obtained from ChIP-Seq experiments is to distinguish between regions of the genome that are bound by a protein without this binding resulting in the regulation of the neighboring genes, from regions of the genome that are bound and whose neighboring genes are indeed regulated by the protein of interest (Kaufmann et al., 2010; Wuest et al., 2012; O'Maoileidigh et al., 2013). To this aim, one would compare the results of ChIP-Seq experiments to those of transcriptomics analyses, for example upon loss of function of the gene of interest. Unfortunately, such experiments are difficult to carry out using a *ufo-2* line in the background of the floral induction system, as introduction of *ufo-2* in a *apl cal* mutant background results in the loss of the typical inflorescence meristem overproliferation in these lines. In order to overcome these problems, I have attempted to isolate an amiRNA that would efficiently target *UFO* mRNA, but was not able to find one among 4 constructs tested (data not shown). In order to by-pass this problem, I will cross-compare the binding sites found for UFO to known LFY target genes, which have been identified using ChIP-Seq and transcriptomics experiments (Moyroud et al., 2011; Winter et al., 2011). I will also attempt to carry out transcriptomics experiments using inflorescences from *ufo-2* mutant plants (not in the floral induction system) and will use reagents that were generated by Dr. Emmanuelle Graciet and that allow ectopic expression of *UFO* under the control of and inducible system.

4. Transcriptional co-activation by SCF complexes is a widespread mechanism to control gene expression

4.1. Introduction

It has been shown that SCF complexes can act as transcriptional co-activators in plants (Section 1.1.3.2), but also in other organisms. As mentioned in the introduction, the questions I aim at answering are whether this kind of transcriptional regulation by SCF complexes is a widespread mechanism to control gene expression, and how many and which genes are regulated in this manner. To this aim, I intend to identify biological processes that require transcriptional co-activation by ASK1, a subunit of SCF Ub ligases, and to uncover the identity of the genes whose expression is transcriptionally co-regulated by this protein. I used 2 different approaches: (i) a perturbation of *ASK1* function, by expressing a fusion of ASK1 with a dominant repressor domain; (ii) ChIP-Seq experiments to identify the genomic regions bound by ASK1 and likely ASK1-containing SCF complexes.

4.2. Results

4.2.1. Identification of physiological processes that are transcriptionally co-regulated by SCF complexes

4.2.1.1. Perturbation of ASK1 function using a dominant repressor domain

In order to disrupt ASK1's function as a transcriptional co-activator without affecting its other roles, ASK1 was expressed as a fusion with 2 different dominant repressor domains, namely the SRDX and the WUSB peptides, which have been shown to be sufficient to change a transcriptional activator into a dominant repressor (Sections 1.4.1 and 3.1) (Chae et al., 2008; Ikeda et al., 2009). Our rationale is that if ASK1-containing SCF complexes regulate physiological processes by binding (directly or indirectly) to DNA and acting as transcriptional co-activators, the expression of ASK1 fused to the SRDX or WUSB domain should lead to phenotypic alterations due to the disruption of these physiological processes.

♦ ***Absence of phenotypic abnormalities in lines expressing SRDX-ASK1 under the control of the ASK1 promoter***

To avoid potential artefacts due to ectopic and/or overexpression of ASK1, I first sought to express this protein fused to the SRDX domain under the control of its endogenous promoter region (Section 2.5.2.1). The latter was chosen based on previously published data which indicated that the chosen promoter region was sufficient to rescue an *ask1-1 ask2-1* double mutant (Liu et al., 2004). I generated an N-terminal translational fusion of ASK1 to SRDX (noted SRDX-ASK1), as it was shown that fusion of a tag to the C-terminus of ASK1 affected the function of the protein (Wang and Yang, 2006). The resulting fusion was placed under the control of the *ASK1* promoter region (noted *ASK1_{pro}:SRDX-ASK1*) and was introduced into wild-type *L-er* plants (Section 2.2.2.2). Ten independent transformants encoding the *ASK1_{pro}:SRDX-ASK1* transgene were obtained, but none of them showed phenotypic abnormalities (data not shown). To test if the SRDX-ASK1 fusion was indeed expressed in these lines, I carried out RT-qPCR experiments (Figure 4.1A) using 2 sets of primers. One set was designed to specifically amplify the *SRDX-ASK1* cDNA, while the other set was used to detect levels of endogenous *ASK1* only. The 10 lines carrying the *ASK1_{pro}:SRDX-ASK1* transgene were tested. Although, *SRDX-ASK1* was found to be expressed in these transgenic plants, the mRNA levels of the *SRDX-ASK1* fusion were much lower than those of the endogenous *ASK1* gene. Therefore, it is possible that the absence of phenotypic abnormalities in these lines is due to the low expression level of the *SRDX-ASK1* fusion, which can lead to insufficient competition of the SRDX-ASK1 protein with endogenous ASK1.

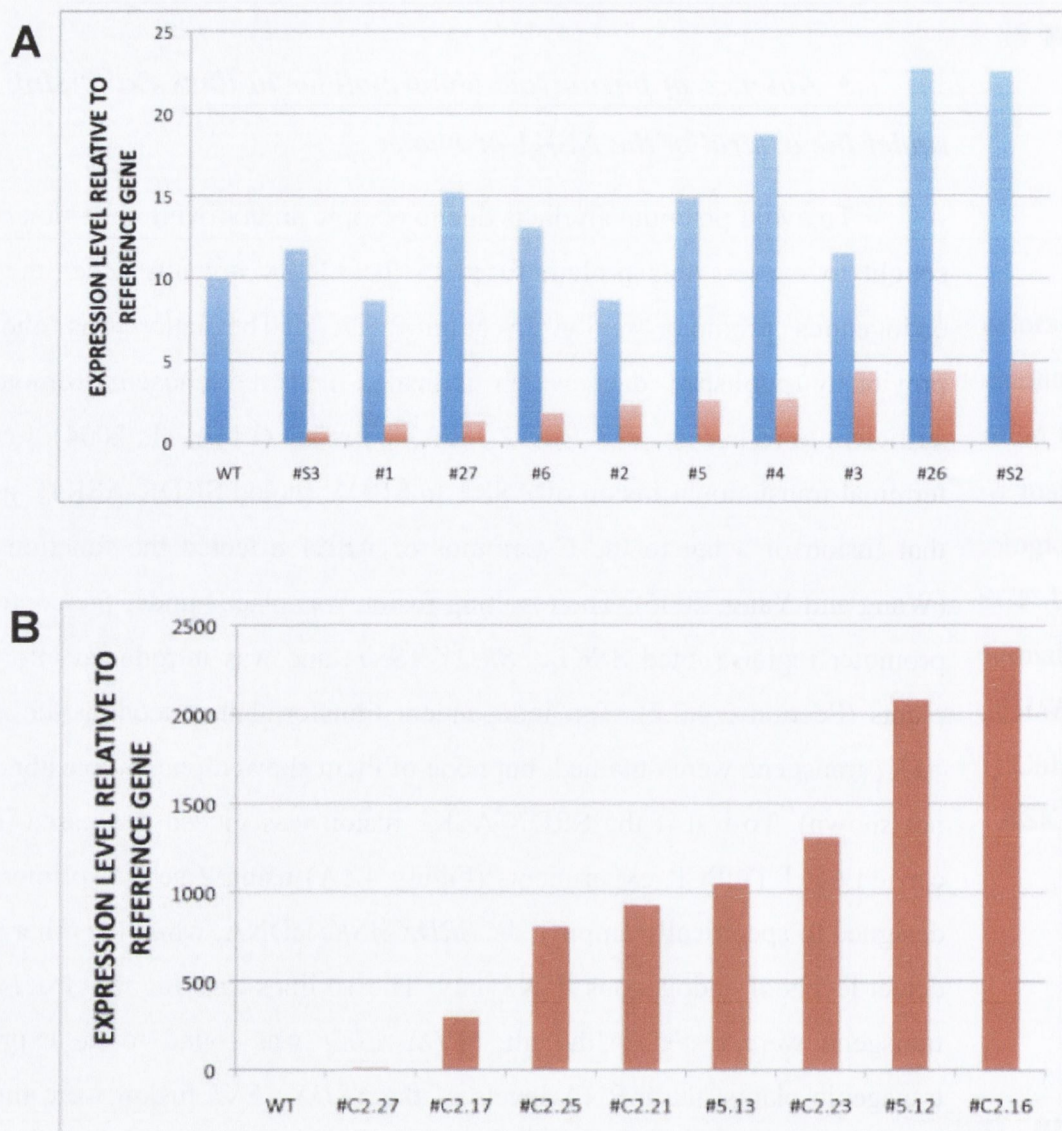


Figure 4.1 Results of RT-qPCR experiments for lines expressing the *SRDX-ASK1* fusion. (A) Results for lines encoding the *ASK1_{pro}:SRDX-ASK1* construct. Lines were tested for the level of endogenous *ASK1* (blue bars) and *SRDX-ASK1* (red bars) mRNAs. **(B)** Results for lines expressing *SRDX-ASK1* from the 35S promoter. Lines were tested for the levels of *SRDX-ASK1* mRNA. The results are presented relative to the reference gene. RNA was extracted from 4-week-old rosette leaves of plants that showed an ‘intermediate’ phenotype (see main text for definition), except for lines C2.27 and C2.17, which showed no phenotypic defects and weak phenotypic defects, respectively (see main text for definition). No error bars are presented because the values were obtained from technical duplicates only.

♦ *Ectopic expression of the *SRDX-ASK1* fusion leads to a wide range of phenotypic abnormalities*

In order to express the *SRDX-ASK1* fusion at higher levels than with the *ASK1* promoter, the *SRDX* repressor domain was translationally fused to the N-terminus of *ASK1* cDNA and the fusion was placed under the control of a strong constitutive

promoter, the Cauliflower Mosaic Virus 35S promoter (Section 2.5.2.2). This construct was introduced into wild-type plants and 17 transformants were obtained.

To determine if the use of the 35S promoter indeed led to higher expression levels than the *ASK1* promoter, I carried out RT-qPCR experiments using rosette leaves from 8 different *35S_{pro}:SRDX-ASK1* lines I obtained (I was not able to carry out this experiment with the remaining 9 transformants because they were either seedling-lethal or sterile so they were not propagated into the T2 generation) (Figure 4.1B). In most lines, the level of expression of the *SRDX-ASK1* fusion was much higher when compared to the levels of endogenous *ASK1* mRNA. Comparing the best *35S_{pro}:SRDX-ASK1* line (line C2.16) to lines carrying the *ASK1_{pro}:SRDX-ASK1* transgene indicated that expression of *SRDX-ASK1* was over 400 times higher when the 35S promoter was used. This result indicates that expression of *SRDX-ASK1* from this promoter is more likely to lead to outcompetition of endogenous ASK1 in SCF complexes and to the inclusion of the SRDX-ASK1 fusion protein, which could result in phenotypic defects.

To determine if expression of SRDX-ASK1 from the 35S promoter indeed led to phenotypic abnormalities, I characterized in detail the offspring of the different transformants I obtained in the T2 and T3 generations. I found that the T2 and T3 populations segregated plants with a range of phenotypes. I assigned the different lines obtained to a specific phenotypic category, based on the phenotype of the majority of the plants in the segregating population I characterized (Table 4.1).

Table 4.1 Assignment of different lines to phenotypic categories. T2 and T3 populations of different primary transformants were grown and characterized phenotypically. Lines were assigned a phenotypic category based on the phenotype of the majority of the plants in the population. Primary transformants that died at the seedling stage or that did not produce seeds were characterized only in the T1 generation.

Line	Phenotypic category
C2.27	No phenotype
C2.17	Weak
C2.25	Intermediate
C2.21	Weak
5.13	Weak to intermediate
C2.23	Intermediate
5.12	Weak to intermediate
C2.16	Intermediate
J2	Weak to intermediate
C2.22	Intermediate*
5.14	Intermediate*
5.15	Strong
5.16	Strong

5.17	Strong
5.18	Strong
5.19	Strong
5.20	Strong

* These lines showed an intermediate phenotype, but were sterile. Hence, only the T1 generation could be characterized.

One line was found to be indistinguishable from the wild type (Figures 4.2A, D, G and J), while other lines, which are categorized as ‘weak’, had plants with mild morphological defects (Figures 4.2B, E, H and K). The latter had smaller leaves and flowers, as well as a shorter stem compared to the wild type. Plants were also fertile (Figure 4.2K).

Lines qualified as ‘intermediate’ were severely affected during both vegetative and reproductive development (Figures 4.2C, F, I and L). Plants from these lines survived past the seedling stage and were characterized by severe abnormalities in both vegetative and reproductive organs (Figures 4.2C, F, I and L). The first phenotypic abnormalities were visible in 4-week old plants (Figure 4.2C), at which stage the morphology and the size of the leaves were affected. In 7-week-old plants (Figure 4.2F), leaves appeared to be smaller and rounder than control wild-type plants. Moreover, based on the number of rosette leaves formed, the transition from the vegetative to the reproductive phase appeared to occur earlier compared to the wild type (data not shown). In 11-week-old plants of this category, it became apparent that the stems did not elongate properly (Figure 4.2I), and flowers showed morphological abnormalities that affected mostly the second and third whorls. In particular, these flowers had a reduced number of stamens, often lacked petals and chimeric petal-stamen organs were also observed (Figure 4.2L). Flowers were often sterile due to a problem with stamen elongation and/or lack of pollen. Interestingly, the floral defects observed in these lines were reminiscent of those found in weak mutant alleles of *ufo* (such as *ufo-6* and *ufo-13*, see Figures 5.5B and C), which is in good agreement with the idea that the F-box protein UFO and ASK1-containing SCF complexes could act as transcriptional co-activators during flower development (Chae et al., 2008; Souer et al., 2008).

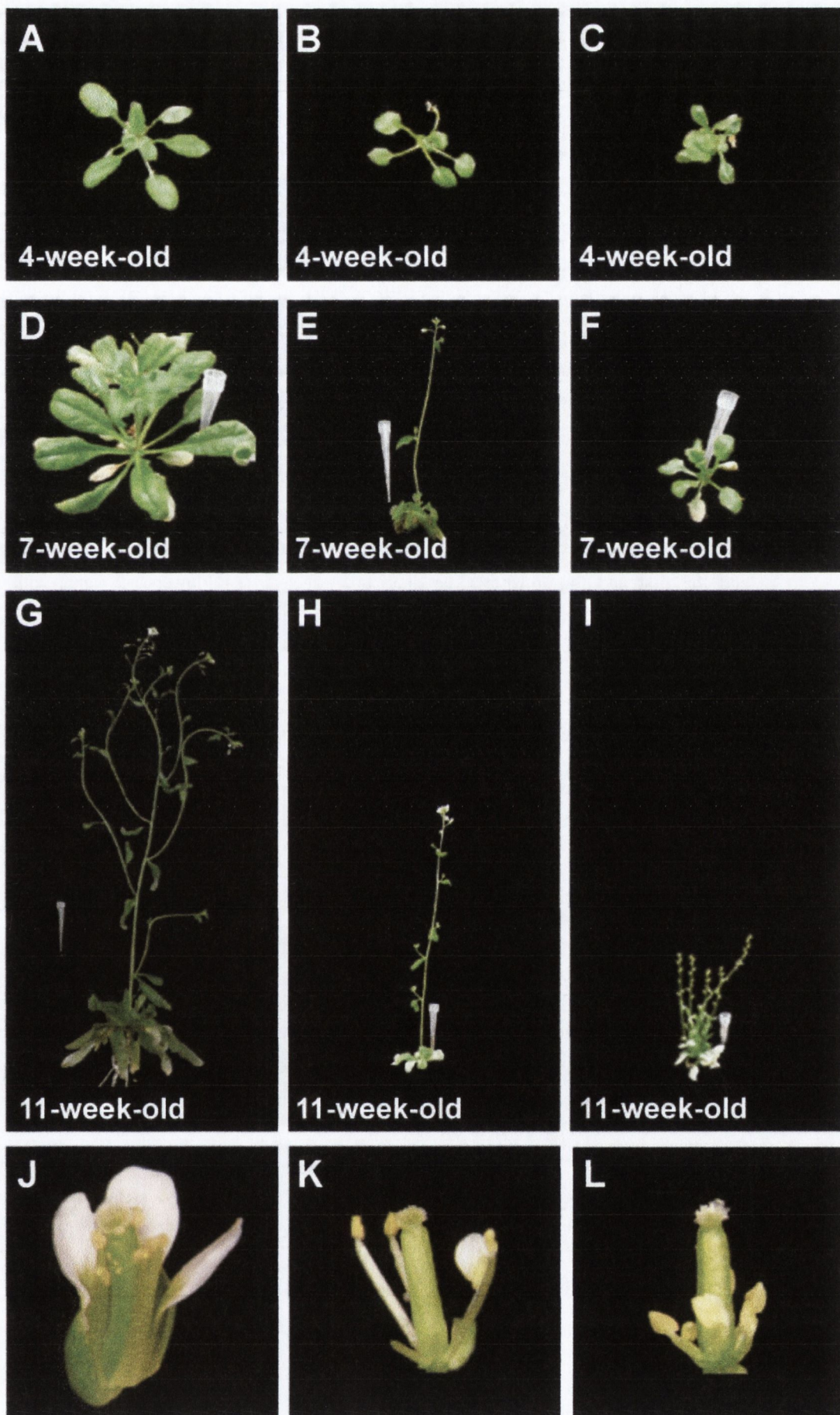


Figure 4.2 Mutant phenotypes observed in plants expressing the SRDX-ASK1 fusion from the 35S promoter. (A-C) Rosettes of 4-week-old plants carrying the *35S_{pro}:SRDX-ASK1* transgene with (A) no phenotypic abnormalities, (B) weak and (C) intermediate phenotypes. (B) Plants with a weak phenotype produced small and round leaves, whose number is reduced compared to that of wild-type plants. (C) The leaves of plants with an intermediate phenotype have short petioles, as well as smaller and more severely deformed leaves. (D-F) Morphological defects of 7-week-old plants with (D) no, (E) weak or (F) intermediate phenotypes. (E) Transition from the vegetative to reproductive phase is accelerated in this line. (F) Plants with an intermediate phenotype are smaller and have shorter stems. (G-I) Characterization of 11-week-old plants expressing the *SRDX-ASK1* fusion showing (G) wild-type, (H) weak or (I) intermediate phenotypes. (H) Plants with a weak phenotype produced a short inflorescence stem. Moreover, axillary branches were initiated but did not developed properly. (I) Plants with an intermediate phenotype were smaller, their inflorescence stems were shorter and produced fewer flowers. A loss of apical dominance was also observed. (J-L) Flower defects observed in plants expressing the *SRDX-ASK1* fusion with (J) no, (K) weak and (L) intermediate phenotypes. The outer organs were removed to reveal inner organs. (K) Flowers of plants with a weak phenotype are smaller than a wild type flower, have a reduced number of stamens and form a chimeric petal-stamen organs. Moreover, no petals were produced and the first and fourth whorls remained not affected. (L) Flowers of plants with an intermediate phenotype are small, lack petals and show stamen defects (*i.e.* elongation and maturation are affected). All plants shown are representative of each category.

Finally, the most severely affected transformants died at the seedling stage in the T1 generation (data not shown) and could not be characterized further. They are termed ‘strong’ lines. This phenotype is similar to that observed in *ask1-1 ask2-1* double mutant plants (Liu et al., 2004) and could suggest that in these lines the function of SCF complexes comprising not only ASK1, but also other ASKs could be affected, possibly because the expression levels of *SRDX-ASK1* could be very high. Unfortunately, because of the early lethality phenotype, I was not able to check the expression levels of *SRDX-ASK1* in these lines and verify this hypothesis. However, seedling lethality in these lines could suggest that some crucial developmental processes require binding of ASK1 and/or ASK1-containing SCF complexes for their regulation.

Although additional biological replicates need to be carried out in order to have a more accurate idea of the expression level of *SRDX-ASK1* in the different lines obtained, I compared the mRNA level of *SRDX-ASK1* (Figure 4.1B) and the strength of the phenotype in different lines (Table 4.1). This comparison suggests that, overall, there is a correlation between the level of *SRDX-ASK1* expression and the severity of the phenotype. For example, the expression level of *SRDX-ASK1* in line C2.27, which was indistinguishable from wild-type plants, was low and in fact comparable to that found in *ASK1_{pro}:SRDX-ASK1* lines (Figure 4.1A). Moreover, line C2.17 which fell in the

‘weak’ phenotypic category showed medium expression level of the SRDX-ASK1 transgene. Finally, the remaining lines showed ‘intermediate’ phenotypic defects and they indeed expressed the SRDX-ASK1 to higher levels. As mentioned above, I was unfortunately not able to check if the most severely affected lines expressed SRDX-ASK1 at higher levels, because they died at early stages of development.

Overall, the morphological defects observed in plants carrying the *35S_{pro}:SRDX-ASK1* transgene were reminiscent of either the *ask1-1 ask2-1* double mutant or plants ectopically expressing an *ASK1_{RNAi}* construct (Zhao *et al.* (2003)), which resulted in the down-regulation of *ASK1*, as well as *ASK2*. In order to check that the phenotypic alterations observed were not due to a reduced expression of endogenous *ASK1* and *ASK2*, I performed RT-qPCRs using mRNA extracted from seedlings and two sets of primers that allowed amplification of either endogenous *ASK1* or *ASK2* mRNAs (Figure 4.3). Although all lines had overall similar phenotypes, I found that lines C2.16, C2.21 and C2.23 exhibited about wild-type levels of the *ASK1* and *ASK2* mRNAs, while lines 5.12, 5.13, C2.25 and C2.29 had reduced expression of *ASK1* and *ASK2*. This result suggests that there is no correlation between the phenotypic alterations observed and the expression levels of *ASK1* and *ASK2*.

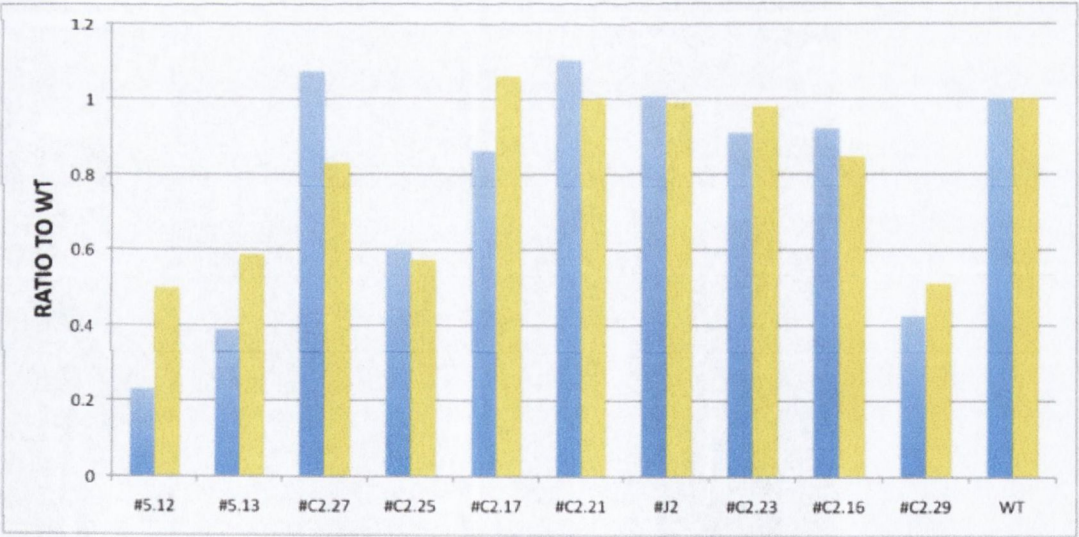


Figure 4.3 Expression levels of endogenous *ASK1* and *ASK2* in selected *35S_{pro}:SRDX-ASK1* lines. Ten out of 17 *35S_{pro}:SRDX-ASK1* lines were tested for the levels of endogenous *ASK1* (blue bars) and *ASK2* (yellow bars) mRNAs. Tissue could not be collected from the remaining 7 lines because they either died at the seedling stage or were too sick. Expression levels are presented relative to the expression level of *ASK1* and *ASK2* in wild-type seedlings, respectively. Total RNA was extracted from 10-day-old seedlings. No error bars are presented because the values represent only technical duplicates only.

♦ *Effects of ectopic expression of ASK1 fused to an inactive mSRDX domain*

To verify that the mutant phenotypes observed are indeed a consequence of transcriptional repression due to the presence of the SRDX domain and are not due to dominant-negative effects caused by the addition of the SRDX peptide, lines expressing ASK1 fused to an inactive version of the SRDX domain (mSRDX) from the 35S promoter were generated (noted *35S_{pro}:mSRDX-ASK1*). Twenty seven transformants carrying the *35S_{pro}:mSRDX-ASK1* transgene were isolated and did not show any phenotypic abnormalities in the T1 generation, similarly to previously published *35S_{pro}:ASK1* lines (Zhao et al., 2003a).

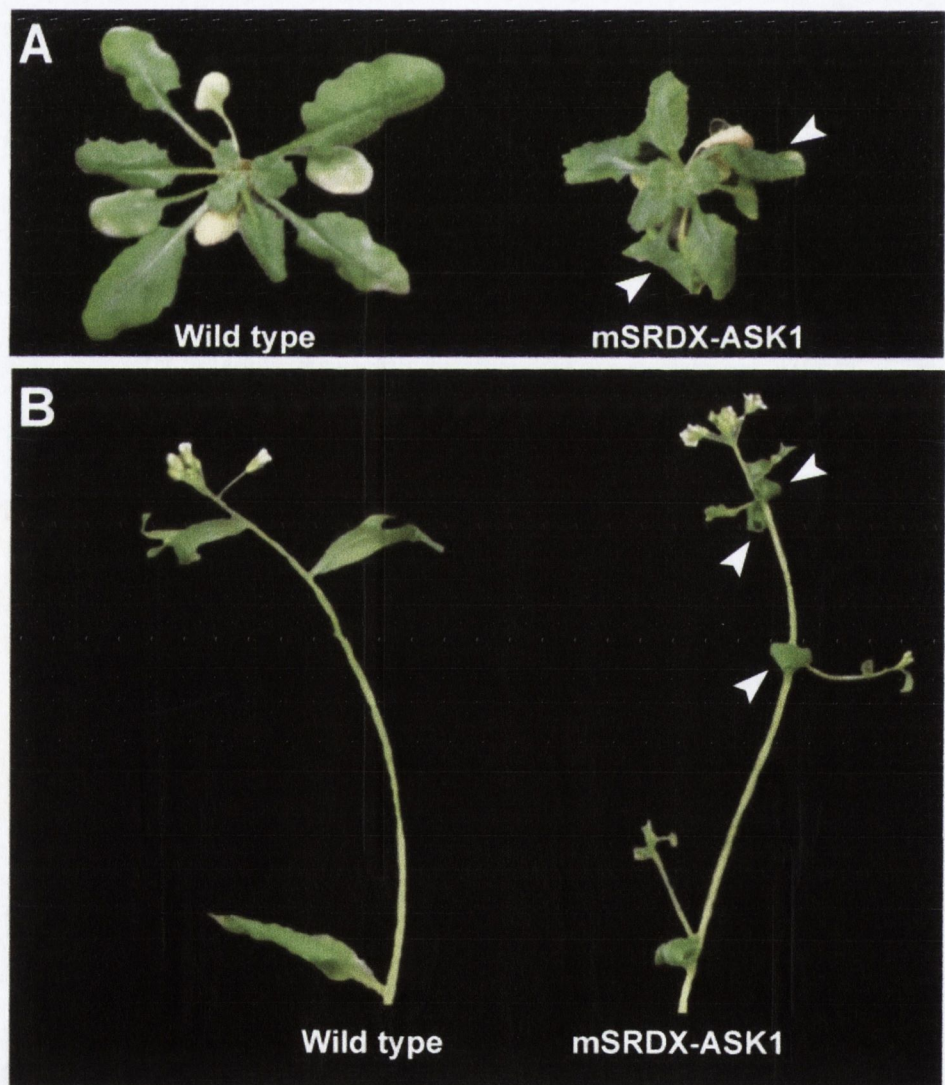


Figure 4.4 Phenotypic alterations observed in line J25 expressing the mSRDX-ASK1 fusion from the 35S promoter. (A) Rosettes of a ~ 3-week-old wild-type plant and of a plant from line J25. The

rosette of the mSRDX-ASK1-expressing plant is smaller and the leaf morphology is also affected (indicated by arrowheads). **(B)** The cauline leaves of plants from line J25 are misshapen (indicated by arrowheads). The pictures are representative of the 2 lines (J3 and J25) carrying the $35S_{pro}:mSRDX-ASK1$ transgene that showed phenotypic defects.

Twelve of these transformants were characterized more in detail. In the T2 generation, 10 of these lines had no phenotypic abnormalities in either vegetative or floral organs. However, the remaining two lines showed minor phenotypic defects resembling those found in the mild $35S_{pro}:SRDX-ASK1$ lines, namely the presence of misshapen rosette and cauline leaves and a slight reduction in leaf size (Figure 4.4).

The results of RT-qPCR experiments indicated that neither *ASK1* nor *ASK2* were significantly downregulated in any of the tested mSRDX-ASK1 lines. Furthermore, in all lines tested, the *mSRDX-ASK1* transcript was detected and, surprisingly, lines J3 and J25, which showed phenotypic alterations, appeared to have the lowest levels of *mSRDX-ASK1* expression (Figure 4.5B).

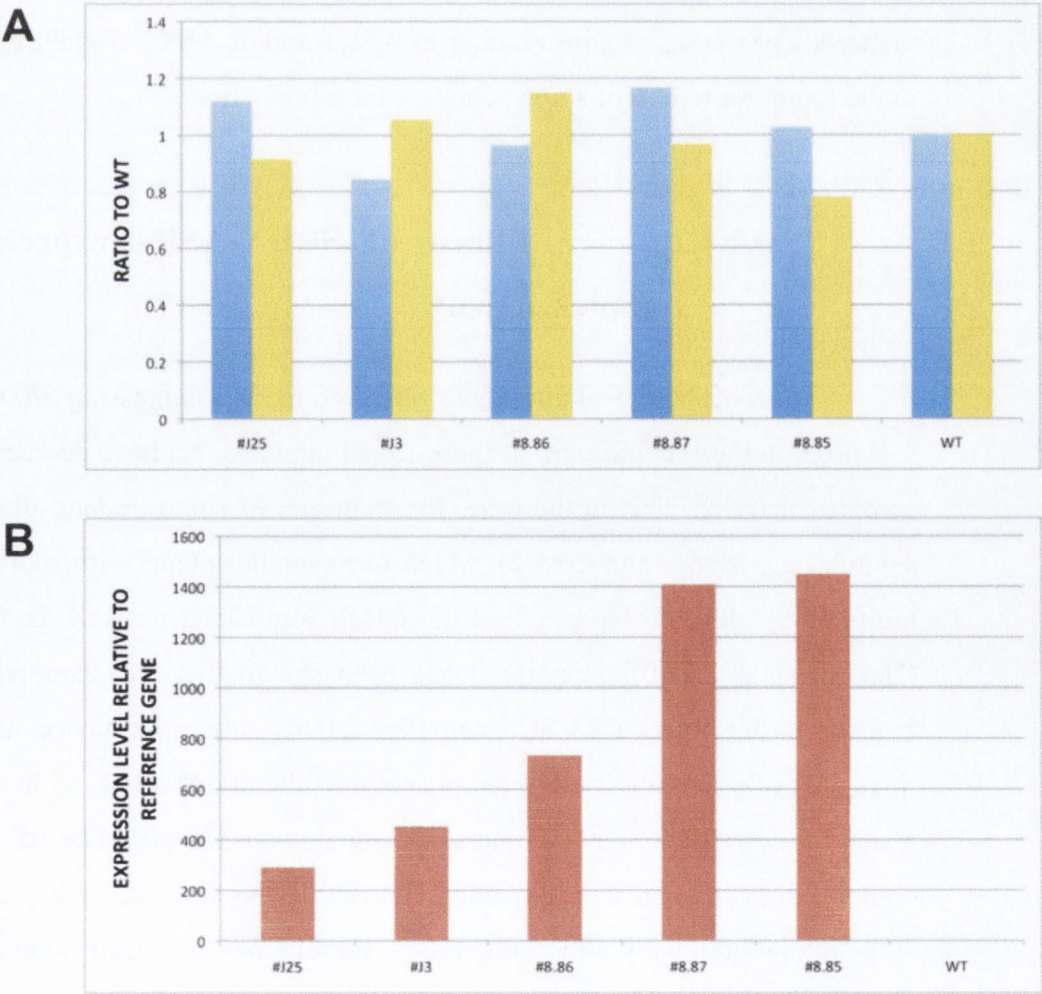


Figure 4.5 Results of RT-qPCR experiments for lines expressing the mSRDX-ASK1 fusion from the 35S promoter. (A) Five 35S_{pro}:mSRDX-ASK1 lines were tested for the levels of endogenous *ASK1* (blue bars) and *ASK2* (yellow bars) mRNAs. Results are presented relative to the levels of *ASK1* and *ASK2* in wild-type plants, respectively. Lines J3 and J25 were in the Col-0 accession, whereas lines 8.85, 8.86 and 8.87 were in the L-er accession. RNA was extracted from 10-day-old seedlings. No error bars are presented for the expression assays because these values represent only technical duplicates. (B) Expression levels of the *mSRDX-ASK1* fusion in 5 independent lines carrying the 35S_{pro}:*mSRDX-ASK1* transgene (red bars). The results are presented relative to the reference gene.

Another possible explanation for the weak phenotype observed in 2 of the lines is that the location of the transgenes in lines J3 and J25 could affect a gene and lead to phenotypic alterations observed. However, neither the segregation ratio of the mutant phenotype, nor the location of the T-DNA in these lines have been determined at this stage. These experiments, as well as the detailed characterization of the remaining 15 transformants are in progress.

Although additional control experiments are required (see below), our preliminary results suggest that the phenotypes observed in the 35S_{pro}:SRDX-ASK1 could indeed be due to the dominant repressor activity of the SRDX domain and that numerous processes require binding of ASK1 and/or ASK1-containing SCF complexes to the promoter region of some genes for their regulation.

4.2.1.2. Effects of SRDX-ASK1 expression on SCF complex activity

The morphological alterations observed in lines expressing *SRDX-ASK1* from the 35S promoter are reminiscent of those found in plants that have decreased levels of SCF complex activity. This is the case, for example, of some mutant alleles of the CUL1 subunit (*e.g. axr6-1* and *axr6-2*), which form smaller plants with short stems, have leaf morphology defects, have a loss of apical dominance as well as fertility problems (Hobbie et al., 2000). Because it has been shown that the phenotype of these *axr6* mutants is due to reduced SCF complex activity, the resemblance with SRDX-ASK1 lines raises the possibility that the phenotypic alterations observed in our plants are the result of decreased SCF complex activity due to the presence of an active SRDX domain. For example, assembly of an HDAC/SRDX-ASK1 complex may prevent ASK1 from interacting with either the F-box protein or the cullin subunit. Alternatively, SRDX-ASK1 may be part of an SCF complex, but its interaction with the HDAC

complex may lead to inactivation of the E3 ligase. Given that SRDX-ASK1 is overexpressed, it is possible that the SRDX-ASK1 protein acts as a competitive inhibitor of other ASK subunits, which would affect SCF complex activity in general.

Showing that expression of the SRDX-ASK1 fusion still results in active SCF complexes would therefore be essential to rule out the possibility that the phenotypes observed are an artefact due to the use of the SRDX domain. To this end, 2 strategies were used. The first one relies on the use of a cell-free degradation assay to monitor the degradation of a known SCF complex substrate (*i.e.* a transcription factor involved in the response to the phytohormone giberellins). The other assay monitors the *in vivo* the degradation of another known substrate of SCF complexes, the Aux/IAA protein IAA17.

♦ *Testing SCF complex activity using a cell-free degradation assay*

The first approach used to test SCF complex activity in plants expressing *SRDX-ASK1* from the 35S promoter is based on a cell-free degradation assay using REPRESSOR OF *gal-3* (RGA) as a substrate. Briefly, RGA is a DELLA protein that undergoes rapid degradation in the presence of the phytohormone giberellic acid (GA3) (Fu et al., 2002; Feng et al., 2008). Moreover, it is also known that an SCF complex that comprises ASK1 is necessary for this process. Wang *et al.* (2009) developed an assay to recapitulate RGA protein degradation *in vitro*. In this assay, RGA fused to the maltose-binding protein (MBP) is expressed in *E. coli* and affinity purified. After addition of the purified MBP-RGA fusion to a protein extract prepared from *Arabidopsis* 10-day-old seedlings, degradation of the purified MBP-RGA protein is monitored using Western blot analysis with an MBP-specific antibody. Using this assay, the MBP-RGA fusion protein was shown to be fully degraded within 60 min (Wang et al., 2009). I applied this approach to test if a line with an intermediate phenotype that expressed SRDX-ASK1 (line 5.13), still retained the SCF complex activity required to degrade MBP-RGA. If incorporation of SRDX-ASK1 allows the assembly of a functional SCF complex, I expect to observe the rapid degradation of the MBP-RGA protein, as previously described (Wang, et. al. 2009). As a control for this approach, I carried out similar cell-free degradation assays with protein extracts obtained from wild-type seedlings, as well as from seedlings expressing the mSRDX-ASK1 fusion (line 8.86).

I first expressed in *E. coli* (Section 2.2.4.2) and purified the MBP-RGA fusion (Section 2.2.4.3). I then determined the sensitivity of the anti-MBP antibody, by loading different amounts of the purified MBP-RGA protein and determining the minimum amount of protein required for detection by the anti-MBP antibody. I found that the anti-

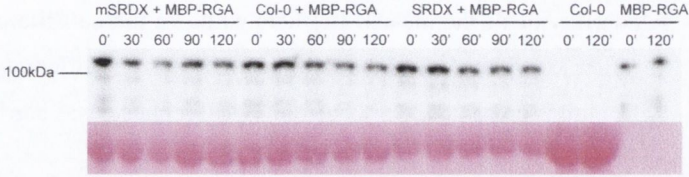
MBP antibody could be used to detect a minimum of 1 ng of the MBP- RGA fusion protein. Hence, in the experiments detailed below I used that amount of MBP-RGA per lane. I then attempted to reproduce the results obtained by Wang *et al.* (2009) and carried out some preliminary degradation assays. To this end, I extracted proteins from 10-day old wild-type seedlings using the same conditions as those described in (Wang *et al.*, 2009). However, I did not observe degradation of the MBP-RGA protein after 30 min, as was reported by Wang *et al.*. Furthermore, even after 120 min the MBP-RGA could still be detected (data not shown). I hence attempted to optimize the assay and tested different conditions.

I carried out a cell-free degradation assay using a degradation buffer supplemented with an ATP regeneration system, as it is known that ATP is important for RGA degradation. However, this change did not improve degradation of MBP-RGA (data not shown) and was therefore not used in subsequent experiments. Another possibility to explain the slow degradation rate of MBP-RGA in our assay is that the level of endogenous Ub could be too low and, consequently, degradation could be stopped or slowed down because free Ub is rate limiting. To overcome this potential problem, I supplemented the assay with 0.1 $\mu\text{g}/\mu\text{l}$ Ub. However, the addition of Ub did not lead to any increase in degradation rate (data not shown), so exogenously added Ub was not used in subsequent experiments. I also tested the effect of using different amounts of the MBP-RGA fusion in the assay, combined with different concentrations of plant protein extracts. I obtained the best results when using a plant protein extract at a final concentration of 2.1 $\mu\text{g}/\mu\text{l}$ together with a final concentration of purified MBP-RGA of 1.1 $\text{ng}/\mu\text{l}$. These conditions were therefore retained in subsequent experiments.

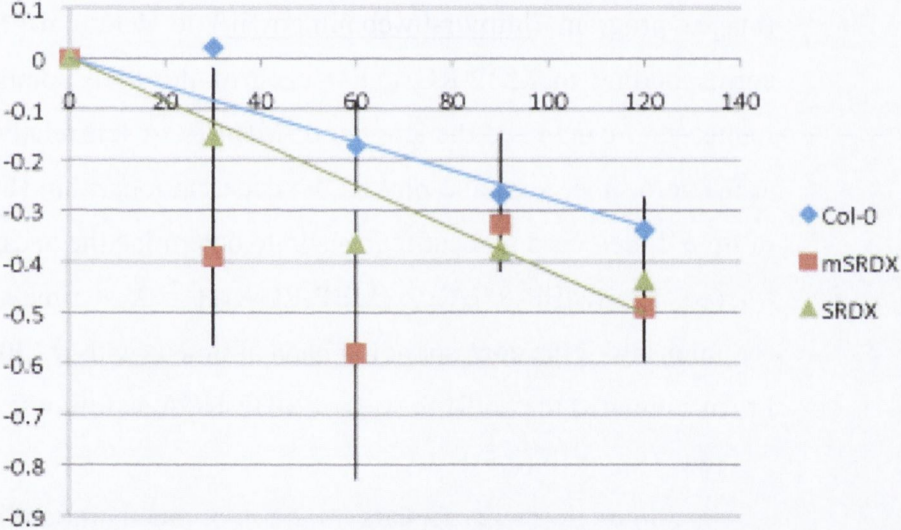
In order to determine if MBP-RGA was degraded at the same rate in protein extracts from the wild type, SRDX-ASK1 or mSRDX-ASK1 seedlings, the different plant protein extracts were mixed with the purified MBP-RGA protein, incubated at room temperature and samples were collected at the following time points: 0, 30, 60, 90 and 120 min. In order to detect cross-reacting bands with the MBP-specific antibody, plant protein extracts were also incubated for 120 min in the absence of MBP-RGA. Moreover, to ensure that the MBP-RGA protein was not degraded in the absence of plant protein extract (*e.g.* because of *E. coli* proteases that might have co-purified with the fusion protein), I examined the stability of the MBP-RGA protein in the degradation buffer alone. All samples were subjected to Western blot analysis using an anti-MBP serum to detect the levels of the MBP-RGA protein (Figure 4.6A) and Ponceau staining was also carried out to ensure that equal amounts of total proteins had been loaded for

each sample. In the case of the wild-type sample alone (*i.e.* to detect cross-reacting proteins), an excess of the plant protein extract was used, as indicated by the more intense bands after Ponceau staining. Although it is difficult to determine if MBP-RGA levels decreased in the control experiment in which the DELLA protein was incubated with degradation buffer alone (Figure 4.6), the decreasing intensity of the band corresponding to MBP-RGA when the fusion was incubated with the different plant protein extract suggests that the MBP-RGA fusion could be slowly degraded in the presence of plant protein extracts.

A



B



C

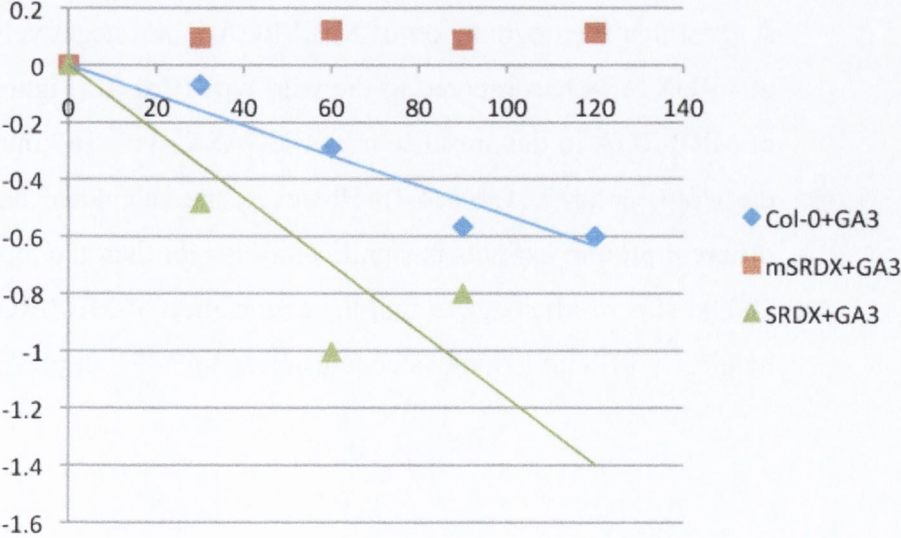


Figure 4.6 Cell-free degradation assays to test the functionality of SCF complexes. (A) Western blot analysis showing the turnover of the MBP-RGA fusion in the presence of protein extracts from 10- day-old seedlings of wild-type (labelled Col-0), SRDX-ASK1 (labelled SRDX) and mSRDX-ASK1 (labelled mSRDX) lines. The MBP-RGA fusion is expected to migrate around 110 kDa, which corresponds to the size of the protein detected using the anti-MBP antibody. Col-0 corresponds to the control used to determine cross-reacting proteins with the anti-MBP antibody (*i.e.* protein extract alone). MBP-RGA corresponds to MBP-RGA incubated with degradation buffer alone (*i.e.* no plant protein extract). (B) Estimation of the half-life of MBP-RGA. Three biological replicates were carried out before quantification using the ImageJ software. The neperian logarithm ($\ln$) of the ratio of MBP-RGA at different time points relative to the amount of MBP-RGA at t_0 is presented. Error bars indicate the standard error of the means. (C) Estimation of the half-life of MBP-RGA in the presence of GA3. The neperian logarithm ($\ln$) of the ratio of MBP-RGA at different time points relative to the amount of MBP-RGA at t_0 is presented. Only one biological replicate was carried out, hence the absence of error bars.

In order to measure more accurately the degradation of the MBP-RGA protein in the cell-free degradation assays, I carried out 3 independent replicates and used the ImageJ program (<http://rsbweb.nih.gov/ij/>) to determine the intensities of the bands corresponding to MBP-RGA. For each of the time points in the different biological replicates, I calculated the amount of MBP-RGA left relative to the level of MBP-RGA at the zero time point and plotted the neperian logarithm ($\ln$) of this value as a function of time. I then used a linear regression to determine the decay constant (λ). Indeed:

$$(\text{eq. 1}) \ln[(\text{MBP-RGA})_i / (\text{MBP-RGA})_{t_0}] = \lambda \times \text{time}, \text{ where } (\text{MBP-RGA})_i \text{ is the intensity of the corresponding band at time } i \text{ (with } i = 30, 60, 90 \text{ or } 120 \text{ min)}$$

I then estimated the half-life ($t_{1/2}$) of MBP-RGA as follows:

$$(\text{eq. 2}) t_{1/2} = [\ln(0.5)] / \lambda$$

Although there appears to be too much variability in the experiment with mSRDX-ASK1 to estimate properly the decay constant λ , the results of this analysis suggest that the degradation of MBP-RGA is not negatively affected by the expression of SRDX-ASK1, compared to the wild type (Col-0) (Figure 4.6B). Indeed, the half-life of MBP-RGA in the presence of SRDX-ASK1 is ~ 165 min, compared to ~ 247 min in the Col-0 control (Table 4.2). However, the calculated half-life of MBP-RGA in the different protein extracts is significantly longer than the reported 3 min in (Wang et al., 2009). This result suggests that the degradation of MBP-RGA is inhibited in my assays, making it difficult to make a conclusion as to SCF complex activity.

Table 4.2 Summary of the different half-lives obtained for MBP-RGA. The different half-lives measured for MBP-RGA are presented in min. The half-lives in the presence of a protein extract expressing mSRDX-ASK1 could not be determined because the results were too variable in the absence of GA3, and the assay did not work properly in the presence of GA3. n.d.: not determined.

Line	No GA3	100 µM GA3	Reference
Wild type	3	<< 3	(Wang et al., 2009)
Wild type	247	130	This work
SRDX-ASK1	165	59	This work
mSRDX-ASK1	n.d.	n.d.	This work

In an attempt to validate my results, I tested if addition of 100 µM GA3 to the degradation buffer would increase the degradation rate of MBP-RGA in my assay. Indeed, as outlined above, degradation of RGA (and MBP-RGA) is known to be accelerated in the presence of GA3 (Fu et al., 2002; Feng et al., 2008). The results obtained were plotted and analyzed as described above (Figure 4.6C). Despite the fact that the assay did not work properly in the presence of the protein extract containing the mSRDX-ASK1 fusion protein, I analyzed the data obtained in the presence of protein extracts from wild-type and SRDX-ASK1-expressing seedlings. The half-life of MBP-RGA in a wild-type protein extract was estimated to be ~ 130 min (Table 4.2), while in the presence of a protein extract with SRDX-ASK1, this half-life was estimated to be ~ 59 min. Hence, as would be expected if the observed decrease in MBP-RGA was due to its proteasome-dependent degradation, adding GA3 to the assay resulted in a more rapid degradation of the DELLA protein. This result suggests that despite the differences found in the half-life of MBP-RGA compared to previously published results (Wang et al., 2009), the cell-free degradation assay is working to some extent. Furthermore, similarly to the results obtained in the absence of GA3, the half-life of MBP-RGA appears to be shorter in the presence of protein extracts containing SRDX-ASK1.

In sum, the results obtained both in the absence and presence of GA3 suggest that degradation of MBP-RGA is faster in protein extracts of plants expressing SRDX-ASK1 compared to protein extracts from wild-type seedlings. This would indicate that the expression of SRDX-ASK1 does not impair SCF complex activity, and instead results in an increase of SCF complex activity, possibly because of the oversupply of ASK1 subunits in the plant, due to ectopic expression of SRDX-ASK1.

In parallel to these cell-free degradation assays, another approach was used to

test SCF complex activity *in vivo* in lines expressing either SRDX-ASK1 or mSRDX-ASK1. Because these experiments worked very well, I did not attempt to further optimize the cell-free degradation assays in order to reach similar half-lives as those published in the literature.

♦ ***Testing SCF complex activity by monitoring IAA17-LUC degradation in vivo***

In order to test if SCF complex activity was impaired in plants expressing SRDX-ASK1, we sought to use a different assay, which is based on the degradation of another known SCF complex substrate, the Aux/IAA protein IAA17. The experiments described below were carried out by Dr. Emmanuelle Graciet, but the data are presented here as it is an important control to validate the use of the SRDX-ASK1 fusion protein for my project.

It has been shown that the degradation of IAA17 is important for auxin response and that functional ASK1-containing SCF complexes are necessary for its degradation (Ulmasov et al., 1997; Tiwari et al., 2001; Tiwari et al., 2004). In particular, the half-life of IAA17 has been determined (Ramos et al., 2001) using *Arabidopsis* lines carrying a transgene that encodes (i) a fusion between IAA17 and the firefly luciferase (LUC) expressed from the UBQ10 promoter (noted *UBQ10_{pro}:IAA17-LUC*) and (ii) a β -glucuronidase (GUS) reporter gene under the control of the 35S promoter (noted *35S_{pro}:GUS*) (Figure 4.7A). The expression of the IAA17-LUC fusion allows to quantify the amount of IAA17-LUC in the plant by measuring LUC activity, while the GUS reporter is used to normalize LUC activities measured in different experiments and/or in a population that is segregating the T-DNA.

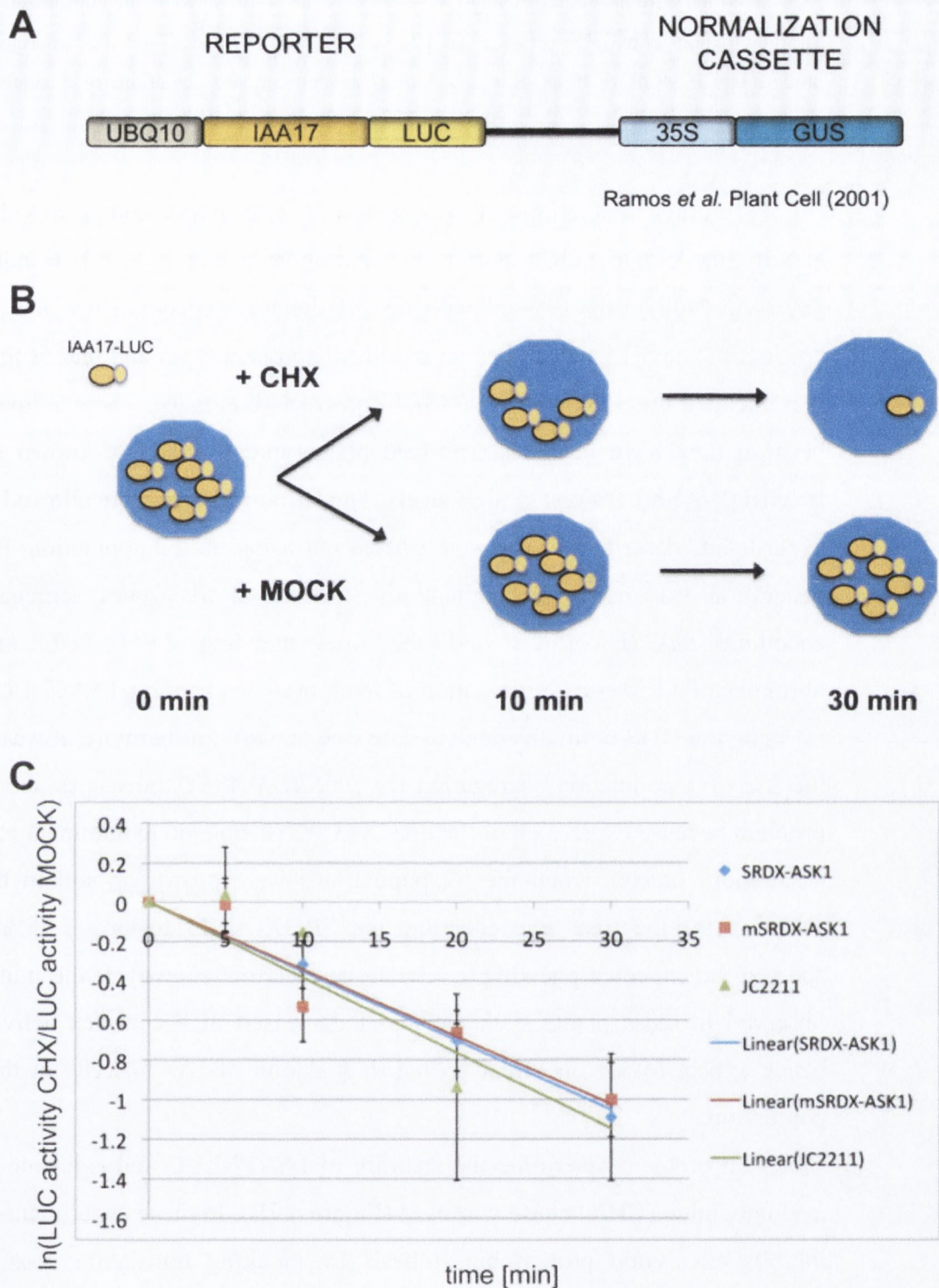


Figure 4.7 Determination of IAA17-LUC half-life in the presence of (m)SRDX-ASK1 fusion proteins. (A) A schematic representation of the T-DNA encoding the *IAA17-LUC* fusion under the control of the *UBQ10* promoter combined with the GUS normalization cassette. The line carrying this transgene was generated by Ramos *et al.* (2001) (B) Principle of a cycloheximide chase. Upon addition of cycloheximide (CHX), protein synthesis is inhibited, hence “fresh” IAA17-LUC proteins are not produced any more. Consequently, the amount of the IAA17-LUC fusion decreases over time due to its Ub-dependent degradation and measurement of LUC activity allows to measure the decrease in IAA17-LUC abundance. In the absence of CHX (*i.e.* MOCK treatment), no changes in IAA17-LUC levels should be observed. (C) Results of cycloheximide chases in the wild type and in lines expressing (m)SRDX-ASK1.

LUC activities were normalized using GUS activities. The ratio between the normalized LUC activities in samples treated with CHX versus a MOCK solution was calculated and the neperian logarithms were plotted as a function of time. The error bars represent standard deviations of 3 independent biological replicates.

In order to determine if expression of SRDX-ASK1 compromised SCF complex activity, the IAA17-LUC reporter line used by Ramos *et al.* (2001) (kindly provided by Dr. Judy Callis) was crossed with one of the lines encoding the $35S_{pro}:SRDX-ASK1$ construct (line 5.13, which has an intermediate phenotype) and one of the control lines that encoded the $35S_{pro}:mSRDX-ASK1$ construct (line 8.86). These 2 lines were chosen because they have been characterized phenotypically and are known to express the (m)SRDX-ASK1 fusions to high levels. The F1 populations were allowed to self and the experiments described below were carried out using the F2 population. Because the F2 generation was used, the populations still had 2 transgenes segregating (the one encoding (m)SRDX-ASK1, and the one encoding IAA17-LUC and the GUS normalization cassette). Segregation of the transgene encoding IAA17-LUC is corrected by using the GUS normalization cassette (see above). Furthermore, it was estimated that the use of a population segregating the (m)SRDX-ASK1 transgenes should not pose a problem because expression of the SRDX-ASK1 fusion led to dominant effects in the F2 population. Indeed, when the F2 populations were grown on soil in the absence of selection for the transgene encoding the SRDX-ASK1 fusion, ~ 75% of the plants showed the expected phenotypic alterations (data not shown). If the mutant phenotype observed in these plants is the result of decreased SCF complex activity, we would hence expect to see an effect on the degradation of IAA17-LUC in this segregating population.

In order to determine the stability of IAA17-LUC and calculate its half-life, a cycloheximide (CHX) chase was used (Figure 4.7B). In these experiments, CHX, which inhibits eukaryotic protein biosynthesis by blocking translation, was added to the medium containing seedlings expressing IAA17-LUC. The degradation of IAA17-LUC was then monitored by measuring LUC and GUS activities at different time points after the addition of CHX or a mock solution. The GUS activities were used to normalize the LUC activities and for each time point the ratio of normalized LUC activity in the presence of CHX compared to the normalized LUC activities in the presence of a mock solution were calculated. In order to determine the decay constant (λ), the neperian logarithm of the ratios obtained above were calculated. The results were plotted as a function of time and a linear regression was used to determine the decay constant (λ)

(Figure 4.7C). Indeed:

(eq. 3) $\ln[(\text{norm. LUC})_{\text{CHX}} / (\text{norm. LUC})_{\text{MOCK}}] = \lambda \times \text{time}$, where ‘norm. LUC’ are the GUS-normalized LUC activities at different time points (5, 10, 20 and 30 min)

I then estimated the half-life ($t_{1/2}$) of IAA17/LUC using the previously described equation 2:

(eq. 2) $t_{1/2} = [\ln(0.5)] / \lambda$

The half-life of IAA17-LUC in the wild type (*i.e.* the parental line that had also been used by Ramos *et al.* (2001)), as well as in plants expressing the (m)SRDX-ASK1 fusions and the IAA17-LUC reporter were estimated to be 18.3 min, 19.7 min and 20.3 min, respectively. Although the half-life of IAA17-LUC obtained in the wild type background is higher than the one calculated by Ramos *et al.*, it falls within the same range, indicating that the cycloheximide chase was working properly. Importantly, the calculated half-lives are comparable among all 3 genotypes, independently of the expression of SRDX-ASK1. This result strongly suggests that expression of SRDX-ASK1, and hence probably its incorporation into SCF complexes, does not affect the overall function of these E3 ligases. This experiment thus suggests that the phenotypic alterations observed in plants expressing SRDX-ASK1 are unlikely to be due to a decrease in SCF complex activity. It also further strengthens the validity of the approach used to determine if transcriptional co-regulation by ASK1-containing SCF complexes is required to regulate developmental processes in *Arabidopsis*.

4.2.1.3. Conclusion

The results described above strongly suggest that the phenotypic abnormalities observed in plants expressing the *SRDX-ASK1* fusion under the control of the 35S promoter are indeed due to the activity of the SRDX domain, when fused to ASK1. They further indicate that ASK1 binds, directly or indirectly, to DNA and that it is likely to control the expression of some genes throughout plant development, possibly as part of an SCF complex. However, in order to determine if the phenotypes observed require the binding of an SCF complex, additional control experiments are being carried out to demonstrate that SRDX-ASK1 is able to interact with the other subunits of an SCF complex.

In order to identify genes whose regulation is affected by expression of SRDX-ASK1, I plan on using gene expression profiling on lines that express the SRDX-ASK1 fusion. This transcriptomics analysis could be carried out using the 35S_{pro}:SRDX-ASK1

lines that were generated, but this would lead to a certain number of problems. For example, the first defects in these lines are observed at very early stages of plant development, so that they are phenotypically very different from wild-type or mSRDX-ASK1-expressing lines already at the seedling stage. Global gene expression analysis on these lines would hence result in the identification of a large number of differentially expressed genes, whose regulation is unlikely to directly depend on ASK1 acting as a transcriptional co-regulator. Instead, the gene expression changes are likely to reflect the strong differences in the morphology of the plants or organs used for the analysis. In order to by-pass this problem, I decided to generate plants that would allow expression of SRDX-ASK1 under the control of an inducible promoter, so that tissue can be collected at different time points after expression of SRDX-ASK1 has been induced. This would not only allow the use of plants that are morphologically similar to the wild type, but would also help enrich the datasets for genes whose regulation depends directly on binding of ASK1 to their promoter element. Furthermore, as mentioned in section 4.2.1.1, lines that express constitutively the SRDX-ASK1 fusion do not yield phenotypically homogenous populations, and that there is no correlation between the level of *SRDX-ASK1* mRNA and the severity of the phenotype (will be tested soon). One possibility to explain these observations is that the constitutive expression of SRDX-ASK1 in the parental plant is detrimental to the next generation. The use of an inducible promoter for expression of SRDX-ASK1 would also help to avoid such problems.

4.2.2. Identification of genes responding to an inducible expression of SRDX-ASK1

4.2.2.1. Generation of plants allowing an inducible expression of SRDX-ASK1

Different inducible systems are available for the expression of SRDX-ASK1 in *Arabidopsis*. Based on the experience of the Wellmer laboratory with different systems, a DEX-inducible promoter appeared to be the most suitable one for the downstream gene profiling experiments. Indeed, other systems, such as the ethanol-inducible one, have been shown to generate significant artefactual gene expression changes (data not shown), while systems depending on the use of a estradiol-inducible promoter are not efficient enough to express SRDX-ASK1 at high levels throughout the plant (data not shown).

♦ **Characterization of lines expressing the (m)SRDX-ASK1 under the control of a DEX-inducible promoter**

In the DEX-inducible system used, the gene of interest is placed downstream of 6 copies of *lac* operator (noted *pOp(6x)*) and a minimal 35S promoter (Craft et al., 2005). To activate transcription of the gene of interest expression of a chimeric transcription factor, noted LhG4, is also required. LhG4 is composed of the high affinity DNA-binding domain of the *lac* repressor and the transcription activation domain II of *GAL4* from *Saccharomyces cerevisiae* (Moore et al., 1998). In order to allow inducible expression, the LhG4 transcription factor is translationally fused to the hormone-binding domain of the rat glucocorticoid receptor (GR) and placed under the control of the 35S promoter (Craft et al., 2005). In the absence of a steroid ligand, LhG4-GR associates with the heat shock protein HSP90. Consequently, the LhG4-GR/HSP90 complex is excluded from the nucleus (Figure 4.8A). Upon DEX treatment, HSP90 dissociates and LhG4-GR enters the nucleus (Figure 4.8B). Finally, LhG4-GR binds to the *lac* promoter and transcription of the gene of interest is activated (Figure 4.8C).

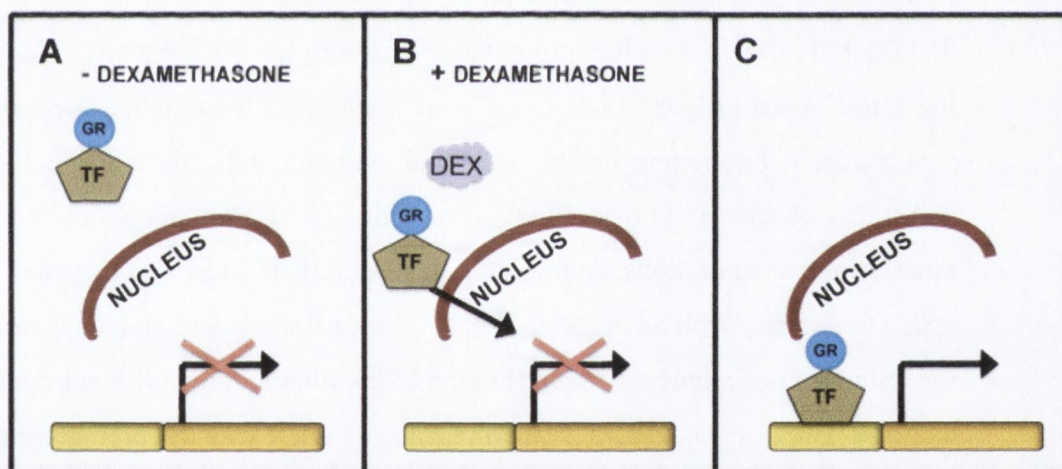


Figure 4.8 The DEX-inducible system used in this study. This system is based on the fusion between a transcription factor (noted TF; LhG4 in our case) and the hormone-binding domain of the rat glucocorticoid receptor (GR). **(A)** In our case, expression of the gene of interest is under the control of the *pOp(6x)* chimeric promoter that consists of 6 *lac* operators cloned upstream of a truncated version of the 35S promoter. The LhG4-GR fusion is maintained in the cytoplasm through its interaction with the heat shock protein HSP90 (Picard, 1993), thus preventing activation of its target genes. **(B)** Upon addition of a steroid ligand, such as DEX, GR binds DEX which triggers dissociation of HSP90 from LhG4-GR. Next, the released LhG4-GR enters the nucleus. **(C)** The transcription factor LhG4-GR binds to the *pOp(6x)* promoter and activates the transcription of the gene of interest.

The *35S_{pro}:LhG4-GR/pOp(6x)_{pro}:SRDX-ASK1* construct was generated and

introduced into wild-type plants by Dr. Emmanuelle Graciet, who also characterized the first 2 lines obtained (noted 523-1 and 523-2), one of which was used for a preliminary microarray analysis. In parallel, I selected for additional lines and have isolated 23 additional independent lines, but I did not have sufficient time to characterize them at this stage.

In order to check if expression of *SRDX-ASK1* in the presence of DEX could lead to phenotypic alterations in lines 523-1 and 523-2, plants were grown on 0.5xMS agar plates containing either 10 μ M DEX or a mock solution (ethanol). Line 523-2 showed no phenotypic alterations in the presence of DEX (data not shown). In contrast, the development of plants from line 523-1 was severely affected (Figure 4.9) in the presence of DEX, but not on plates with a mock solution. The phenotypic alterations observed were reminiscent of those found in intermediate lines carrying the *35_{pro}:SRDX-ASK1* transgene (Figures 4.2K-L). More specifically, in the presence of DEX, the rosette of the plants was small, the transition from the vegetative to the reproductive stage occurred earlier, stems did not elongate properly and flowers had an abnormal morphology (Figures 4.9A and B). In addition, the floral defects appeared to be stronger in the DEX-inducible line compared to those observed in intermediate lines expressing *SRDX-ASK1* from the 35S promoter. For example, similarly to *35S_{pro}:SRDX-ASK1* lines, in flowers of line 523-1 with a weak phenotype, the number of petals and stamens was reduced, misshapen petals, abnormal stamens and chimeric petal-stamen organs were also observed (Figure 4.9D). In addition, line 523-1 had flowers with stronger morphological alterations that were not observed in lines carrying the *35S_{pro}:SRDX-ASK1* transgene, such as deformed sepals, lack of petals and stamens, aerial ovules and the outgrowth of stigmatic tissue (Figure 4.9E). Finally, an additional mutant phenotype that was found in line 523-1 in the presence of DEX was the premature termination of the floral meristem into a structure that resembled a flower with an overproliferation of female reproductive tissue (Figure 4.9F).

The expression level of *SRDX-ASK1* in these 2 lines was then determined using RT-qPCR. To this aim, 10-day-old seedlings from lines 523-1 and 523-2 grown in the presence and absence of DEX were collected, followed by RNA extraction. I carried out RT-qPCRs using the set of oligos that was designed to amplify the *SRDX-ASK1* cDNA and found that line 523-1 expressed the *SRDX-ASK1* fusion to high levels in the presence of DEX, whereas *SRDX-ASK1* mRNA was not detectable in line 523-2, which explains the lack of phenotype in this line, even in the presence of DEX (data not shown).

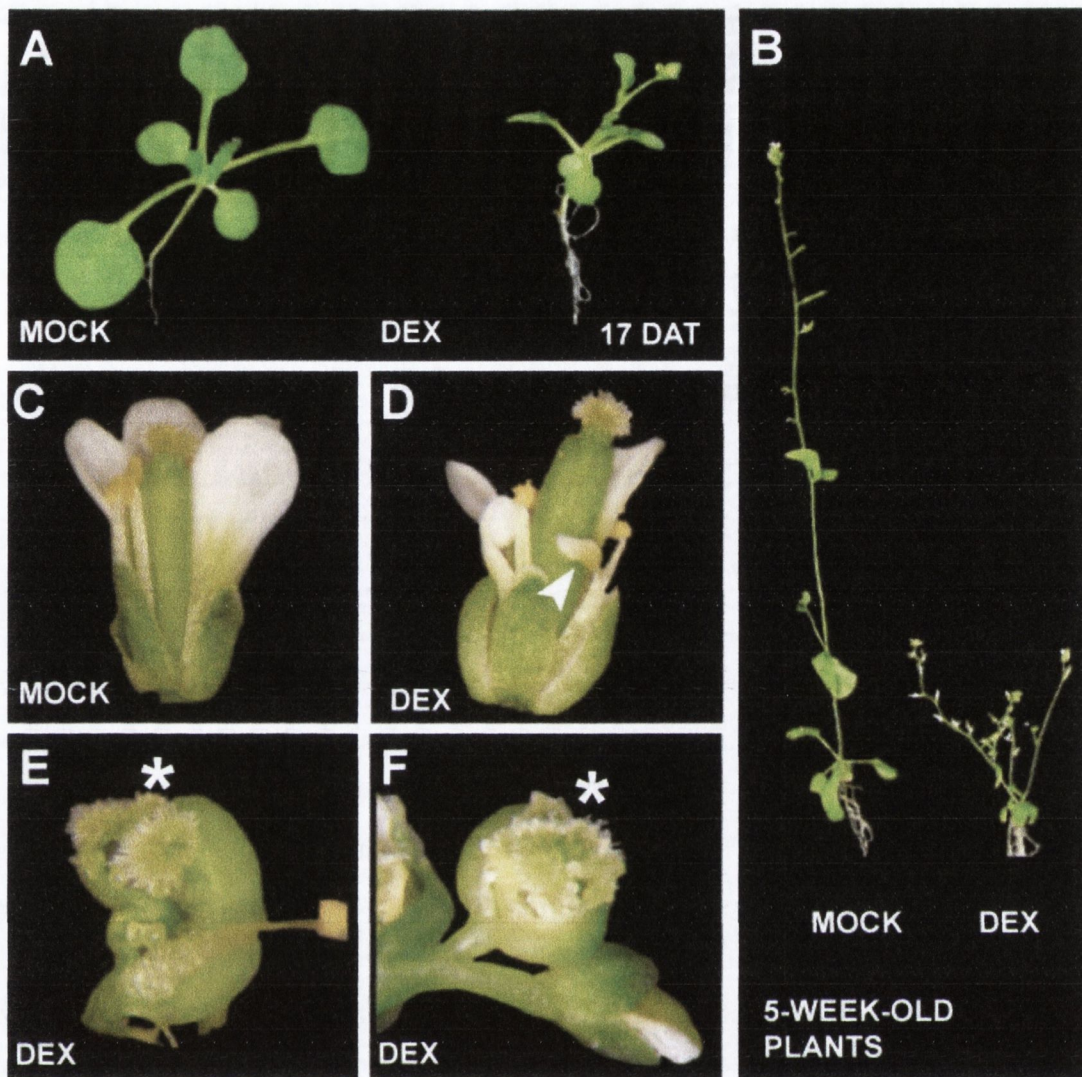


Figure 4.9 Phenotypes observed in line 523-1 carrying the *35S_{pro}::LhG4-GR/pOp(6x)_{pro}::SRDX-ASK1* transgene. **(A)** 17-day-old plants grown in the presence of a mock solution (left) or 10 μ M DEX (right). The DEX-treated plant showed phenotypic defects: the size of the rosette leaves is reduced and the transition from the vegetative to the reproductive stage appears to be accelerated compared to the mock-treated plant. **(B)** Mock- and DEX-treated 5-week-old plants. The mock-treated plant (left) did not exhibit the phenotypic abnormalities that were found in plants grown in the presence of DEX (right) such as short stems, sterility and flowers showing a wide range of morphological abnormalities. **(C)** Flower from a mock-treated plant. One sepal and one petal were removed to reveal inner organs. **(D)** Flower from a plant grown in the presence of DEX with a weak phenotype, which includes a reduction in the number of petals and stamens, misshapen petals, short stamens and chimeric petal-stamen organs (indicated by arrowhead). **(E)** Flower with a strong phenotype from a plant grown on DEX-containing plates. All 4 floral whorls are affected: no sepals and petals were observed and flowers only formed one abnormal stamen, as well as ectopic ovules and stigmatic tissue (indicated by an asterisk). **(F)** Premature termination of the floral meristem in plants grown in the presence of DEX. No wild-type floral organs are produced, except for ectopic stigmatic tissue (indicated by asterisk). For all panels, seeds were germinated on 0.5xMS agar plates supplemented with BASTA and either ethanol (mock) or 10 μ M DEX. Seedlings were then transferred to cups containing 0.5xMS medium supplemented with either ethanol (mock) or 10 μ M DEX. DAT: days after transfer from the cold room to long-day growth conditions.

In summary, the large overlap between the phenotypes observed in lines expressing *SRDX-ASK1* from the 35S and in line 523-1 indicates that the DEX-inducible system is likely suitable to examine gene expression changes related to the induction of *SRDX-ASK1* expression in order to identify genes that respond to changes in ASK1 functions as a transcriptional co-regulator.

♦ *Kinetics of induction of SRDX-ASK1 expression*

In order to determine which time-points could be used for the microarray analysis, following treatment with DEX, I monitored the expression of the *SRDX-ASK1* fusion in 2 homozygous lines for the *35S_{pro}:LhG4-GR/pOp(6x)_{pro}:SRDX-ASK1* transgene that were derived from line 523-1 (these homozygous lines are noted 523-1-2 and 523-1-8). For this experiment, seedlings were grown on 0.5xMS agar plates in the absence of DEX for 10 d and were then transferred to either plates containing ethanol (mock treatment) or 10 μ M DEX. Seedlings were collected after 30 min, 1 hr, 2 hr, 4.25 hr and 6 hr. After RNA extraction, I carried out RT-qPCRs (Figure 4.10) using the set of oligos that was designed to amplify the *ASK1-SRDX* mRNA. The response to DEX was similar in both homozygous lines. More specifically, expression of the *SRDX-ASK1* fusion could be detected starting 2 hr after the beginning of the DEX treatment, at which stage *SRDX-ASK1* expression level reached $\sim 25\%$ of the maximum expression level (as determined by measuring the level of *SRDX-ASK1* expression in seedlings that had been grown continuously on plates containing DEX). The maximum expression level of the *SRDX-ASK1* fusion was reached between the 4-hr and 6-hr time points.

Based on these results, I decided to carry out a preliminary microarray analysis at the 2-hr and 5-hr time points after transferring the seedlings to a DEX-containing plate. Indeed, because the expression level of *SRDX-ASK1* is quite low before 2 hr, it is unlikely that significant expression changes can be detected. The use of the 2-hr time point could however enrich the dataset in direct target genes of ASK1, as we limit the possibilities for secondary events. The 5-hr time point was also chosen because it would allow us to monitor the progression of gene expression changes from the beginning of a significant expression of *SRDX-ASK1* (at 2 hr) until expression reaches its maximum (around 5 hr).

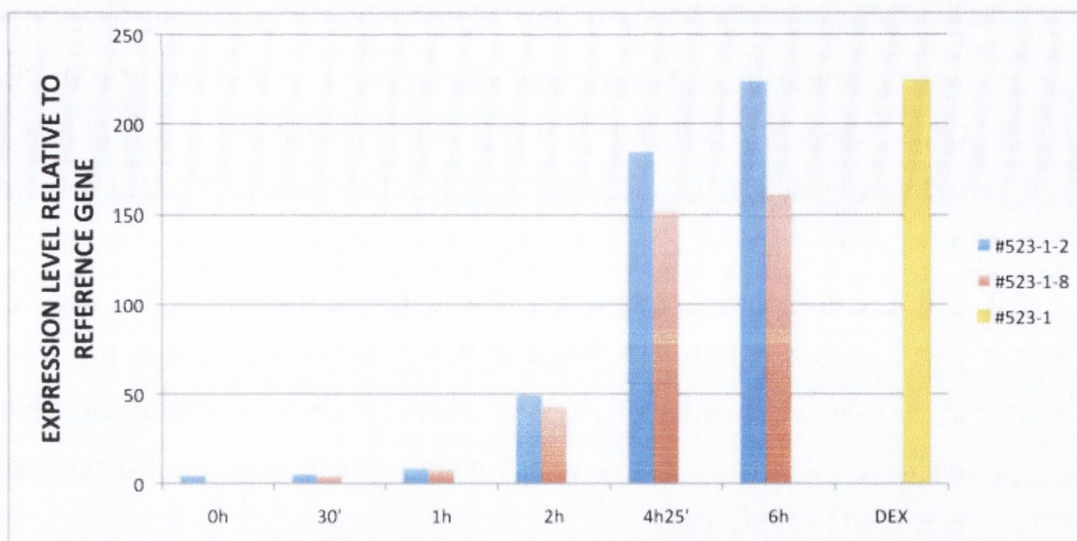


Figure 4.10 Kinetics of SRDX-ASK1 induction following DEX treatment. Lines were tested for the level of *SRDX-ASK1* expression at different time points after the beginning of a DEX treatment. Results are presented for a segregating population of line 523-1 grown continuously on plates containing DEX (yellow bars), as well as for 2 homozygous lines (523-1-2 and 523-1-8, represented by blue and red bars, respectively) isolated from 523-1 in the T3 generation. The results are presented as fold changes relative to the reference gene. No error bars are presented because these values represent technical duplicates. In case of lines 523-1-2 and 523-1-8, seeds were germinated on 0.5xMS agar plates supplemented with BASTA. After 10 d, seedlings were transferred onto 0.5xMS agar plates supplemented with 10 μ M DEX, incubated for 30 min, 1 hr, 2 hr, 4.25 hr and 6 hr followed by RNA extraction. The seeds of the 523-1 line were grown on 0.5xMS agar plates supplemented with BASTA and 10 μ M DEX. RNA was extracted from 10-day-old seedlings. DEX – plants grown continuously in the presence of DEX.

4.2.2.2. Identification of genes regulated by SRDX-ASK1

In order to identify biological pathways that are affected by the expression of the SRDX-ASK1 fusion, a preliminary microarray experiment was carried out, in which the transcript profiles of mock and DEX-treated seedlings of a *35S_{pro}:LhG4-GR/pOp(6x)_{pro}:SRDX-ASK1* line (line 523-1; see description above) were compared at different time points after inducing the expression of *SRDX-ASK1* on a DEX-containing growth medium. For this experiment, seeds were germinated on 0.5xMS agar plates. After 10 d, the seedlings were transferred onto 0.5xMS agar plates containing either 10 μ M DEX or ethanol (mock treatment) followed by incubation at room temperature for 2 hr and 5 hr, after which seedlings were collected and frozen in liquid nitrogen for RNA extraction. Seedlings of the wild type were treated in the same way for 5 hr in order to test the effects of a DEX treatment on gene expression. Moreover, to test the effect of

the continuous expression of *SRDX-ASK1* on gene expression, seeds from line 523-1 were germinated on 0.5xMS agar supplemented with either 10 μ M DEX or ethanol (mock sample). After 10 d, seedlings were collected and frozen in liquid nitrogen for RNA extraction. RNA was then amplified, labelled with the Cy3 and Cy5 fluorescent dyes (Table 4.3) and then hybridized to custom-designed *Arabidopsis* microarrays (Agilent Technology) as per manufacturer’s instructions by Kamila Kwaśniewska, a post-graduate student in Dr. Frank Wellmer’s laboratory. Because this was a preliminary experiment to determine if the time points chosen were appropriate, only 2 biological replicates with dye swaps were carried out. A summary of the different hybridizations is presented in Table 4.3.

Table 4.3 Summary of the hybridizations carried out for the gene expression profiling of inducible SRDX-ASK1 lines. Lines, treatments and labeling are indicated. Red: samples labeled with Cy5; green: samples labeled with Cy3.

Experiment	Replicate 1	Replicate 2
2 hr	EG523-1 – DEX vs. EG523-1 – Mock	EG523-1 – Mock vs. EG523-1 – DEX
5 hr	EG523-1 – DEX vs. EG523-1 – Mock	EG523-1 – Mock vs. EG523-1 – DEX
continuous	EG523-1 – DEX vs. EG523-1 – Mock	EG523-1 – Mock vs. EG523-1 – DEX
Wild type control (5 hr)	L-er – DEX vs. L-er – Mock	L-er – Mock vs. L-er – DEX

The datasets obtained were then processed by Patrick Ryan, a post-graduate student in Dr. Frank Wellmer’s group. Genes that had a fold-change of > 1.5 and an adjusted *P*-value < 0.05 were considered as differentially expressed (Table 4.4). Based on these criteria, I found that there were no differentially expressed genes when wild-type seedlings were treated with DEX for 5 hr, compared to a mock treatment, thus indicating that DEX treatment alone does not greatly alter gene expression and that the changes observed in line 523-1 are likely the result of the presence of the transgene in these plants. Analysis of the datasets obtained with line 523-1 revealed that there were 72, 275 and 515 differentially expressed genes in seedlings treated with DEX for 2 hr, 5 hr or continuously, respectively. The fact that the number of differentially expressed genes increases with the length of the DEX treatment was expected, as we had hypothesized that longer treatments would likely result in gene expression changes related to both the direct and indirect effects of the SRDX-ASK1 fusion (*i.e.* the mis-

regulation of genes that are directly regulated by ASK1 could lead to additional changes in the transcriptome, independently of the binding of ASK1 to the promoter of these genes).

Table 4.4 Number of differentially expressed genes. ‘2 hr’ and ‘5 hr’ indicate the microarray datasets obtained for SRDX-ASK1 seedlings treated with DEX for 2 hr or 5 hr, respectively. ‘DEX’ indicates the microarray dataset for SRDX-ASK1 seedlings grown continuously on plates supplemented with DEX. ‘L-*er*’ indicates wild type L-*er* seedlings grown for 5 hrs on DEX-containing medium.

	Up	Down	Total
2 hr	69	3	72
5 hr	242	33	275
DEX	265	250	515
L-<i>er</i>	0	0	0

Because in this experiment, ASK1 was fused to a dominant repressor domain, we expected to observe predominantly down-regulation of the differentially expressed genes, especially for the short incubation times with DEX, as these datasets are expected to be enriched in direct target genes of ASK1. However, the result we obtained was the opposite: after both 2 hr and 5 hr of DEX treatment, the majority of the mis-expressed genes were up-regulated (96% and 88% of the total number of differentially expressed genes, respectively). When seedlings were grown continuously on a DEX-containing medium, ~ 50% of the genes were up-regulated. However, upon continuous DEX treatment, this large proportion of up-regulated genes could be the result of indirect effects of the expression of SRDX-ASK1.

A possible explanation for the large proportion of up-regulated genes at the 2 and 5-hr time points, is that overexpression of SRDX-ASK1 could cause up-regulation of some of the genes identified in this dataset. Another possibility is that the use of the DEX-inducible system leads to artefactual gene activation, probably due to the transcriptional activity of the LhG4-GR transcription factor in the presence of DEX. In order to rule out these possibilities, I have been isolating lines that express a *mSRDX-ASK1* fusion under the control of the pOp promoter (this line (514-2) is noted *35S_{pro}:LhG4-GR/pOp(6x)_{pro}:mSRDX-ASK1*). However, despite screening a large number of seeds, I was only able to obtain one transformant, and because of time limitations, I have not been able to finish the characterization of this line in order to use it for preliminary microarray experiments.

In order to assess quickly whether the large proportion of up-regulated genes could be due to the expression of the LhG4-GR transcription factor, I cross-compared the microarray datasets generated using our DEX-inducible *35S_{pro}:LhG4-GR/pOp(6x)_{pro}:SRDX-ASK1* line with another dataset that had been obtained using floral tissue of a line that encoded an artificial microRNA (amiRNA) designed to target the mRNA of the gene *AGAMOUS* under the control of the pOp promoter. This line also encoded the LhG4-GR transcription factor under the control of the 35S promoter for induction of the pOp promoter upon DEX treatment (this line is noted *35S_{pro}:LhG4-GR/pOp(6x)_{pro}:AG-amiRNA*) (O'Maoileidigh et al., 2013). In this experiment, inflorescences were treated with 10 μ M DEX and floral tissue was collected 24 hr later. In this experimental setting 926 genes were found to be differentially expressed. Despite the fact that very different types of tissue were used and that the genes under the control of the pOp promoter were unrelated, I found that $\sim 38\%$, $\sim 24\%$ and $\sim 14\%$ of the genes that were differentially expressed at 2 hr, 5 hr, or upon continuous DEX treatment, respectively, in *35S_{pro}:LhG4-GR/pOp(6x)_{pro}:SRDX-ASK1* seedlings were also present in the dataset obtained using floral tissue of the *35S_{pro}:LhG4-GR/pOp(6x)_{pro}:AG-amiRNA* line. Such a large overlap is unlikely to happen by chance. Because the only common factor between the 2 lines used in these microarray experiments is the expression of the LhG4-GR transcription factor, our results suggest that expression of LhG4-GR and its transcriptional activity leads to important artefactual gene expression changes.

This result has never been reported in the literature and I am currently generating new reagents to further test this possibility and generate datasets that can be used to correct those that have been obtained with our *35S_{pro}:LhG4-GR/pOp(6x)_{pro}:SRDX-ASK1* line. These reagents include a line that expresses only LhG4-GR under the control of the 35S promoter, as well as a line that expresses ASK1 (without the SRDX domain) under the control of the pOp promoter. The latter will allow us to rule out the possibility that some of the mis-expressed genes are simply due to overexpression of ASK1 itself, independently of the activity of the SRDX domain. As mentioned above, I am also selecting lines that express mSRDX-ASK1 under the control of the same DEX-inducible system, which will also allow us to detect artefactual gene expression changes in our experiments.

Because I did not have the reagents required to correct the microarray datasets obtained in these experiments, I used the dataset obtained with the *35S_{pro}:LhG4-GR/pOp(6x)_{pro}:AG-amiRNA* line and removed from the list of differentially expressed genes those that were common to the 2 experiments (Table 4.5).

Table 4.5 Number of differentially expressed genes after correction. ‘2 hr’ and ‘5 hr’ indicate the microarray datasets obtained for SRDX-ASK1 seedlings treated with DEX for 2 hr or 5 hr, respectively. ‘DEX’ indicates the microarray dataset for SRDX-ASK1 seedlings grown continuously on plates supplemented with DEX.

	Up	Down	Total
2 hr	43	2	45
5 hr	183	27	210
DEX	218	223	441

Even after correction, most of differentially expressed genes were still up-regulated in the datasets obtained 2 and 5 hr after the beginning of the DEX treatment. Despite this, I attempted to validate the data obtained using RT-qPCR on different lines.

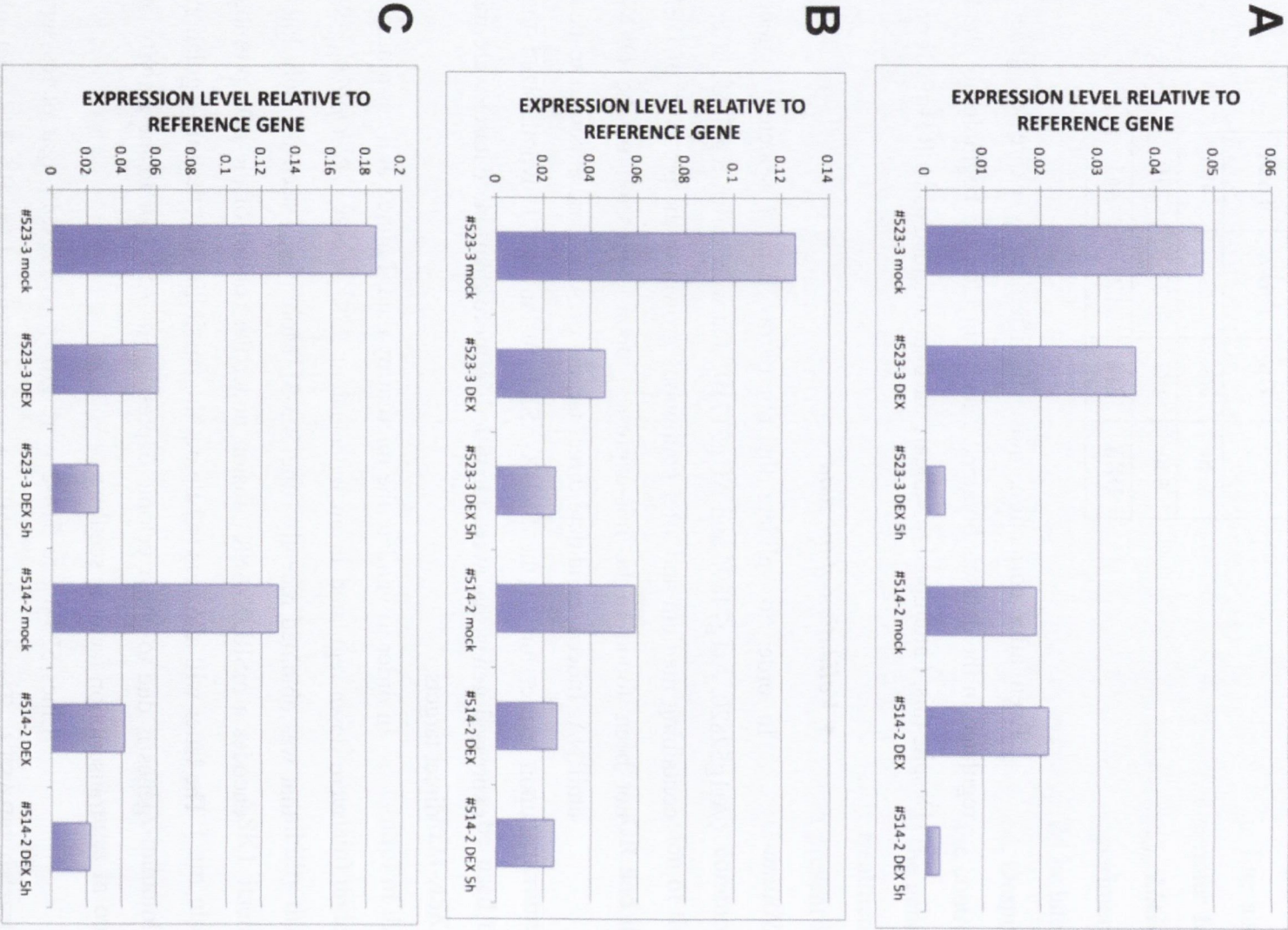
♦ *Validation of the data*

In order to validate the microarray data, I selected 4 genes (At4g39795, At1g52820, At1g74890 and At1g31710) that were down-regulated after 5 hr on a DEX-containing medium and after continuous growth in the presence of DEX, and that had not been found to be mis-expressed upon expression of the DEX-inducible AG-amRNA. Indeed, candidate direct targets of ASK1 are expected to be down-regulated upon expression of the SRDX-ASK1 fusion and I hypothesized that differentially expressed genes that were identified in more than one dataset were more likely to be direct targets.

In order to validate the microarray data, I aimed at determining if these 4 genes were down-regulated in an independent *35S_{pro}:LhG4-GR/pOp(6x)_{pro}:SRDX-ASK1* line that was obtained recently (line 523-3). Moreover, I used the only line I obtained that encodes a mSRDX-ASK1 fusion under the control of the pOp promoter (line 514-2). The latter will allow us to rule out the possibility that the mis-regulation of the selected genes is due to either ectopic expression of ASK1 or to the activity of the LhG4-GR transcription factor in seedlings.

Lines 523-3 and 514-2 were grown on 0.5xMS agar plates supplemented with BASTA. Ten-day-old seedlings were transferred on 0.5xMS agar plates containing either 10 µM DEX or a mock solution (ethanol), and incubated for 5 hr at room temperature. Moreover, seeds of the same line were geminated and grown on plates containing either 10 µM DEX or a mock solution. Finally, I carried out RT-qPCR using

cDNA samples from all lines and oligonucleotides designed to amplify cDNA from the 4 control genes mentioned above (Figure 4.11).



D

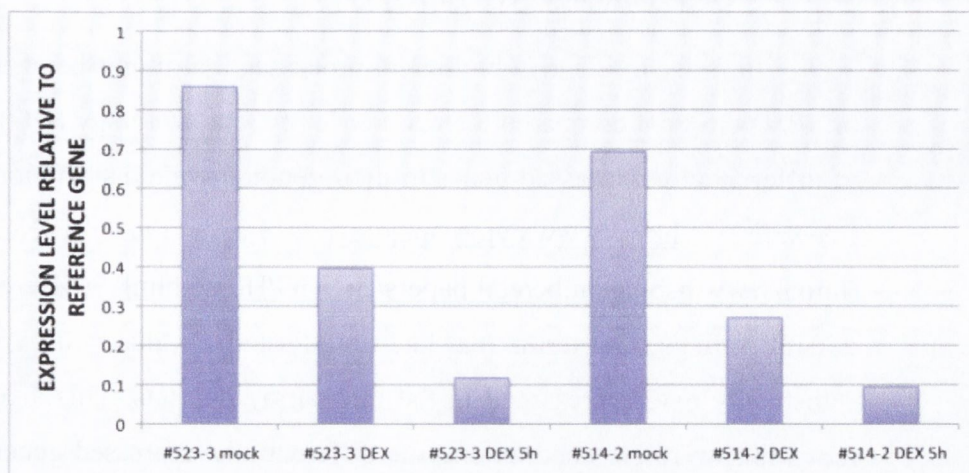


Figure 4.11 Results of RT-qPCR experiments on selected genes to validate the microarray data. Lines 523-3 and 514-2 that carry transgenes encoding $35S_{pro}:LhG4-GR/pOp(6x)_{pro}:SRDX-ASK1$ and $35S_{pro}:LhG4-GR/pOp(6x)_{pro}:mSRDX-ASK1$, respectively, were tested for the expression of 4 genes that were identified as down-regulated in line 523-1 in our preliminary microarray experiments. (A) *Atlg74890*, (B) *Atlg52820*, (C) *At4g39795* and (D) *Atlg31710*. Experiments labeled ‘mock’ and ‘DEX’ correspond to seedlings that were grown continuously on 0.5xMS plates containing either a mock solution (ethanol) or 10 μ M DEX. Experiments labeled ‘DEX 5h’ correspond to seedlings grown on 0.5xMS plates for 10 d. Seedlings were then transferred to 0.5xMS agar plates with 10 μ M DEX and incubated for 5 hr. The results are presented as fold changes relative to the reference gene. No error bars are presented for the expression assays because these values represent only technical duplicates.

The preliminary data obtained indicate that the 4 control genes are down-regulated in the presence of both SRDX-ASK1 (line 523-3) and mSRDX-ASK1 (line 514-2). Hence, these results suggest that the down-regulation of the selected genes is not related to the activity of the SRDX domain when fused to ASK1, but instead is likely to be due to either ectopic expression of ASK1 or to the activity of the LhG4-GR transcription factor. However, because of time limitations, I was not able to carry out independent biological replicates of this experiment in order to confirm this result.

All together, our data suggest that the datasets obtained contain a large number of genes whose mis-expression is not the result of the activity of the SRDX-ASK1 fusion and to the presence of an active SRDX domain. Although it remains unclear at this stage which genes are mis-expressed as a result of the presence of SRDX-ASK1, I proceeded with a very short analysis of the preliminary microarray datasets I obtained after correction in order to generate a preliminary list of potential biological processes that could be affected by the expression of *SRDX-ASK1*.

◆ Gene ontology (GO) analysis

In order to identify biological processes that might be affected upon expression of *SRDX-ASK1*, I compared the occurrence of Gene Ontology (GO) terms within the lists of differentially expressed genes to their genome-wide distribution using the Cytoscape freeware (<http://www.cytoscape.org/>) and the BINGO 2.44 plugin (<http://www.psb.ugent.be/cbd/papers/BiNGO/Home.html>) (Maere et al., 2005) with default settings. GO terms that had an adjusted P -value < 0.05 were judged as being statistically over-represented in the microarray datasets. This analysis revealed 29 GO terms that were enriched among the differentially expressed genes in the *SRDX-ASK1* lines that were exposed to DEX for 5 hr or were continuously grown in the presence of DEX. In contrast, no GO terms were found to be enriched when seedlings were incubated with DEX for 2 hr.

Most GO terms identified as over-represented after 5 hr on a DEX-containing medium and after continuous growth in the presence of DEX were common to both datasets. The main GO categories that were found are related to the response of plants to hormones, including auxin, as well as the regulation of glycoside metabolic processes and the response to biotic stimulus (Figure 4.12). In addition, in the dataset obtained after 5 hr on a DEX-containing medium, genes involved in cell wall modification were also enriched.

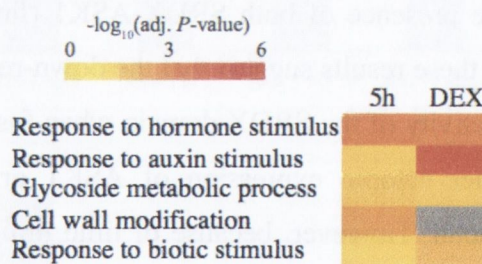


Figure 4.12 GO terms enriched among differentially expressed genes. Negative decadal logarithms of Benjamini and Hochberg-adjusted P -values for enriched GO terms are presented through a color code. ‘5h’ represents the data obtained after 5 hr of DEX treatment; ‘DEX’ corresponds to the data obtained after continuous growth in the presence of DEX.

◆ Conclusion

Our preliminary microarray experiments suggest that plant hormone signaling pathways might require the activity of ASK1 as a transcriptional co-regulator. Given the known role of ASK1-containing SCF complexes in the signal transduction pathways of multiple hormones in plants, this result could be of potential interest. However, there are

important caveats, as it appears that many of the differentially expressed genes identified in our datasets are not related to the expression of ASK1 fused to a functional SRDX domain. Indeed, the comparison of our datasets to that obtained upon expression of an AG-amiRNA placed under the control of the pOp promoter suggests that the use of the LhG4-GR transcription factor leads to significant artefactual gene expression changes. The results of our RT-qPCR analysis using a DEX-inducible mSRDX-ASK1 line further corroborates the idea that the use of the LhG4-GR transcription and/or overexpression of ASK1 contribute to the gene expression changes detected.

Experiments are currently under way, that will allow us to determine if activation of LhG4-GR in the absence of a target gene indeed leads to similar gene expression changes in seedlings. Furthermore, I will determine if overexpression of ASK1 alone also results in detectable gene expression changes. More specifically, as mentioned above, I will carry out preliminary microarray analyses on lines expressing the LhG4-GR transcription factor alone and on lines expressing ASK1 and mSRDX-ASK1 under the control of the pOp promoter.

4.2.3. Identification of a functional RNAi construct to target ASK1 mRNA

To validate the datasets obtained from the global expression profiling for the *35S_{pro}:LhG4-GR/pOp(6x)_{pro}:SRDX-ASK1* lines, I aim at using another approach that allows to perturb ASK1 function, namely a RNAi construct designed to target *ASK1* mRNA.

The use of RNAi is based on the conversion of inverted repeats of a DNA sequence into long double-stranded RNA (dsRNA) complexes that are ultimately converted into small interfering RNAs (siRNAs). The integration into the genome of inverted repeats complementary to the sequence of a gene of interest has been shown to be efficient to perturb expression of this gene. Moreover, the introduction of an intron between the inverted repeats appears to increase the efficiency of gene silencing (Wesley et al., 2001). Well-established vectors have been generated and inducible systems have also been designed (Guo et al., 2003; Wielopolska et al., 2005).

An RNAi construct that allowed expression of a double-stranded RNA molecule that was homologous to a portion of the *ASK1* mRNA was generated by Dearbhaile McEvoy, an undergraduate student I supervised. The choice of the *ASK1* region that was

targeted was based on a previously published RNAi construct that had been shown to efficiently disrupt *ASK1* function (Zhao et al., 2003b) (Figure 4.13A). In order to allow ectopic expression of this RNAi, it was placed under the control of the 35S promoter (Figure 4.13B; this construct is noted 35S_{pro}:*ASK1*_{RNAi}).

Because *Arabidopsis* transformation followed by selection of stable transformants and phenotypic characterization takes a long time, I first used a transient assay to determine if the 35S_{pro}:*ASK1*_{RNAi} construct I made was functional. To this aim, I used the FAST method (Section 2.2.2.3), which allows the transient expression of transgenes in *Arabidopsis* seedlings (Li et al., 2009). Briefly, seedlings of an *ask1-1* mutant that also expresses an epitope-tagged version of ASK1 under the control of the *ASK1* promoter (line noted *ASK1*_{pro}:6xmyc-*ASK1*, see below) were transiently transformed with *Agrobacterium* carrying either a derivative of the plasmid pML-BART that encodes the 35S_{pro}:*ASK1*_{RNAi} transgene or an empty pML-BART plasmid. If the *ASK1*_{RNAi} construct cloned efficiently targets *ASK1* mRNA, the level of the 6xmyc-ASK1 fusion protein should be reduced, which can be monitored by immunoblotting using an antibody specific for the myc tag (Section 2.2.4.7). Protein extracts from wild-type seedlings (*i.e.* that do not encode the 6xmyc-ASK1 fusion protein) were also loaded in order to detect proteins that cross-react with the anti-myc antibody. Two replicates of this experiment were performed and yielded the same results. More specifically, the use of protein extracts from wild-type seedlings indicated that the anti-myc antibody did not cross-react with other plant proteins (Figure 4.13C, lane 1). Furthermore, when *ask1-1 ASK1*_{pro}:6xmyc-*ASK1* seedlings were transformed with an empty pML-BART plasmid, a protein with an apparent molecular weight of ~ 50 kDa, was detected by anti-myc antibodies, as expected for 6xmyc-ASK1 (Figure 4.13C, lane 2). Importantly, when *ask1-1 ASK1*_{pro}:6xmyc-*ASK1* seedlings were transformed with a plasmid encoding the 35S_{pro}:*ASK1*_{RNAi} transgene, the 6xmyc-ASK1 protein no longer accumulated (Figure 4.13C, lane 3), indicating that the *ASK1*_{RNAi} construct efficiently targets *ASK1* mRNA.

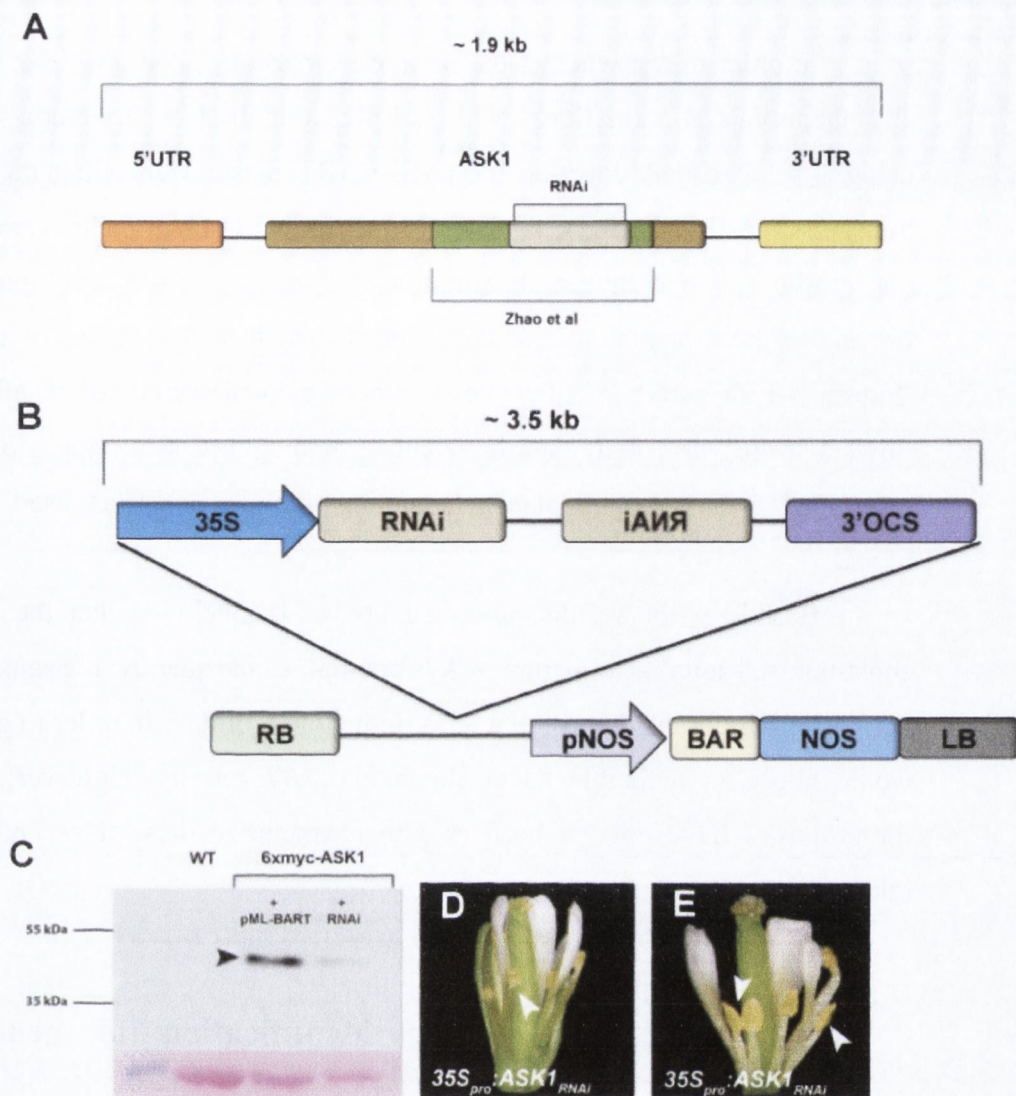


Figure 4.13 Characterization of an RNAi construct specific for *ASK1*. (A) A schematic representation of the genomic region containing the *ASK1* gene. The *ASK1* coding region targeted by the RNAi construct designed by Zhao *et al.* (2003) is shown in green, while the region used to generate the RNAi construct used in this work is represented in grey. Note that the target sequence used in this work is contained within the region that had been used by Zhao *et al.* (2003). (B) A schematic of the T-DNA mediating expression of an *ASK1_{RNAi}* under the control of the 35S promoter. The binary vector pML-BART was used in conjunction with *Agrobacterium tumefaciens* to transform wild-type plants. (C) Results of Western blot analysis of protein extracts of *ask1-1 ASK1_{pro}:6xmyc-ASK1* seedlings that transiently expressed the *ASK1_{RNAi}* transgene (indicated as '+ RNAi') or that were transiently transformed with an empty plasmid (indicated as 'pML-BART'). Molecular weight markers are indicated on the left, and Ponceau staining of the membrane is shown to control for loading of equal amounts of total proteins. (D-E) Flower phenotype of plants ectopically expressing the *ASK1_{RNAi}* transgene. The arrowheads indicate affected stamens. (D) A flower with short filaments, but with stamens that form viable pollen. (E) A flower with 5 stamens that have short filaments, as well as mildly misshapen petals. No viable pollen was observed in this flower. 3'OCS, octopine synthase terminator sequence; RB, right border of T-DNA; LB, left border of T-DNA; pNOS, nopaline synthase promoter sequence; BAR, BASTA® resistance gene; NOS, nopaline synthase terminator sequence; UTR, untranslated region; WT, wild type.

In order to test whether the ectopic expression of the *ASK1_{RNAi}* construct triggers any phenotypic abnormalities, wild-type plants of the *L-er* accession were transformed using *Agrobacterium* carrying the pML-BART plasmid encoding the *35S_{pro}:ASK1_{RNAi}* transgene (Section 2.2.2.2). To date, 2 independent transformants were isolated that did not exhibit any phenotypic abnormalities during vegetative development. In contrast, flowers of these plants had misshapen petals and formed stamens with short filaments (Figure 4.13D and E). Pollen development also appeared to be affected and these flowers were also male sterile. Hence, plants expressing the *ASK1_{RNAi}* construct resembled *ask1-1* mutant plants, indicating that the construct used indeed works *in planta*.

Based on the results presented above, I concluded that the *35S_{pro}:ASK1_{RNAi}* construct was suitable to disrupt *ASK1* function. Consequently, the same RNAi construct was cloned under the control of a DEX-inducible promoter in order to generate lines that would allow an inducible knock down of *ASK1* activity. However, because of time limitations, I have not yet been able to characterize these lines and use them for a microarray analysis.

4.2.4. Genome-wide identification of genomic regions bound by ASK1

4.2.4.1. Isolation of lines expressing a functional epitope-tagged version of ASK1

As outlined above, the use of dominant repressor domains fused to ASK1 indicated that a variety of physiological processes require the binding of ASK1 (and likely ASK1-containing SCF complexes) to DNA for their regulation. To gain further insights into the mechanisms underlying the role of ASK1, and probably associated SCF complexes, in this type of transcriptional regulation, I aim at identifying the direct target genes of ASK1 on a genome-wide scale. To this end, I carried out ChIP-seq experiments using seedlings. To this end, an antibody that efficiently and specifically recognizes the DNA-binding proteins of interest must be available. This antibody can be specific to the endogenous protein or to an epitope-tag that may be fused to the protein. To maximize the likelihood of finding an antibody that would be suitable for ChIP-Seq, both strategies were used in parallel.

♦ *Characterization of lines expressing ASK1 fused to GFP*

The Wellmer laboratory has successfully carried out ChIP-seq experiments using transcription factors that were expressed from endogenous promoters and that had been fused to GFP. In order to take advantage of the protocols developed in the Wellmer laboratory, I generated a vector containing 1 kb upstream of ASK1 coding region, followed by the GFP sequence cloned in frame with the ASK1 coding region (including introns) and 59 bp downstream of the stop codon (Figure 4.14A; this construct is noted *ASK1_{pro}:GFP-ASK1*). Because homozygous *ask1-1* mutants are male sterile (Yang et al., 1999), this construct was used to transform an *ask1-1/+* segregating population to determine if expression of GFP-ASK1 from the *ASK1* promoter was sufficient to rescue the *ask1-1* mutant phenotype, and hence test the functionality of the GFP-ASK1 fusion *in vivo*. Out of 15 independent transformants, 3 plants were heterozygous (*ask1-1/+*) in the T1 generation. The T2 generation of these 3 plants was examined for rescue of the *ask1-1* male sterility phenotype, but in all 3 independent lines, plants were indistinguishable from *ask1-1* mutants. Specifically, the plants had smaller rosette leaves and their stems were shorter compared to the wild-type. Furthermore, petals were smaller and both stamen and pollen development were affected, resulting in male sterility.

To check if the GFP-ASK1 fusion was indeed expressed, I performed Western blot analysis using protein extracts from 10-day-old seedlings that carried the *ASK1_{pro}:GFP-ASK1* transgene. Although *ASK1* should be expressed at high levels from its endogenous promoter, no proteins could be detected using an anti-GFP antibody (data not shown). It is possible that the fusion protein is not expressed or is expressed at a level that is undetectable by Western blot analysis, suggesting that expression level of the GFP-ASK1 fusion in these 3 lines is insufficient to rescue *ask1-1* mutants.

In order to overcome this problem, I generated a new rescue construct that consists of a fusion between GFP and *ASK1*'s cDNA under the control of the constitutive 35S promoter (noted *35S_{pro}:GFP-ASK1*) (Figure 4.14B). Two independent transformants were obtained, but they did not carry the *ask1-1* mutation. Because a functional rescue line with an alternative tag was isolated in parallel (see below), I did not continue to work with the *35S_{pro}:GFP-ASK1* lines.

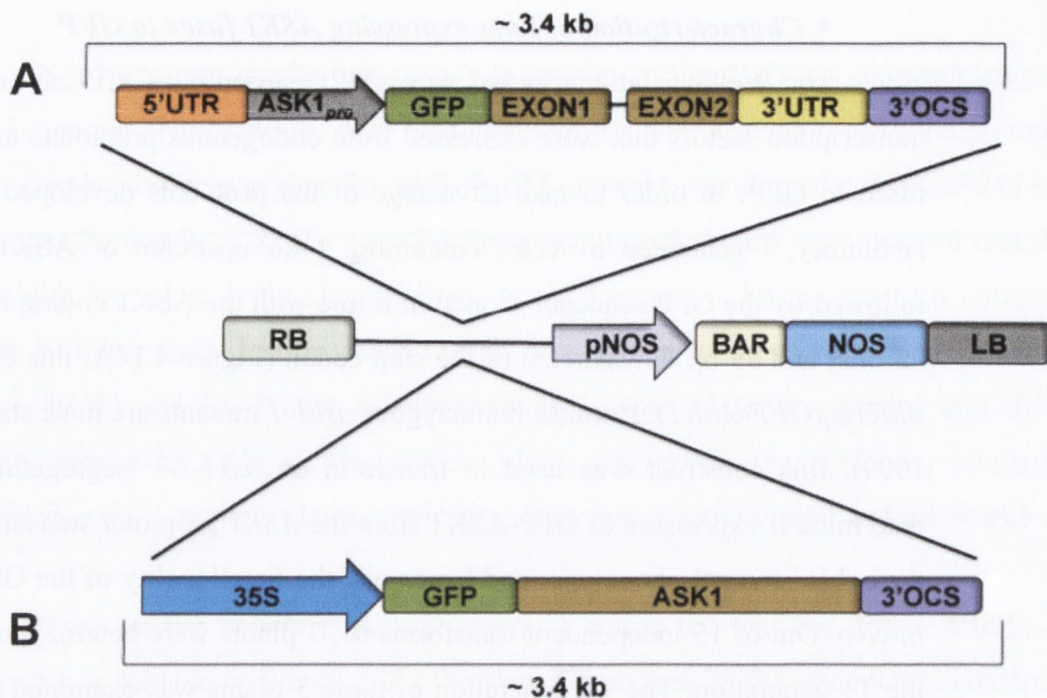


Figure 4.14 Configuration of the *GFP-ASK1* rescue constructs. A schematic of the T-DNA insertion carrying the *GFP-ASK1* transgene under the control of (A) the endogenous promoter and (B) the 35S promoter. The binary vector pML-BART was used in conjunction with *Agrabacterium tumefaciens* to transform a population of *ask1-1* segregating plants. 3'OCS, octopine synthase terminator sequence; RB, right border of T-DNA; LB, left border of T-DNA; pNOS, nopaline synthase promoter sequence; BAR, BASTA® resistance gene; NOS, nopaline synthase terminator sequence; UTR, untranslated region.

♦ *Rescue of an ask1-1 mutant by expression of 6xmyc-ASK1*

Because it is difficult to predict which epitope tag will lead to a functional fusion protein, while isolating plants expressing GFP-ASK1, I also tested the use of an alternative epitope tag. To this end, I generated a rescue construct encoding *ASK1*'s endogenous promoter (1 kb upstream of the start codon), followed by 6 repeats of the myc epitope tag (6xmyc) and the genomic region coding for *ASK1*, followed by 59 bp downstream (this construct is noted *ASK1_{pro}:6xmyc-ASK1*) (Section 2.5.3.5) (Figure 4.15A). The resulting vector was used to transform an *ask1-1/+* segregating population (Section 2.2.2.2) in order to check for functionality of the 6xmyc-ASK1 fusion protein.

Two independent transformants (noted 5.2 and 5.3) were obtained and genotyped for the presence of both the rescue transgene and the transposon in *ask1-1* plants. This genotyping revealed that both transformants were wild-type for *ASK1* and hence could not be used to check functionality of the construct. To overcome this problem, I crossed these 2 lines with an *ask1-1* mutant. The F1 generations of these 2 crosses were allowed to self. In the resulting F2 generation from the cross between line 5.2 and the *ask1-1*

mutant, no *ask1-1* homozygous plants could be isolated. In contrast, all plants of the F2 population resulting from the cross of line 5.3 with *ask1-1* were indistinguishable from the wild-type including those that were homozygous for the transposon in *ASK1*, as verified by genotyping. Seeds were collected from the individual *ask1-1 ASK1_{pro}:6xmyc-ASK1* plants and they were used for further analyses (Figures 4.15B and C).

In order to check that the 6xmyc-ASK1 fusion protein is indeed present *in planta*, I performed a Western blot analysis using protein extracts from 10-day-old seedlings carrying *ASK1_{pro}:6xmyc-ASK1* transgene. In both lines (5.2 and 5.3), a protein that is recognized by the anti-myc antibody was detected. Although the apparent molecular weight was higher than expected, the absence of any cross-reacting bands in the control (*i.e.* wild-type seedlings) strongly suggests that this protein corresponds to the 6xmyc-ASK1 fusion. (Figure 4.15D). This result suggests that the 6xmyc-ASK1 fusion protein is present in these lines at a detectable level.

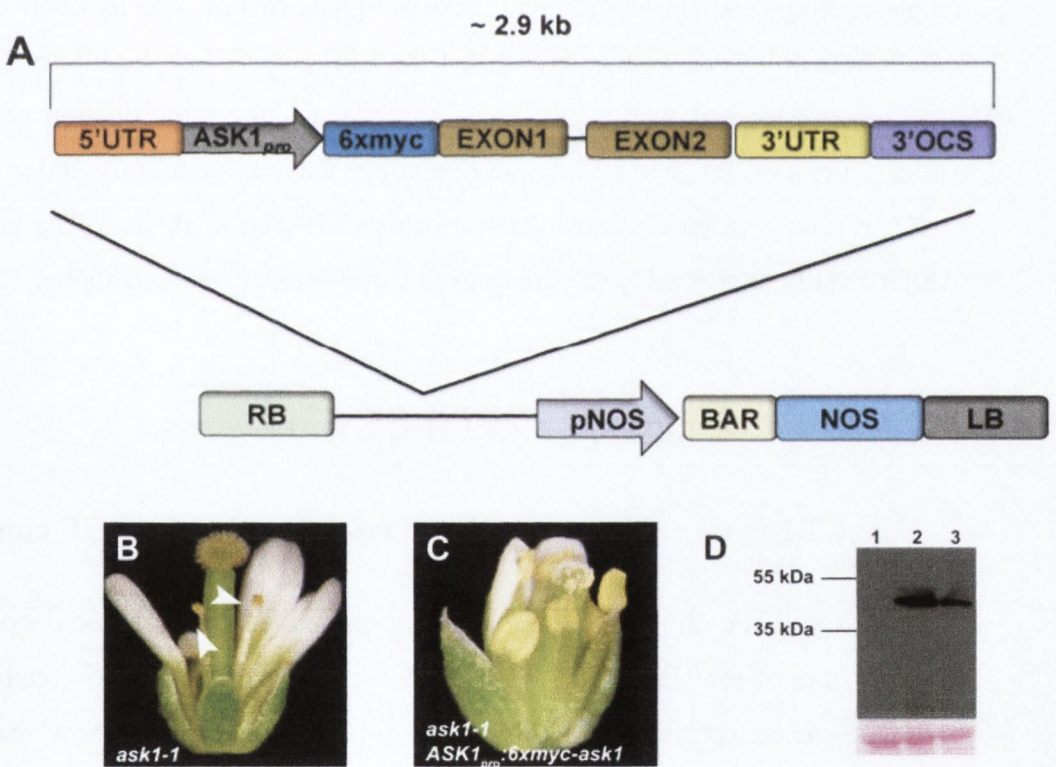


Figure 4.15 Characterization of a 6xmyc-ASK1 rescue line. (A) A schematic representation of the T-DNA encoding the *ASK1_{pro}:6xmyc-ASK1* rescue construct. The binary vector pML-BART was used in conjunction with *Agrobacterium tumefaciens* to transform a population of *ask1-1* segregating plants. (B) Male-sterile flower of an *ask1-1* mutant produces short stamens (indicated by arrowheads) that do not carry viable pollen. (C) Flower from an *ask1-1 ASK1_{pro}:6xmyc-ASK1* plant resulting from the cross of line 5.3 and *ask1-1*, which shows that stamen growth and pollen viability are restored in the presence of the *ASK1_{pro}:6xmyc-ASK1* transgene. (D) Western blot analysis of 6xmyc-ASK1 rescue lines. Proteins were

extracted from 10-day-old seedlings. Lane 1: wild type control; lane 2: line 5.2; lane 3: line 5.3. The expected size for the 6xmyc-ASK1 fusion protein is approximately 29 kDa. However, the protein detected migrates at ~ 50 kDa. Two independent biological replicates were carried out and gave similar results. 3'OCS, octopine synthase terminator sequence; RB, right border of T-DNA; LB, left border of T-DNA; pNOS, nopaline synthase promoter sequence; BAR, BASTA® resistance gene; NOS, nopaline synthase terminator sequence; UTR, untranslated region.

4.2.4.2. Identification of genomic regions bound by ASK1 in seedlings

In parallel to the generation and characterization of lines expressing a functional epitope-tagged version of ASK1 for ChIP-Seq experiments, Dr. Emmanuelle Graciet raised polyclonal rabbit antibodies against recombinant ASK1. The characterization of these antibodies for immunoblotting and immunoprecipitation indicated that they should be suitable for ChIP-Seq. Hence, Dr. Graciet carried out ChIP using 10-day-old wild-type seedlings and Dr. Diarmuid O'Maoileidigh carried out quality control checks on the sequences obtained, which suggest that the ChIP, as well as the library preparation and the sequencing had worked. Because analysis and the identification of genes located near regions of the genome that are bound by ASK1 are currently under way, I was not able to cross-compare the results from this ChIP-Seq analysis to the genes that were differentially expressed in the microarray experiments discussed above.

4.3. Discussion and future work

4.3.1. Transcriptional co-activation by SCF complexes

My work on ASK1 aimed at determining if transcriptional co-regulation by components of the Ub system, such as SCF complexes, is a widespread mechanism to regulate gene expression. To this end, I aimed at determining how many and which genes are transcriptionally co-regulated by ASK1. In the first part of this work, I fused 2 dominant repressor domains, SRDX and WUSB, to ASK1 and placed the fusions under the control of the 35S promoter. Unfortunately, lines expressing WUSB-ASK1 were obtained only recently, and hence I did not have sufficient time to characterize them. However, given that UFO-SRDX and UFO-WUSB plants exhibited similar phenotypic abnormalities, I expect to find similar morphological defects in WUSB-ASK1 and

SRDX-ASK1 lines. The latter showed different phenotypic defects (Figure 4.2), ranging from wild-type looking plants to plants that were severely affected in both vegetative and reproductive development, or that died at the seedling stage in the T1 generation. The control experiments I carried out (*i.e.* expression of a mSRDX-ASK1 fusion and measurement of SCF complex activity) indicate that these phenotypes are unlikely to be the result of a change in ASK1 function due to the presence of the SRDX domain, or to a decrease in overall SCF complex activity.

Hence, the range of phenotypes observed in SRDX-ASK1 plants suggests that SRDX-ASK1 does indeed bind either directly or indirectly to DNA, which then leads to developmental defects, likely because of changes in gene expression. Based on these results, it appears that ASK1 regulates different physiological and developmental processes throughout the life cycle of a plant. Because it appears that expression of SRDX-ASK1 does not negatively affect SCF complex activity, but instead increases overall SCF activity (Figures 4.6 and 4.7), I assume that the SRDX-ASK1 fusion is indeed incorporated into functional SCF complexes. However, biochemical data is still required to show that this is the case, and *in vitro* SCF complex reconstitutions, as well as co-immunoprecipitation experiments will be carried in order to confirm that SRDX-ASK1 can indeed participate in SCF complexes.

In order to obtain further insights into the molecular mechanisms underlying *ASK1* function as a transcriptional co-regulator, a preliminary global gene expression profiling analysis was carried out using seedlings from a line that expresses the SRDX-ASK1 fusion under the control of a DEX-inducible promoter (*35S_{pro}:LhG4-GR/pOp(6x)_{pro}:SRDX-ASK1*; Section 4.2.2.2). In this experiment, I expected to find predominantly down-regulated genes due to the activity of the SRDX domain, but instead the datasets obtained after 2 and 5 hr in the presence of a DEX-containing growth medium mostly contained genes that were up-regulated. I hypothesized that this could be the result of ASK1 overexpression (independently of the activity of the SRDX domain) or to ectopic expression of LhG4-GR. The best control to test these hypotheses would be to carry out a similar gene expression profiling analysis using a line expressing the mSRDX-ASK1 fusion under the control of the pOp promoter. Unfortunately, I have only obtained one such transformant to date and did not have sufficient time to characterize it and carry out this analysis.

In order to check if the gene expression changes could be the result of the activity of LhG4-GR, I compared my datasets to those obtained upon expression of an AG-miRNA placed under the control of the pOp promoter. Although the tissue used

and the gene under the control of the pOp promoter were very different in these experiments, I found a large overlap between the different datasets. This preliminary analysis hence revealed that the use of the LhG4-GR transcription factor leads to important artefactual gene expression changes that are mis-leading in global gene expression profiling experiments. Such effects of LhG4-GR have not been described in the literature and I am hence investigating this further (see below).

In order to obtain some insights into the biological processes that are controlled by ASK1 as a transcriptional co-regulator, I carried out a preliminary microarray analysis using seedlings of a line that expresses SRDX-ASK1 under the control of the 35S promoter (line 5.13; Section 4.2.1.1). Although this would lead to a large number of differentially genes that are unlikely to be direct targets of ASK1, I thought this approach would be a good control for the results obtained upon expression of SRDX-ASK1 under the control of the DEX inducible promoter. The data is currently being analyzed by Patrick Ryan and will be available later for cross-comparisons.

The second aspect of my work on ASK1 was the identification of its direct target genes using ChIP-Seq. To this end, I generated a line expressing 6xmyc-ASK1 under the control of the *ASK1* promoter, which can be used for such experiments. In parallel, Dr. Emmanuelle Graciet raised rabbit polyclonal antibodies specific for ASK1. The latter were used in a preliminary ChIP-Seq experiment using seedlings as a starting material, as our phenotypic analysis of SRDX-ASK1-expressing plants suggests that ASK1 should act as a transcriptional co-regulator at this developmental stage. The data is currently being analyzed by Patrick Ryan. In the absence of this data and given the problems revealed by our preliminary microarray analysis of SRDX-ASK1-expressing seedlings, it is difficult to draw any biological conclusions regarding the role of ASK1, and associated SCF complexes, as transcriptional co-regulators.

4.3.2. Future experiments

♦ *Additional controls for the gene expression analysis using a DEX-inducible promoter*

First, I aim at generating lines that will allow me to validate the dataset obtained from the global expression profiling analysis of the $35S_{pro}:LhG4-GR/pOp(6x)_{pro}:SRDX-ASK1$. These include lines that will express the mSRDX-ASK1 fusion under the control of the pOp promoter ($35S_{pro}:LhG4-GR/pOp(6x)_{pro}:mSRDX-ASK1$), as well as lines that

express LhG4-GR alone from the 35S promoter, or untagged ASK1 under the control of the pOp promoter. Using these reagents, I will be able to understand if many of the differentially expressed genes that were identified in the microarray datasets discussed above are the result of LhG4-GR or ASK1 activity.

Furthermore, as mentioned above, I will compare the gene expression changes detected in a line expressing SRDX-ASK1 from the 35S promoter to the datasets that will be obtained with the DEX-inducible system.

♦ *Complementary experiments using ask1-1 or ASK1_{RNAi} lines*

In order to complement the gene profiling experiments using SRDX-ASK1 lines, as well as the ChIP-Seq analysis on ASK1-containing complexes, I have generated reagents that will allow me to compare the data from these experiments with microarray data I will obtain in seedlings that lack ASK1 activity or that have reduced ASK1 levels. Because the *ask1-1* mutant is sterile, it is difficult to perform such experiments using this mutant line, as I would have to work with segregating populations and will not be able to select seedlings that are homozygous *ask1-1* for the analysis. To by-pass this problem, after confirming the efficiency of the ASK1_{RNAi} construct (Section 4.2.3), I have been generating lines carrying a *35S_{pro}:LhG4-GR/pOp(6x)_{pro}:ASK1_{RNAi}* transgene (Section 4.2.3). However, we now know that the use of the LhG4-GR transcription factor can lead to important artefacts. Hence, instead, I will use lines that express an ASK1_{RNAi} construct under the control of the 35S promoter. In this case, primary transformants in which ASK1 activity is significantly down-regulated will be sterile, and hence I will not be able to propagate them in the next generation for microarray analysis. Primary transformants will therefore be pollinated with pollen from a wild-type plant. Seedlings of the F1 generation will be grown on 0.5xMS agar with BASTA in order to select seedlings that carry the *35S_{pro}:ASK1_{RNAi}* transgene. Because the effects are dominant, I will be able to use these seedlings for a microarray experiment to determine gene expression changes in seedlings that have reduced levels of ASK1 activity.

The use of an ASK1_{RNAi} line will have several advantages and will complement both the transcriptomics experiments with SRDX-ASK1-expressing lines and the ChIP-Seq experiments. Indeed, because the SRDX-ASK1 lines used express the fusion under the control of a 35S promoter, it would be possible to argue that (i) the effects are due to overexpression of the fusion, above physiological levels, which could lead to non-physiological binding of SRDX-ASK1 to the promoter of some genes; and (ii) the effect on transcription might not be physiological but simply due to the presence of the SRDX

domain (*e.g.* in a physiological context, ASK1 can bind to a promoter region without affecting transcription of the gene, but binding of SRDX-ASK1 to the same genomic region will lead to transcriptional repression, because the SRDX domain can recruit HDAC complexes).

◆ *Data comparison*

Using seedlings as a starting material, I will obtain 3 different datasets: (i) genes that are differentially expressed upon expression of SRDX-ASK1 compared to either wild-type or mSRDX-ASK1-expressing seedlings; (ii) genes that are differentially expressed as a result of ASK1 loss of function (*i.e.* upon expression of ASK1_{RNAi}); and (iii) genes whose upstream and/or downstream genomic regions are bound by ASK1 (generated by ChIP-Seq). The comparison of these 3 datasets should then lead to the identification of high-confidence target genes of ASK1 (Figure 4.16). The validation of the ChIP-Seq data using the microarray data is essential to distinguish between regions of the genome that are bound by ASK1 without this binding resulting in the regulation of the neighboring genes, from regions of the genome that are bound and whose neighboring genes are indeed regulated by the protein of interest (Kaufmann et al., 2010; Wuest et al., 2012; O'Maoileidigh et al., 2013).

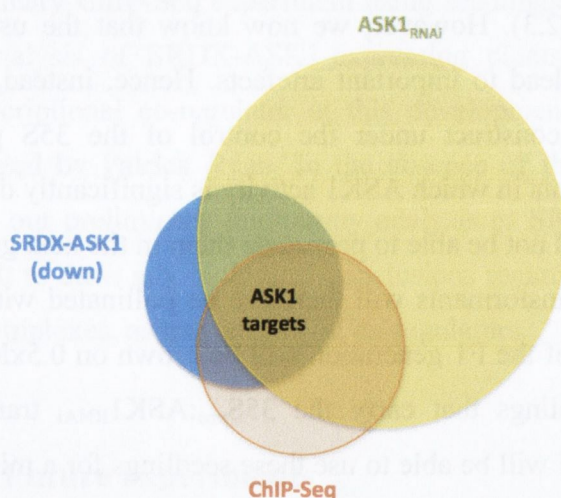


Figure 4.16 Strategy to identify high-confidence ASK1 direct targets. The circles (not to scale) represent either differentially expressed genes or genes whose promoter regions are bound by ASK1. The overlap of all 3 datasets corresponds to high-confidence target genes of ASK1. SRDX-ASK1 (down): genes that are found as being negatively regulated in the presence of SRDX-ASK1 compared to either the wild type or mSRDX-ASK1-expressing seedlings; ASK1_{RNAi}: genes that are differentially expressed upon expression of this construct; ChIP-Seq: genes associated with binding of ASK1 either 3 kbp upstream of the initial methionine residue or 1 kb downstream of the stop codon.

These experiments will allow us to provide further insights into the roles of ASK1 and SCF complexes as transcriptional co-regulators during early stages of development in *Arabidopsis* by identifying not only the direct target genes of ASK1, but also the biological pathways that are regulated in this manner. This project will hence determine if this type of transcriptional is widespread in plants and will provide molecular details into the role of ASK1, and likely ASK1-containing SCF complexes, as a transcriptional co-regulator.

♦ *Role of ASK1 as a transcriptional co-regulator during flower development*

The initial aim of this project was to provide insights into the role of ASK1 as a transcriptional co-regulator not only at the seedling stage, but also during flower development. Indeed, based on previously published papers on UFO (Chae et al., 2008) and the phenotype of plants that express SRDX-ASK1, we expect that ASK1 and associated SCF complexes regulate at least some of LFY target genes in this manner. In order to address this possibility, I have generated reagents that will allow me to carry out experiments similar to those described using seedlings, on floral tissue. To this aim, I have crossed some of the reagents I generated into the floral induction system. These include the *ask1-1* mutant, the DEX-inducible SRDX-ASK1 line, as well as the 6xmyc-ASK1 rescue line. Using these reagents, I intend to carry out similar gene expression profiling and ChIP-Seq experiments to identify direct targets of ASK1 at early stages of flower development. Furthermore, I will be able to compare this data to that obtained after ChIP-Seq using UFO-GFP (Chapter 3), which will provide further insights into the roles of both UFO and SCF complexes during flower development.

To conclude, I hope that the experiments described above will yield a comprehensive view on the processes and genes whose expression relies on ASK1 acting as a transcriptional co-regulator at different stages of development. They will reveal to what extent the regulation of different biological pathways depends on this type of transcriptional regulation by components of the Ub system.

5. Characterization of closely related F-box proteins

NAN and *GRA*

5.1. Introduction

It is known that orthologs of *UFO* in *Petunia hybrida* and *Antirrhinum majus* (snapdragon) are not only involved in the establishment of petal and stamen identity, but also in the specification of the floral meristem itself (Souer et al., 2008). Based on the study of *Arabidopsis* mutants, such a role could not be assigned to *UFO*, despite the fact that it is expressed early in the floral meristem. One possibility is that the role of *UFO* in floral meristem identity has been overlooked in *Arabidopsis* due to functional redundancy with unknown genes. In an attempt to identify *Arabidopsis* genes that might act redundantly with *UFO* in the specification of floral meristem identity, I used the Expression Angler program (http://bar.utoronto.ca/ntools/cgi-bin/ntools_expression_angler.cgi) (Toufighi et al., 2005) which calculates the correlation coefficients for expression of all genes for which expression data is available compared to that of the gene of interest. It was assumed that a protein that acts redundantly with *UFO* would also belong to the family of F-box proteins. Using the version of Expression Angler that was available in 2009, 2 F-box proteins (At4g19865 and At3g23260) were found to have similar expression patterns as *UFO* (Table 5.1). I chose to focus first on the F-box protein encoded by At4g19865 and named it *NANNY* (*NAN*). To test for the presence of a paralog of *NAN* in the *Arabidopsis* genome, I used BLASTP analysis and detected another F-box protein that shared 62% sequence identity at the protein level with *NAN*. It was found that the gene (At4g19870) encoding this protein was also located besides *NAN* in the genome and was named *GRANNY* (*GRA*).

Table 5.1 Results obtained using Expression Angler with *UFO* as a query. AGI numbers, score (E-value) and name of protein encoded are indicated. In bold: NAN.

Gene	E-value	Protein name and family
At1g30950	1.000	UFO (UNUSUAL FLORAL ORGANS)
At3g60390	0.782	HAT3 (HOMEBOX-LEUCINE ZIPPER PROTEIN 3)
At5g16850	0.777	ATTERT (TELOMERASE REVERSE TRANSCRIPTASE)
At1g51960	0.751	IQD27 (IQ-DOMAIN 27)
At1g12620	0.730	Pentatricopeptide (PPR) repeat-containing protein
At3g62370	0.722	Unknown protein
At2g18500	0.688	ATOF7 (ARABIDOPSIS THALIANA OVATE FAMILY PROTEIN 7)
At1g02840	0.672	SRP34
At4g19865	0.670	Kelch-repeat-containing F-box family protein
At1g03100	0.667	Pentatricopeptide (PPR) repeat-containing protein
At3g23740	0.666	Unknown protein
At2g46250	0.656	Myosin heavy chain-related
At5g38150	0.654	PMI15 (PLASTID MOVEMENT IMPAIRED 15)
At3g54630	0.648	Unknown protein
At1g07780	0.645	TRP6 (PHOSPHORIBOSYLANTHRANILATE ISOMERASE 1)
At1g51580	0.642	KH domain-containing protein
At2g01120	0.631	ATORC4 (ORIGIN RECOGNITION COMPLEX SUBUNIT 4)
At5g11020	0.630	ATP binding protein
At1g27120	0.626	Galactosyltransferase family protein
At1g63470	0.619	DNA-binding family protein
At3g23260	0.618	F-box protein
At4g25160	0.612	Protein kinase family protein
At1g05055	0.608	ATGTF2H2 (GENERAL TRANSCRIPTION FACTOR II H2)
At3g09660	0.607	MCM8 (MINICHROMOSOME MAINTENANCE 8)

To confirm that *UFO* and *NAN* are expressed in similar domains I used the *Arabidopsis* eFP Browser (<http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>), which allows the visualization of gene expression based on the dataset produced by (Schmid et al., 2005). As expected, *UFO* and *NAN* both appeared as being expressed in the floral

meristem, but also in flowers (Figure 5.1A). Although data for *GRA* was not available in this database, I used the results of previously published microarray experiments and determined that *GRA* was also expressed at early stages of flower development (Wellmer et al., 2006) (Figure 5.1B).

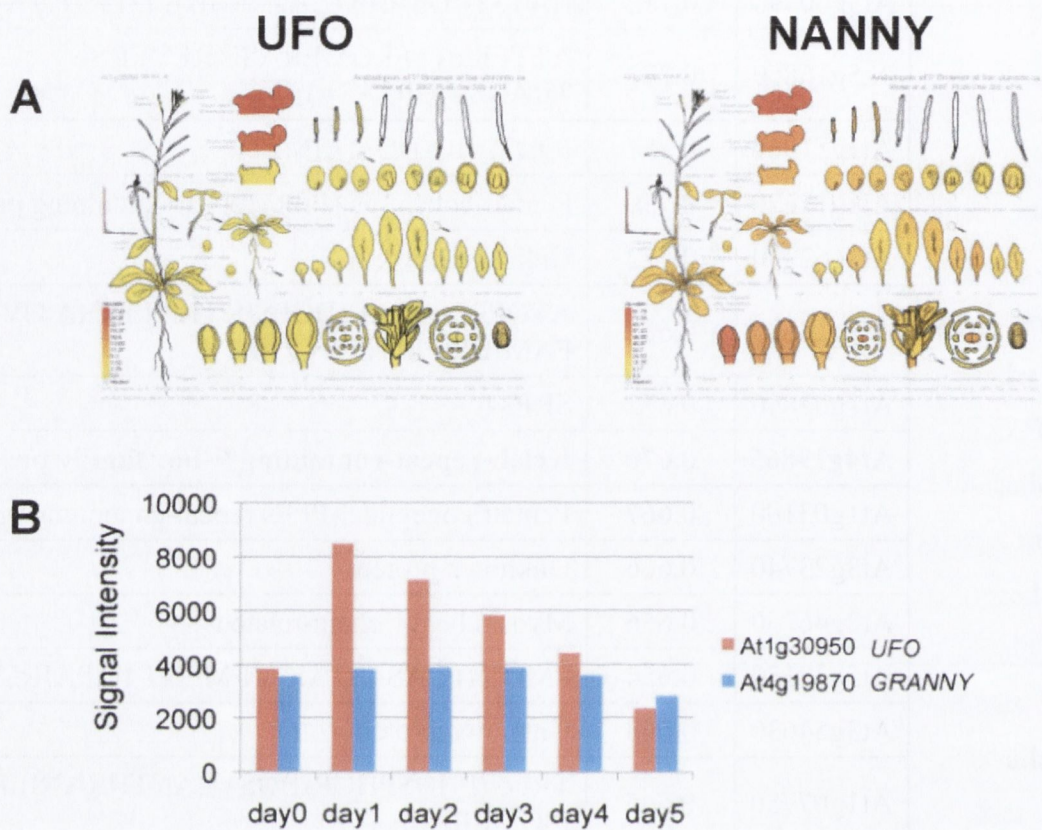


Figure 5.1 Identification of candidate genes that act redundantly with *UFO*. (A) Expression patterns of *UFO* and *NAN* obtained using the *Arabidopsis* eFP Browser. Note the maximum of expression in floral meristems for both *UFO* and *NAN*, as well as expression in flowers for *NAN*. (B) *GRA* (blue) and *UFO* (red) expression at early stages of flower development (Wellmer et al., 2006). In this experiment, the floral induction system was used (Section 1.4.1) and tissue was collected before DEX treatment and then, 1, 2, 3, 4 and 5 d after the induction of flower development using a DEX-containing solution. RNA samples from tissues on 2 consecutive days were co-hybridized on a microarray. Then the direct ratios were calculated for each gene represented on the array using its normalized signal intensities at the individual time points.

Because *NAN* and *GRA* had not been studied before, I searched for the presence of known motifs in these proteins. I found only 2 known motifs: the F-box domain and repeats of the Kelch motif, which is known to be involved in mediating protein-protein interactions (Adams et al., 2000). As described previously, the F-box domain contains ~ 50 amino acids and is involved in the interaction between an F-box protein and ASK1. This domain is located approximately between residues 25 and 75 in *NAN* and *GRA*.

The Kelch motif is composed of ~ 50 amino acid residues forming a 4-stranded β -sheet propeller and is often found at the C-terminus of F-box proteins. It appears to confer substrate-specificity to the SCF complex (Petroski and Deshaies, 2005). These repeats are located approximately between residues 140 and 220 in NAN and GRA. In sum, the domains found in both NAN and GRA suggest that these 2 proteins could be part of SCF complexes and target other proteins for degradation through the Ub system.

To gain further information on *NAN* and *GRA*, I used the *GRA* protein sequence as a query in a BLASTP analysis to determine if there were additional homologs of *NAN* and *GRA* in the *Arabidopsis* genome. I found that there was a large number of proteins encoded in the *Arabidopsis* genome that showed significant sequence similarities to *GRA*. These similarities correlated with the presence of the F-box domain and that of the Kelch repeats. Based on previously published papers (Andrade et al., 2001; Schumann et al., 2011), it appears that proteins encompassing these 2 domains constitute a large family. However, although *NAN* and *GRA* bear similarities throughout their sequence, their C-termini differ from C-terminal regions found in other proteins that combine an F-box domain and Kelch repeats. Because the C-terminus of F-box proteins is known to be often involved in the recognition of the substrate protein, it is possible that *NAN* and *GRA* are not functionally redundant with the other proteins identified in this BLASTP analysis. I next carried out a BLASTP analysis using *GRA* as a query to determine if orthologs of *GRA* and *NAN* were encoded in the genomes of other plants or of other organisms. The analysis was made difficult due to the fact that many proteins that combined F-box proteins and Kelch repeats could be identified in other plant genomes, but it was not clear whether they were related to *NAN* and *GRA*. However, I did not find orthologs of *NAN* and *GRA* outside of the plant kingdom. Hence these 2 F-box proteins appear to be plant-specific.

Despite the fact that *NAN* and *GRA* are expressed in floral meristems, as well as in other parts of the plant at earlier developmental stages, their function has never been studied. Because *NAN* and *GRA* are likely the result of a recent gene duplication event, I hypothesized that they would likely act in a functionally redundant manner. Hence, to examine the role of these genes, I first aimed at isolating plants in which the function of both *NAN* and *GRA* would be affected. Because I hypothesized that *NAN* and *GRA* might act redundantly with *UFO*, I analyzed the genetic interaction between lines with reduced levels of *NAN* and *GRA* in combination with *ufo* mutants. If the hypothesis is true, the phenotype of the mutant for *UFO*, *NAN* and *GRA* will be stronger than in the *ufo* single mutants with defects also during the specification of floral meristem as it is

the case in other plant species (see above). Alternatively, if *UFO*, *NAN* and *GRA* are not functionally redundant, there will be 2 possibilities. First, there is no genetic interaction between these genes. Then, the phenotypic defects of mutants for *UFO*, *NAN* and *GRA* will be the sum of defects found in both parental lines. Second, *UFO*, *NAN* and *GRA* are not functionally redundant but they may genetically interact with each other. If this is true, the novel phenotypic defects (but not related to the floral meristem specification) will be observed in the mutant for *UFO*, *NAN* and *GRA*.

Furthermore, to gain additional insights into the roles of *NAN* and *GRA*, I generated and characterized lines that ectopically express *GRA*. Finally, I have tested for potential genetic interactions with other known regulators of flower development.

5.2. Results

5.2.1. Characterization of plants mutant for *NAN* and *GRA*

5.2.1.1. Isolation of lines with reduced levels of *NAN* and *GRA* mRNAs

In order to test if one gene was predominantly expressed over the other, I carried out RT-qPCRs using RNA extracted from young floral buds and sets of oligonucleotides designed to amplify specifically endogenous *NAN* or *GRA* mRNAs (Figure 5.2). I found that both genes were expressed in flowers and that the expression level of *GRA* was approximately twice as high as that of *NAN*.

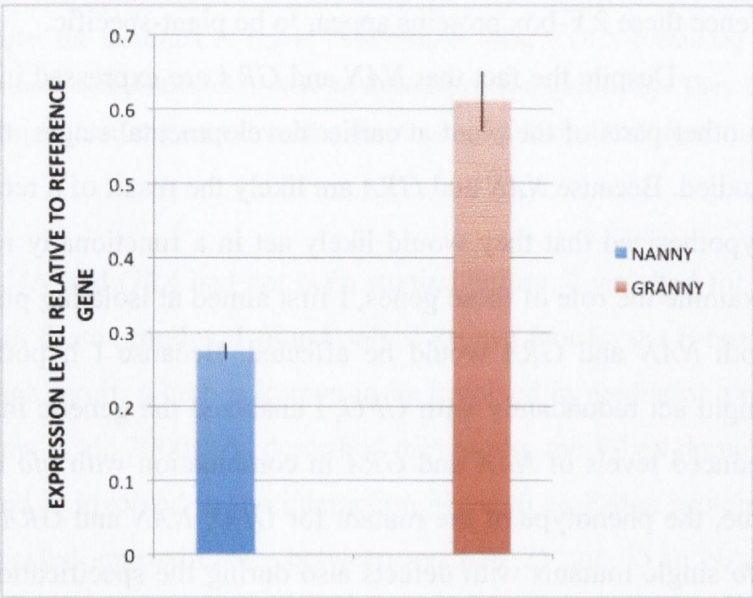


Figure 5.2 Expression levels of endogenous *NAN* and *GRA* in wild-type plants. The results are presented as expression level relative to the reference gene. RNA was extracted from whole inflorescences containing only young floral buds (up to stage 9). Error bars indicate the standard errors of the means of 3 biological replicates.

In order to test whether *NAN* and *GRA* act redundantly with *UFO* in floral meristem specification, and possibly during flower development, plants with lower levels of *GRA* and *NAN* mRNAs were isolated. Because *NAN* and *GRA* are located next to each other in *Arabidopsis* genome (Figure 5.3A), isolating a double mutant for both genes is not straightforward. To circumvent this problem and isolate lines with lower levels of both *GRA* and *NAN* mRNAs, I used 2 approaches.

I first searched publicly available T-DNA and transposon collections for lines that would carry a T-DNA or a transposon in either *NAN* or *GRA*, or in-between the 2 genes. Three lines were selected: SALK_076200 (denoted SALK), which carries a T-DNA insertion in the 3' UTR region of *GRA*; GT_5_111847 (denoted GT), which has a DS transposable element inserted in the 3'UTR region of *NAN*; and SAIL_272_A02 which has a T-DNA insertion in the intergenic region between *GRA* and *NAN* (Figure 5.3A). Plants homozygous for each of the insertions were isolated and found to be phenotypically similar to wild-type plants throughout development. A possible explanation for the wild-type phenotype of these lines is that the expression level of *NAN* and *GRA* is not (sufficiently) affected by the T-DNA insertions. To test this hypothesis, the RNA from young floral buds of the 3 insertion lines obtained was extracted to determine the expression levels of *NAN* and *GRA* using RT-qPCR (Figures 5.3B and C). The level of *NAN* mRNA in the GT line was reduced to 60% of that found in the wild type, whereas expression of *GRA* was not affected in this line. In the case of the SALK line, *NAN* mRNA level was unchanged and the mRNA levels of *GRA* were decreased to 40% of those found in the wild type. In the SAIL_272_A02 line, *NAN* and *GRA* mRNA levels were reduced to 10% and 70% of the wild-type levels, respectively. Because the expression of both *NAN* and *GRA* was affected in this line, it is noted *nan-1 gra-1* and was characterized in more detail.

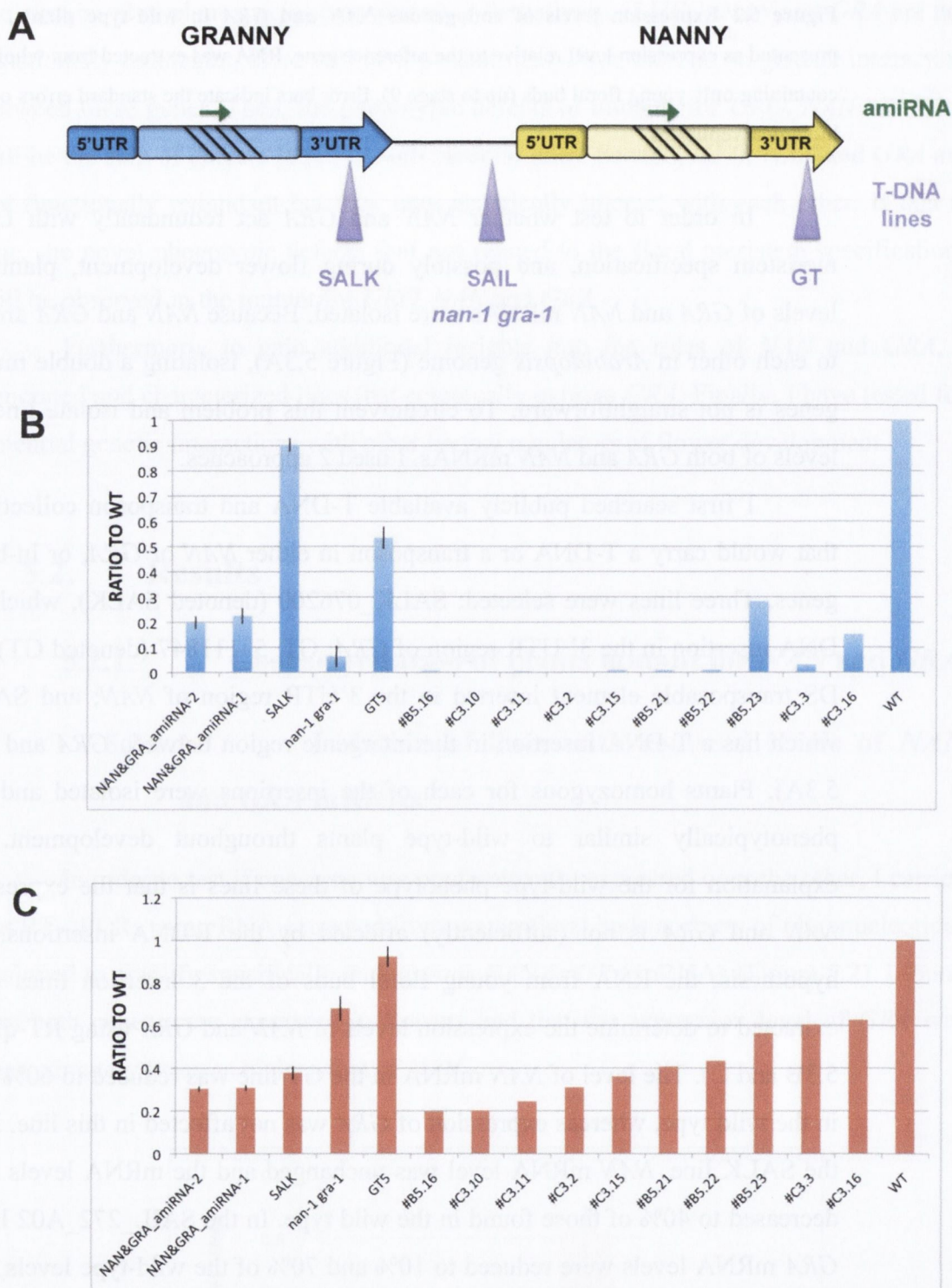


Figure 5.3 Characterization of lines mutant for *NAN* and *GRA*. (A) Schematic representation of the *GRA* and *NAN* genomic locus including the positions of the T-DNAs and the transposon in the lines used in this study. The relative position of the sequences targeted by the NAN&GRA_{amiRNA} construct are indicated by green arrows (not to scale). Hatched areas indicate the conserved regions of *GRA* and *NAN*. mRNA levels of (B) *NAN* (blue bars) and (C) *GRA* (red bars) in plants carrying the 35S_{pro}:NAN&GRA_{amiRNA} transgene, in different T-DNA and transposon insertion lines, as well as in ten independent *nan-1 gra-1* 35S_{pro}:NAN&GRA_{amiRNA} lines. For (B) and (C), RNA was extracted from whole inflorescences that had been dissected to remove older flowers. The results are shown relative to the level of *NAN* and *GRA* mRNA in wild-type plants, respectively. Only one replicate was carried out except for

the NAN&GRA_amiRNA, SALK, *nan-1 gra-1*, GT and wild-type plants. For these lines 3 biological replicates were carried out and error bars indicate the standard errors of the means. SALK: SALK_076200; GT: GT_5_111847; SAIL: SAIL_272_A02 (denoted *nan-1 gra-1* hereafter).

In order to increase the chances of obtaining plants that had reduced *NAN* and *GRA* function simultaneously, I also designed an amiRNA targeting both mRNAs at the same time (Section 2.5.4.1). Such a construct could also be used to further knock down the mRNA levels of *GRA* and *NAN* in the *nan-1 gra-1* mutant background. The amiRNA designed (denoted NAN&GRA_{amiRNA}) was specific to conserved regions found in both *NAN* and *GRA* coding sequences (Figure 5.3A). First, the amiRNA was cloned and expressed in wild-type plants (*L-er*) under the control of the 35S promoter (Section 2.5.4.1). Nineteen independent primary transformants carrying the 35S_{pro}:NAN&GRA_{amiRNA} transgene were obtained, but none of them showed any phenotypic abnormalities. To test the efficiency of the NAN&GRA_{amiRNA}, I tested the levels of the *NAN* and *GRA* mRNA in 8 of these independent lines (data not shown). The best 2 lines expressing NAN&GRA_{amiRNA} (denoted NAN&GRA_{amiRNA}-1 and NAN&GRA_{amiRNA}-2) were chosen for further analysis (Figures 5.3B and C). In these 2 lines, a strong reduction of both *NAN* and *GRA* mRNA levels was observed, with only ~ 20% and ~ 30% of the wild-type levels remaining, respectively. This result indicates that the NAN&GRA_{amiRNA} used indeed targets efficiently the *GRA* and *NAN* mRNAs and line NAN&GRA_{amiRNA}-1 was chosen for further characterization.

Because the use of T-DNA or transposon insertion lines and the expression of an amiRNA did not yield plants with phenotypic defects and these plants still retained some level of *GRA* and *NAN* expression, I hypothesized that the absence of phenotypic abnormalities could be due to residual expression of *NAN* and *GRA*. In order to further decrease the levels of *NAN* and *GRA* expression, I combined the use of the *nan-1 gra-1* T-DNA insertion line with the expression of the NAN&GRA_{amiRNA} from the 35S promoter by super-transforming *nan-1 gra-1* homozygous plants using *Agrobacterium* carrying the 35S_{pro}:NAN&GRA_{amiRNA} transgene (Section 2.5.4.1). Fourteen *nan-1 gra-1* 35S_{pro}:NAN&GRA_{amiRNA} independent transformants were obtained but none of them showed any phenotypic abnormalities. To test whether this strategy had indeed led to a further reduction of *NAN* and *GRA* expression, I carried out RT-qPCR assays for 10 of the isolated lines in the T2 generation using young floral buds (Figures 5.3B and C). Two of the lines tested (B5.16 and C3.10) appear to have a further reduction in *NAN* and *GRA* mRNA levels, which had not been attained with either T-DNAs or the expression

of the *NAN&GRA_{amiRNA}* alone. In particular, *NAN* mRNA was not detectable anymore in these 2 lines.

In summary, I isolated 3 different types of lines that have reduced expression of *NAN* and/or *GRA*. These include T-DNA or transposon insertion lines, plants that express *NAN&GRA_{amiRNA}* in a wild-type background and finally lines that express *NAN&GRA_{amiRNA}* in the *nan-1 gra-1* mutant background. Although the latter lines showed the lowest expression of *NAN* and *GRA*, they were only obtained very recently, and hence could not be used at early stages of the project to dissect the function of *NAN* and *GRA*. The results presented below were therefore obtained using the *nan-1 gra-1* and the *35S_{pro}:NAN&GRA_{amiRNA}-1* lines, which allowed the use of 2 very different methods to decrease the levels of both *NAN* and *GRA* mRNAs. The 2 mutant lines used in this study present different advantages and problems. For example, in *nan-1 gra-1* plants, *GRA* is expressed up to 70% of the level found in wild-type plants (Figure 5.3C) and it is possible that such a low down-regulation of *GRA* is not sufficient to observe full or any effect of *NAN* and *GRA*'s disruption because of their potential functional redundancy. Although the *35S_{pro}:NAN&GRA_{amiRNA}* lines showed higher down-regulation of *GRA* (Figure 5.3C) compared to *nan-1 gra-1* line, the use of an amiRNA could lead to other artefacts, such as the down-regulation of genes encoding other F-box/Kelch repeats proteins (*i.e.* off-target problems). Finally, the 2 lines are in different accessions, the *35S_{pro}:NAN&GRA_{amiRNA}-1* line being in the *L-er* background, while *nan-1 gra-1* is in the Col-0 accession. The use of different accessions was useful when analyzing the results of genetic interactions with other mutants that were either in the *L-er* or the Col-0 backgrounds.

5.2.1.2. Detailed phenotypic characterization of plants with reduced levels of *NAN* and *GRA* mRNAs

I carried out a detailed phenotypic characterization of the vegetative and reproductive stages of development of *nan-1 gra-1*, as well as the *35S_{pro}:NAN&GRA_{amiRNA}-1* and *nan-1 gra-1 35S_{pro}:NAN&GRA_{amiRNA}* lines, but I did not observe any phenotypic abnormalities during vegetative development or after flowering (data not shown). However, this characterization was carried out in continuous light conditions and it has been shown that some mutants (for example, *ufo-1* (Wilkinson and Haughn, 1995)) are more severely affected when grown in short-day conditions (8 hr of

light followed by 16 hr of dark). Therefore, I also characterized the *nan-1 gra-1* and *35S_{pro}:NAN&GRA_{amiRNA}-1* mutants in short-day conditions, but again I found no visible morphological defects during either the vegetative or the reproductive phases of development. The *nan-1 gra-1 35S_{pro}:NAN&GRA_{amiRNA}* double mutant was obtained too late, and hence this line could not be characterized on time under short-day conditions.

In sum, the mutant lines obtained for *NAN* and *GRA* did not show any phenotypic abnormalities. This could be due to the fact that the mRNA transcript of at least *GRA* is detected in all 3 lines and that the residual level of *NAN* and *GRA* expression in the tested mutant lines could still be sufficient to maintain the function of these genes. Furthermore, it is possible that there are additional genes that are functionally redundant with *NAN* and *GRA*. Finally, as mentioned above, another gene that could act redundantly with *NAN* and *GRA* is *UFO*. To address this possibility and test our initial hypothesis, I studied the genetic interactions between mutants for *NAN* and *GRA*, and different mutant alleles of *UFO*.

5.2.1.3. *NAN* and *GRA* act antagonistically to *UFO*

To test whether *UFO*, *NAN* and *GRA* interact genetically, I crossed the *nan-1 gra-1* and the *35S_{pro}:NAN&GRA_{amiRNA}-1* lines with different weak, intermediate and strong *ufo* mutant alleles (Table 5.2). Unfortunately, the *nan-1 gra-1 35S_{pro}:NAN&GRA_{amiRNA}* double mutant could not be included in this analysis, as it was obtained too late.

Table 5.2 Summary of the double mutants generated to study genetic interactions between *NAN*, *GRA* and *UFO*. Two photoperiod conditions were tested – continuous light and short days. To avoid working in a mixed genetic background, *nan-1 gra-1* was crossed into *ufo-1* or *ufo-13* (all in the Col-0 background), whereas the *35S_{pro}:NAN&GRA_{amiRNA}-1* line was crossed into *ufo-2* and *ufo-6*, which are in the L-*er* background. Due to time constraints, the *ufo-2 35S_{pro}:NAN&GRA_{amiRNA}-1* double mutant was not characterized in short-day conditions. ‘Yes’ indicates at least partial rescue of the corresponding *ufo* phenotype; n.d. – not determined.

Condition	ufo allele	Ecotype	Strength of ufo allele	Mutant for NAN&GRA	Rescue in double mutant?
Continuous light	ufo-6	L-er	weak	35S _{pro} :NAN&GRA _{amiRNA} -1	no
	ufo-13	Col-0	intermediate	nan-1 gra-1	yes
	ufo-1	Col-0	strong	nan-1 gra-1	yes
	ufo-2	L-er	strong	35S _{pro} :NAN&GRA _{amiRNA} -	yes

				1	
Short days	ufo-1	Col-0	strong	nan-1 gra-1	no
	ufo-2	L-er	strong	35S _{pro} :NAN&GRA _{amiRNA} -1 1	n.d.

Weak and intermediate *ufo* alleles were used because it is possible that the use of a strong *ufo* allele could mask the functions of *NAN* and *GRA*. The 35S_{pro}:NAN&GRA_{amiRNA}-1 line was crossed into the weak *ufo-6* mutant, which produces flowers with a slightly reduced number of petals and stamens, as well as chimeric petal-stamen organs (Figure 5.4B). In the F2 generation of this cross, plants were genotyped for the *ufo-6* mutation and for the presence of the 35S_{pro}:NAN&GRA_{amiRNA} transgene. Plants that were homozygous for the *ufo-6* mutation and that also carried at least one copy of the 35S_{pro}:NAN&GRA_{amiRNA} transgene were characterized. The *ufo-6* 35S_{pro}:NAN&GRA_{amiRNA}-1 double mutant plants were found to be undistinguishable from the *ufo-6* single mutant in all developmental aspects (data not shown).

In parallel, I crossed the *nan-1 gra-1* mutant with the intermediate allele *ufo-13*. This *ufo* mutant lacks organs in the second whorl whereas the number of stamens is not affected and, occasionally, petal/stamen chimeric organs are formed (Durfee et al., 2003) (Figure 5.4C). I was not able to develop a functional assay to genotype the *ufo-13* mutation, and hence carried out a phenotypic and segregation analysis in the F2 generation. Out of 119 plants that were characterized, only 2 types of phenotypes were observed: 96 plants (81% of the population) appeared wild type in all aspects of their development, whereas 23 plants (19% of the F2 population) showed the phenotypic abnormalities characteristic for the *ufo-13* mutant. If *UFO* and *NAN/GRA* interacted in a synergistic manner, one would expect that ~ 6.25% of the population (~ 7 plants) would show a phenotype that is different from that found in either of the 2 parental lines, which is not the case. Similarly, if *UFO* acted redundantly with *NAN/GRA*, more severe *ufo*-like or novel phenotypes would be expected in ~ 6.25% of the F2 population, which is not the case either. Another possibility would be that there is no genetic interaction between *ufo-13* and *nan-1 gra-1* (i.e. *UFO* and *NAN/GRA* act independently of each other and the *ufo-13 nan-1 gra-1* double mutant is phenotypically similar to the *ufo-13* parent), in which case 25% of the plants would show the *ufo-13* phenotype and 75% of the plants would appear wild type. Again, this is not in agreement with the observed ratios, as there is a higher proportion of plants with a wild-type phenotype (81%). One

possibility to explain this difference would be that a *ufo-13 nan-1 gra-1* triple mutant is phenotypically similar to the wild type, suggesting that decreasing the levels of *NAN* and *GRA* in a *ufo-13* mutant could rescue the *ufo-13* phenotype. Indeed, in this case, ~ 81% of the population should appear wild type, while ~ 19% of the population should be similar to the *ufo-13* parent. This result corresponds to the ratios observed. In order to confirm this observation, I analyzed the results of other crosses, which involved strong mutant alleles of *UFO*.

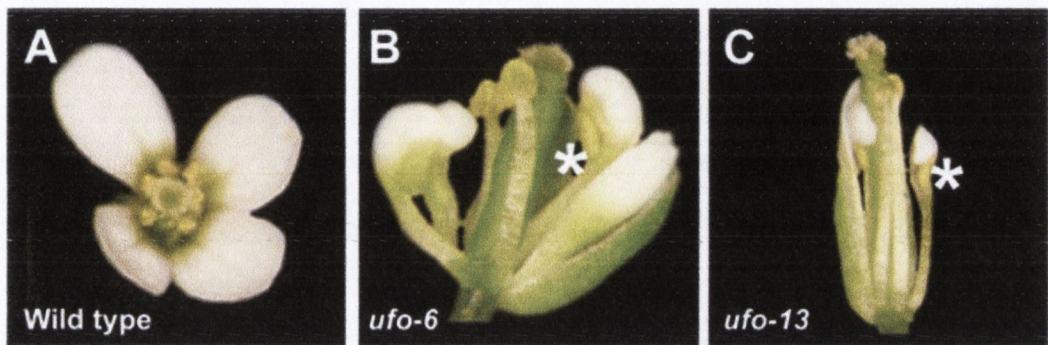


Figure 5.4 The floral phenotypes of weak and intermediate mutant alleles of *UFO*. (A) Top view of wild-type flower. (B) Phenotypic abnormalities observed in flowers of a *ufo-6* mutant. The number of organs in the second and third whorls is reduced and chimeric petal/stamen organs are produced (asterisk). (C) Floral defects found in flowers of a *ufo-13* mutant. The flower lacks petals in the second whorl and petaloid stamens are found in the third whorl (asterisk).

First, I crossed the *nan-1 gra-1* into the strong *ufo-1* mutant. In this mutant allele of *UFO*, the number of petals and stamens is severely reduced and mosaic organs are frequently produced in the second and third whorls (Figure 5.5A) (Wilkinson and Haughn, 1995). In the F₂ generation of this cross, I isolated lines homozygous for all mutations (noted *ufo-1 nan-1 gra-1*) and characterized them in detail. In these *ufo-1 nan-1 gra-1* triple mutant plants, the *ufo-1* mutant phenotype appeared to be partially rescued (Figures 5.5 B-C). However, because the phenotype of different flowers even within the same plant carrying the *ufo-1* allele varied considerably, in order to determine if the differences observed between the *ufo-1* and the *ufo-1 nan-1 gra-1* mutants were statistically significant, I quantified each type of organ produced in both genotypes and in wild-type plants (Figure 5.5D). For that purpose, 20 flowers per plant and per genotype were examined, and at least 2 plants were used. I then carried out a T-test analysis and considered differences between *ufo-1* and *ufo-1 nan-1 gra-1* as significant if the *P*-value was below 0.05 (Figure 5.5D). The results of this analysis indicated that there was indeed a partial rescue of the *ufo-1* phenotype in *ufo-1 nan-1 gra-1* plants, in

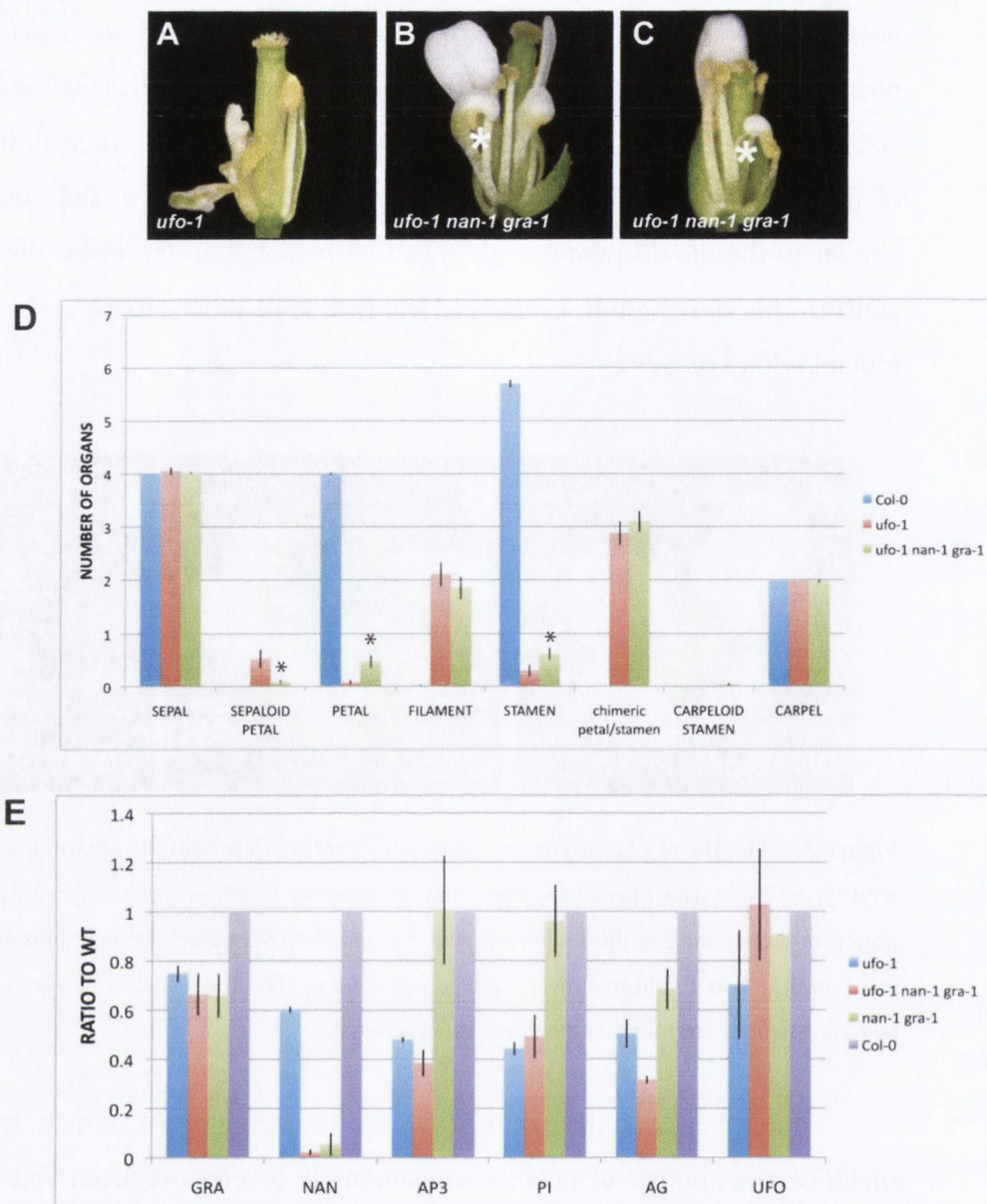


Figure 5.5 Characterization of the *ufo-1 nan-1 gra-1* double mutant. (A) Floral phenotype of a *ufo-1* mutant. The phenotype is variable within flowers of the same plant, but in general, flowers have a reduced number of petals and stamens. In the flower shown, stamens did not produce pollen. (B) Partial rescue of the *ufo-1* phenotype in the *ufo-1 nan-1 gra-1* triple mutant. Two wild-type petals, 2 chimeric petal-stamen organs (asterisk) and 2 pollen-producing stamens are visible. (C) Floral defects in the *ufo-1 nan-1 gra-1* triple mutant. One petal, one chimeric petal/stamen organ (asterisk) and 3 wild-type stamens were observed. (D) Quantification of the different organ types in flowers of *ufo-1*, *ufo-1 nan-1 gra-1* and Col-0. Twenty flowers per plant were scored in at least 2 plants of each genotype. Averages and standard errors are shown. Statistical difference between *ufo-1 nan-1 gra-1* and *ufo-1* was calculated using a Student's *t* test, with $P < 0.05$ indicated by asterisks. (E) Results of RT-qPCR experiments for *ufo-1*, *nan-1 gra-1* and *ufo-1 nan-1 gra-1* mutants and wild-type plants. Lines were tested for the levels of *GRA*, *NAN*, *AP3*, *PI*, *AG* and *UFO* mRNA levels. The results are presented relative to the levels of the respective mRNA in wild-type plants. RNA was extracted from whole inflorescences that had been dissected to enrich for young flower buds. Results are the means of 2 biological replicates and standard errors are shown. Plants from (A-C) are representative examples of observed phenotypes.

particular for defects concerning petals and stamens. The fact that the rescue is only partial may be due to residual expression of *NAN* and *GRA* in the *nan-1 gra-1* mutant.

To investigate further the potential genetic interaction between *UFO* and *GRA/NAN*, I tested the *ufo-1* and *nan-1 gra-1* single mutants and the *ufo-1 nan-1 gra-1* double mutant for expression levels of genes known to be involved in petal and stamen development, as well as *NAN*, *GRA* and *UFO*. To this end, I extracted RNA from young floral buds, synthesized cDNA and carried out qPCR analysis using sets of oligonucleotides designed specifically to amplify the different genes. First, I checked the expression level of *UFO* in the different genetic backgrounds and found that it does not change significantly compared to the wild type. Next, I examined these mutants for the expression levels of *NAN* and *GRA* (Figure 5.5E) and found that these 2 genes were slightly down-regulated in the *ufo-1* mutant. However, it is unclear if this is due to the fact that flowers of the *ufo-1* mutant lack petals and stamens, in which *NAN* and *GRA* could be expressed, or if this result indicates that *UFO* could be involved in the regulation of *NAN* and *GRA* expression.

Because it appears that reducing the levels of *NAN* and *GRA* rescues the *ufo-1* mutant phenotype, I tested if the expression level of genes involved in petal and/or stamen development is restored in *ufo-1 nan-1 gra-1* triple mutants. These genes include the B-function transcription factors *AP3* and *PI* (Section 1.3.2), as well as the C-function gene *AG*. Because petal and stamen development is partially restored in *ufo-1 nan-1 gra-1* triple mutants, I expected that expression of these 3 genes would be higher in *ufo-1 nan-1 gra-1* flowers compared to those of *ufo-1*, but this was not the case. This result is surprising, given that it is known that expression of *AP3* and *PI* is required to rescue the mutant phenotype of a *ufo-1* mutant (Krizek and Meyerowitz, 1996). A possible explanation for this unexpected result is that decreased levels of *NAN* and *GRA* mRNAs could result in higher expression of B-function genes only at early stages of flower development, when *AP3* and *PI* are required to specify petal and stamen primordia (~ stage 3). Although I dissected the inflorescences to remove older flower buds, the tissue collected is still enriched for later stages of development than early ones such as stage 3. It is hence possible that the rescue of *AP3* and *PI* expression at early stages of flower development is masked in the tissue used for these RT-qPCR experiments.

The results obtained with *AG* indicate that its expression is reduced in not only *ufo-1* flowers, but also in *nan-1 gra-1* flowers, suggesting that these 2 F-box proteins could be involved in the regulation of *AG* activity, although this does not result in

phenotypic abnormalities. Furthermore, it appears that *AG* expression is down-regulated further in *ufo-1 nan-1 gra-1* flowers, compared to each of the parental lines, indicating that *UFO* and *NAN/GRA* might be involved in controlling *AG* expression. However, additional biological replicates would be required in order to confirm this result, because the difference is not very strong.

In order to determine if reducing the levels of *NAN* and *GRA* could indeed rescue a *ufo* mutant, I also crossed the *35S_{pro}:NAN&GRA_{amiRNA}-1* line with the *ufo-2* mutant (Figure 5.6C). Flowers from the latter often lack petals and stamens. Moreover, filaments, aerial ovules and carpeloid stamens are frequently observed. In the F₂ generation of this cross, I isolated lines homozygous for the *ufo-2* mutation that also carried at least one copy of the *35S_{pro}:NAN&GRA_{amiRNA}* transgene. Unlike the *ufo-2* single mutant, these plants were fertile. However, similarly to the *ufo-2* mutant, I observed phenotypic variability for different flowers formed by a single plant. These *ufo-2 35S_{pro}:NAN&GRA_{amiRNA}* lines hence showed 3 types of floral phenotypes: (i) flowers that were indistinguishable from those found in the *ufo-2* parental line (data not shown); (ii) flowers producing some petals and more than 2 carpels that did not fuse properly (Figure 5.6D), as well as chimeric petal-stamen organs and filaments (Figures 5.6E and D); (iii) flowers with wild-type petals, stamens and carpels. The results of this genetic interaction hence confirm that reducing expression of *NAN* and *GRA* rescues a *ufo* mutant.

The strong *ufo-1* and *ufo-2* mutant alleles exhibit a less variable phenotype when plants are grown in short-day conditions. I hence also attempted to characterize *ufo-1 nan-1 gra-1* and *ufo-2 35S_{pro}:NAN&GRA_{amiRNA}-1* plants in short-day conditions to determine whether rescue of the *ufo* mutant phenotype was more consistent under these light conditions. However, the results I obtained were different than expected, as the *ufo-1 nan-1 gra-1* plants were indistinguishable from the *ufo-1* single mutants, when grown in short-day conditions. Unfortunately, I did not have sufficient time to characterize the *ufo-2 35S_{pro}:NAN&GRA_{amiRNA}-1* plants under these light conditions.

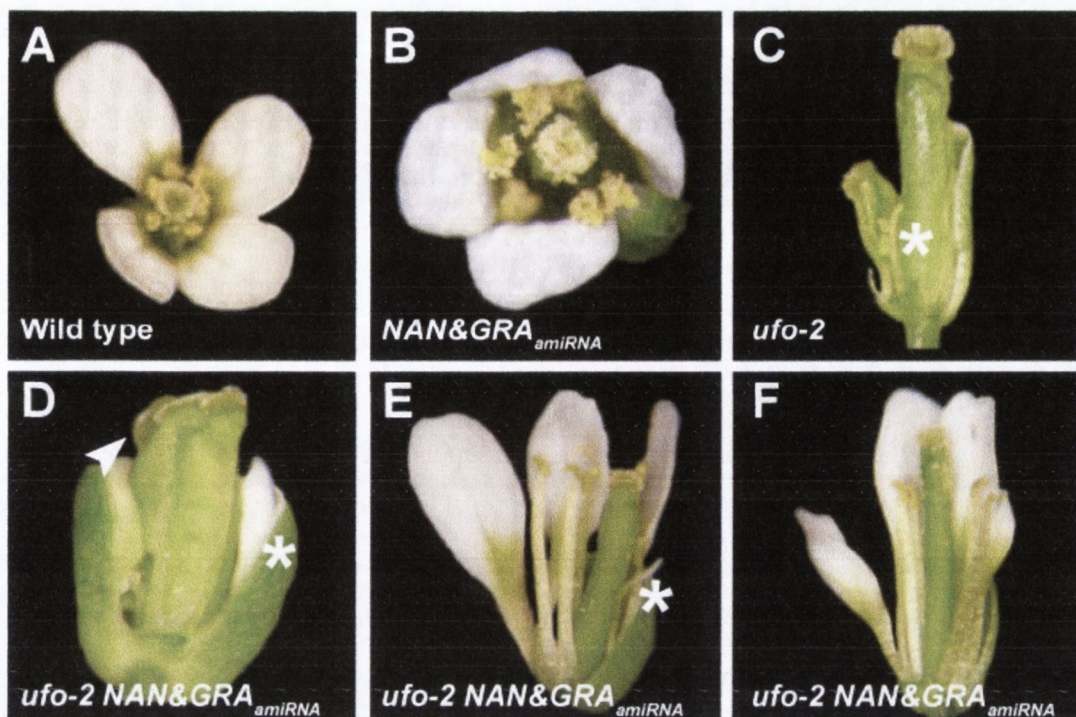


Figure 5.6 Phenotypic characterization of *ufo-2* $35S_{pro}:NAN\&GRA_{amiRNA-1}$ double mutants. (A) A wild-type flower. (B) A representative flower formed by $35S_{pro}:NAN\&GRA_{amiRNA-1}$ plants. These flowers are undistinguishable from wild-type ones. (C) A *ufo-2* flower. The flower lacks petals and stamens. Filaments (asterisk) and aerial ovules are produced. (D) Phenotypic defects found in a *ufo-2* $35S_{pro}:NAN\&GRA_{amiRNA-1}$ double mutant. A small petal is produced in the second whorl (asterisk). More than 2 carpels are formed in the center of the flower (arrowhead). (E) Partial rescue of the *ufo-2* floral phenotype in a *ufo-2* $35S_{pro}:NAN\&GRA_{amiRNA-1}$ double mutant plant. Three wild-type petals are formed in the second whorl and 3 wild-type stamens are present in the third whorl. Filaments instead of stamens are still formed (asterisk). (F) Rescue of the floral phenotype of the strong *ufo-2* allele. Four petals and 5 stamens (only 4 visible on this picture) were produced. Pictures are representative examples of the phenotypes observed in the different genetic backgrounds.

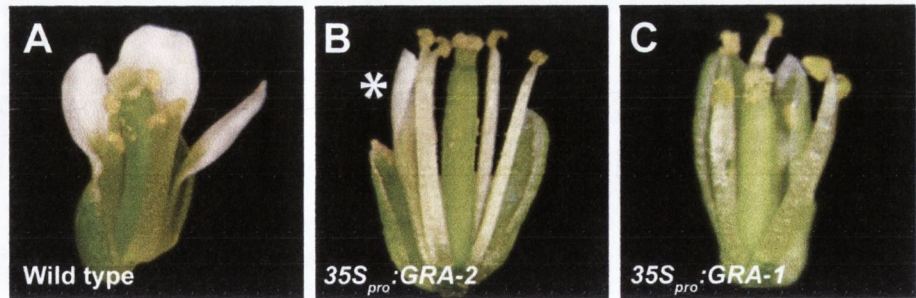
In summary, different mutant alleles of *UFO* (weak, intermediate or strong) were crossed into the *nan-1 gra-1* mutant or lines that express an amiRNA specific for *NAN* and *GRA* (Table 5.2). Contrary to our expectations, based on the hypothesis that *UFO* and *NAN/GRA* might act in a functionally redundant manner in the regulation of the floral meristem identity or of flower development, I found that reducing the levels of *NAN* and *GRA* in some *ufo* mutants leads to a partial rescue of the *ufo* mutant phenotype, at least in continuous light. Hence, overall, the data obtained suggest that *NAN* and *GRA* might act antagonistically to *UFO* in the regulation of petal and stamen development.

5.2.2. Characterization of lines ectopically expressing *GRA*

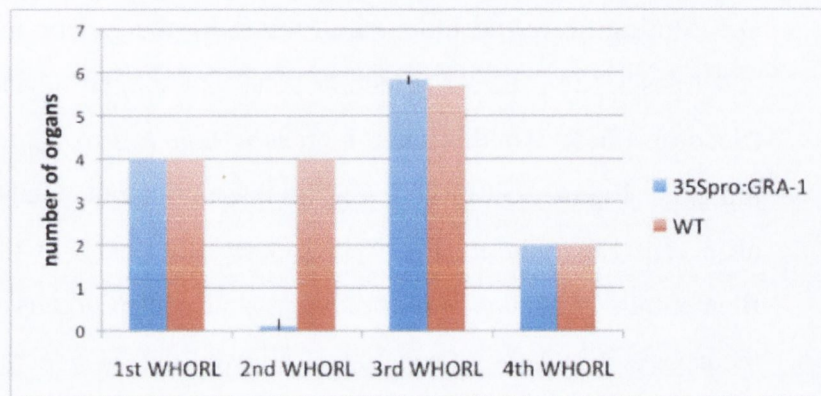
In order to shed more light on the functions of *NAN* and *GRA*, I generated plants ectopically expressing *GRA*. To this end, the coding region of *GRA* was placed under the control of the 35S promoter and the construct was introduced in wild-type plants (accession Col-0) (Section 2.5.4.2). Thirteen independent primary transformants were isolated and characterized in the T2 generation. Although 9 lines were indistinguishable from the wild type, 4 lines showed defects during flower development. More specifically, plants from these 4 independent lines produced flowers with a reduced number of petals (Figures 5.7B and C). The strongest 35S_{pro}:*GRA*-1 line was selected for a more detailed characterization and floral organ quantification (Figure 5.7D). On average, it was found that plants of this specific line produced only 0.12 petals per flower, whereas the first, third and fourth whorls were unaffected.

I then tested if *GRA* was indeed overexpressed in the different 35S_{pro}:*GRA* lines and attempted to determine if there was a correlation between the level of *GRA* expression and the severity of the phenotype observed. To this aim, I carried out RT-qPCR using RNA extracted from 10-day-old seedlings and oligonucleotides designed to amplify specifically *GRA* (Figure 5.7E). Four independent lines were tested and all showed higher levels of *GRA* expression compared to the wild type. *GRA* mRNA levels in lines 35S_{pro}:*GRA*-1 and 35S_{pro}:*GRA*-2, which showed a reduced number of petals were ~ 180 and ~ 50 times higher, respectively, compared to the wild type. In contrast, in lines 35S_{pro}:*GRA*-3 and 35S_{pro}:*GRA*-4, which were indistinguishable from wild-type plants, *GRA* expression was only ~ 20 times higher compared to the wild type. This preliminary result suggests that the observed phenotype is indeed related to the over-expression of *GRA*.

In summary, ectopic expression of *GRA* specifically affects the second whorl and results in a reduction of petal number. Together with the results of our genetic interaction between *ufo* mutants and plants with reduced levels of *NAN* and *GRA*, this data suggest that *GRA*, and possibly *NAN* as well, are involved in the regulation of petal development in an antagonistic manner to *UFO*.



D



E

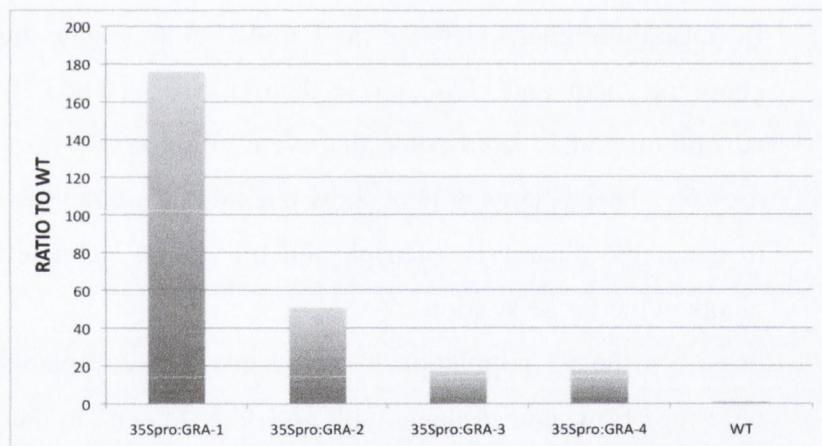


Figure 5.7 Characterization of lines ectopically expressing *GRA*. (A) A wild-type flower. One sepal and one petal were removed. (B) Floral defects observed in a *35S_{pro}:GRA-2* line. Only one petal was formed (asterisk). The first, third and fourth whorls were not affected by *GRA* ectopic expression. One sepal and 2 stamens were removed for better visualization of the phenotype. (C) Flower from the *35S_{pro}:GRA-2* line that does not form any petals. Sepals, stamens and carpels were not affected. One sepal was removed for better visualization of the inner organs. (D) Quantification of different organ types observed in flowers of the *35S_{pro}:GRA-1* line. Twenty flowers per plant were scored in at least 10 plants. The mean number of organs per flower is shown. Error bars represent standard error. (E) RT-qPCR experiments for 4 independent *35S_{pro}:GRA* plants. Lines were tested for the level of *GRA* mRNA. The results are presented relative to the level *GRA* mRNA in wild-type plants. RNA was extracted from 10-day-old seedlings. No error bars are presented because the values were obtained from technical duplicates only.

5.2.3. Dissection of the genetic interactions between *NAN* and *GRA* mutants, and mutant plants for B function genes

The phenotypic characterization of mutants with reduced expression levels of *UFO*, *NAN* and *GRA* suggests that *NAN*/*GRA* participate in the specification and/or development of petals and stamens. To investigate further the potential role of *NAN* and *GRA* during flower development, I tested for the genetic interaction between *NAN*, *GRA* and transcription factors directing petal and stamen development, namely *AP3* and *PI* (Section 1.3.2). To this end, I crossed *nan-1 gra-1* into the strong *ap3-3* and *pi-1* mutants (Figures 5.8B and C). In these mutants petal-to-sepal transformations are observed (Bowman et al., 1991; Jack et al., 1992). In the third whorl carpels and/or filamentous structures are produced and these organs are often fully or partially incorporated into the gynoecium. I allowed the F1 generation to self and analyzed the phenotype of plants in segregating F2 populations. Among these plants, I found some that had floral defects different from those present in either of the parental lines. Because these plants were sterile, I could not propagate them into the next generation to genotype them and characterize them further. Hence, I genotyped plants in the F2 generation and isolated some that were either *nan-1 gra-1 pi-1/+* or *nan-1/+ gra-1/+ ap3-3/+*. These plants did not show phenotypic alterations. In order to characterize more in detail the phenotype of triple mutant plants, I characterized the offspring of these plants in the F3 generation.

In the F3 population of *nan-1 gra-1 pi-1/+* plants, I found plants that formed wild-type flowers and plants with *pi-1*-like flowers, in that in these flowers sepals were produced in the first and second whorls (data not shown). However, no organs were found in the presumptive third whorl and the gynoecium showed novel phenotypic abnormalities that are not present on the *pi-1* mutant. Indeed, the basal part of the gynoecium did not form properly and was very narrow, which enhanced the bulging appearance of the apical part of the gynoecium (Figure 5.8 G-I). Moreover, aerial ovules were also observed (Figures 5.8I). I genotyped 22 plants, 13 of which appeared wild type and 9 of which showed the mutant phenotype described above. I found that all plants with the novel mutant phenotype were *nan-1 gra-1 pi-1* triple mutants, whereas among the plants that appeared wild type, 9 were *nan-1 gra-1 pi-1/+* and 4 were *nan-1 gra-1*. These genotyping results hence confirm that the novel mutant phenotype correlates with plants mutant for the 3 genes.



Figure 5.8 Phenotypic comparison of the *nan-1 gra-1*, *pi-1*, *ap3-3* single mutants and the *nan-1 gra-1 ap3-3* and *nan-1 gra-1 pi-1* double mutants. (A) The flower of the *nan-1 gra-1* single mutant. Note organs with no phenotypic defects. Two sepals and two petals were removed. (B) The phenotypic defects found in the fourth whorl in the *pi-1* mutant. The gynoecium is abnormal in size and shape due to fusion of supernumerary carpels (asterisk). (C) Floral defects observed in the *ap3-3* mutant. In the second whorl petals are transformed into sepals. Carpels are produced at the expense of stamens and they are incorporated into the gynoecium (asterisk). Adapted from (Wuest et al., 2012). (D-F) The gynoecia of the *nan-1 ap3-1* double mutants. The lower parts of styles are narrowed (arrowheads). The numbers of carpels are reduced compared to the *ap3-3* single mutant. (D-E) The filaments are partially fused with the gynoecia (asterisks). (G-I) Phenotypic defects observed in the fourth whorl of the *nan-1 gra-1 pi-1* double mutants. The bases of gynoecia are narrowed (arrowheads). The upper parts of styles are bulged (asterisks). The number of carpels is reduced compared to the *pi-1* single mutants. (I) Additionally, the aerial ovules are produced. The outer organs were removed for better visualization of the phenotype.

In the case of *nan-1/+ gra-1/+ ap3-3/+*, I identified plants with 3 different phenotypes in the F3 population: 50% of the plants did not show any phenotypic abnormalities, 25% were indistinguishable from the *ap3-3* single mutant and, finally, 25% of the plants showed phenotypic defects that were different from those observed in the parental lines (Figures 5.8D-F). More specifically, in the first and second whorls only sepals were produced (data not shown), and in the presumptive whorl 3, extra carpels or filaments were observed, but they were fused to the central gynoecium (Figure 5.8D-E). Furthermore similarly to what was observed in *nan-1 gra-1 pi-1* plants, the basal part of the gynoecium did not develop properly and was narrow, whereas the apical part of the gynoecium was enlarged.

In summary, combining the *nan-1 gra-1* double mutant with plants mutant for *AP3* or *PI* led to the appearance of novel phenotypes, that are absent from both parental lines. This result would hence suggest a synergistic interaction between *NAN/GRA* and *AP3* or *PI*, indicating that *NAN/GRA* and the B-function genes might be controlling similar aspects of flower development.

5.3. Discussion and future work

Based on the phenotype of mutants for the ortholog of *UFO* in other plant species than *Arabidopsis*, I hypothesized that the *Arabidopsis* genome might encode additional F-box proteins that act redundantly with *UFO* in the regulation of floral meristem identity, and possibly during flower development as well. Using gene expression correlation analysis, I identified an F-box protein, *NAN*, with similar expression pattern as *UFO*. Protein sequence similarities searches revealed that *GRA* was a paralog of *NAN*, and hence both proteins were likely to be functionally redundant.

♦ *NAN/GRA act antagonistically to UFO*

In order to test if these 2 F-box proteins could be at least partially functionally redundant with *UFO*, I isolated plants that had reduced levels of both *NAN* and *GRA* mRNAs. Although the resulting plants did not have any mutant phenotype (possibly because they are not null alleles), I tested for their genetic interaction with different mutant alleles of *UFO*. This analysis revealed, that contrary to our initial hypothesis, *NAN* and *GRA* are likely to act in an antagonistic manner to *UFO*. Indeed, decreasing the levels of *NAN* and *GRA* mRNAs partially rescued the *ufo* mutant phenotype found in

the *ufo-1*, *ufo-2* and *ufo-13* mutant alleles. However, no rescue of the *ufo-6* mutant phenotype was observed in these plants.

The *ufo-6* mutant is the weakest of the different mutant alleles of *UFO* used in this study (the number of petals and stamens is only mildly affected), suggesting that the lack of rescue by the *35S_{pro}:NAN&GRA_{amiRNA}* transgene could be due to sufficient *UFO* activity remaining in this mutant background, masking the effect of a down-regulation of *NAN* and *GRA*. Alternatively, the absence of rescue could be related to the nature of the mutation in the *ufo-6* mutant allele. Indeed, whereas mutations in *ufo-1*, *ufo-2* and *ufo-13* all result in a deletion of the C-terminal region of *UFO* (after residues 285, 262 and 390, respectively; *UFO* has 442 residues in total), the *ufo-6* mutation results in the substitution of Pro 299 into Leu, which has been shown to reduce the interaction of the resulting protein with *ASK1* (Durfee et al., 2003). In contrast, the interaction of *UFO* with *ASK1* in the *ufo-13* mutant is not altered (Durfee et al., 2003). Although it is not known whether *UFO* can interact with *ASK1* in the *ufo-1* and *ufo-2* mutants, this observation suggests that either the C-terminal region of *UFO* needs to be deleted in order for a reduced level of *NAN* and *GRA* to rescue the mutant, or that interaction of *UFO* with *ASK1* needs to be maintained.

The other surprising result I found is that rescue of the *ufo* mutant phenotype could depend on the photoperiod. Indeed, although I could observe rescue of the *ufo* mutant phenotype in *ufo-1 nan-1 gra-1* plants grown in continuous light, there was no rescue under short-day conditions. Because the *ufo-1* phenotypic defects are much stronger in short-day conditions, it is possible that the partial reduction of the *GRA* and *NAN* expression levels in the *nan-1 gra-1* mutant is no longer sufficient to rescue the *ufo* mutant phenotype. Alternatively, another pathway regulating petal and stamen development, which is not dependent on *GRA/NAN* could be active under the short-day conditions.

Finally, the *ufo-2 35S_{pro}:NAN&GRA_{amiRNA-1}* double mutants produced supernumerary carpels in the fourth whorl (Figure 5.6D) and this phenotype is not normally found the *ufo-2* mutant, which is a strong mutant allele of *UFO*. This phenotype was not observed in all flowers or plants, possibly because the phenotype in the *ufo-2* mutant is itself very variable even within flowers of the same plant. This phenotype suggests that reducing the expression levels of *NAN* and *GRA* in a *ufo-2* background also affects the fourth whorl, suggesting that *NAN/GRA* might have a role in the development of this whorl. Moreover, I found that the C-function gene *AG*, which specifies carpels and is required to prevent indeterminacy in the flower, is down-

regulated in *nan-1 gra-1* and *ufo-1 nan-1 gra-1* mutants. Hence the phenotype observed could be due to a partial loss of determinacy in the flower. However, this possibility needs to be further investigated.

Because decreasing the levels of *NAN/GRA* in a *ufo-2* mutant background led to rescue of the *ufo* mutant phenotype, I assumed that the expression of *AP3* and *PI* had been restored in triple mutant plants. However, this did not appear to be the case when I carried out RT-qPCRs on these genes. A possible explanation for this result is that *NAN/GRA* act only at early stages of flower development, when second and third whorl organs are being specified, but these stages are under-represented in the tissue collected for the RNA extraction, which might have masked a possible role of *NAN/GRA* in the regulation of *AP3* and/or *PI* expression. In order to test this possibility, I have crossed the *ufo-1 nan-1 gra-1* triple mutant into lines that express *AP3-GFP* and *PI-GFP* fusions under the control of the respective endogenous promoter (Wuest et al., 2012) and I will use confocal microscopy to characterize the expression pattern of *AP3-GFP* and *PI-GFP* in *ufo-1 nan-1 gra-1*, *ufo-1* and *nan-1 gra-1* mutant plants throughout flower development.

In sum, the interaction of *nan-1 gra-1* with different mutant alleles of *ufo* suggest that *NAN/GRA* act antagonistically to *UFO* during flower development, and are negative regulators of petal and stamen development.

♦ *NAN/GRA act as negative regulators of petal and stamen development*

In order to gain further understanding on the role of *NAN/GRA*, I generated plants that ectopically express *GRA* from the 35S promoter. I found that the resulting plants formed flowers that lacked petals, but had normal stamens. This result hence confirms that at least *GRA* acts as a negative regulator of petal development, through a mechanism that remains to be determined.

The genetic interaction of *nan-1 gra-1* with different mutant alleles of *UFO*, also implies that *NAN/GRA* should be involved in stamen development, however, this type of organ was not affected in 35S_{pro}:*GRA* flowers. Possible explanations for this result are that petals might be more sensitive to *GRA* and/or *NAN* activities. The appearance of a mutant phenotype in stamens might require higher levels of *GRA* and/or *NAN*. Alternatively, the presence of *UFO* in the third whorl primordia could mask the activity of *GRA*, and possibly *NAN* as well. In order to address these possibilities, I am

currently generating plants that express *NAN* from the 35S promoter and will characterize them in detail.

♦ *Interaction with B-function genes*

Because *NAN/GRA* appear to be involved in regulating petal and stamen development, I crossed the *nan-1 gra-1* double mutant with strong mutant alleles of *AP3* and *PI*. The resulting triple mutant plants formed flowers that had novel phenotypes, in that the basal part of the gynoecium did not develop properly and was narrow. This result suggests that *NAN/GRA* might also be involved in the development of the fourth whorl, although this role is only revealed when *AP3* and *PI* are inactive, thus providing a sensitized background to reveal this function. It is possible that this interaction is also related to the fact that *AG* appears to be down-regulated in *nan-1 gra-1* flowers, but this hypothesis needs to be further investigated.

♦ *Future experiments*

In order to further understand the role of *NAN/GRA* during flower development, I have crossed the *nan-1 gra-1* mutant with other mutant for genes known to be involved in petal, stamen and carpels development (Table 5.3). I will then characterize the resulting triple mutants.

Table 5.3 Summary of other mutants crossed into the *nan-1 gra-1* mutant. The name of the genes, their function and the mutant allele used are indicated. To test for potential genetic interactions, mutants for these genes were crossed into the *nan-1 gra-1* double mutant. Due to time limitations, the plants in the F2 generation were not characterized in this work.

Gene	Known functions	Mutant allele	Reference
<i>LFY</i>	Controlling of floral morphogenesis, including organ number and identity	<i>lfy-2</i>	(Schultz and Haughn, 1991; Weigel et al., 1992)
<i>PERIANTHIA (PAN)</i>	Determination of floral organ number	<i>pan-3</i>	(Chuang et al., 1999)
<i>RABBIT EARS (RBE)</i>	Maintaining boundaries between petal primordia	<i>rbe-1</i>	(Takeda et al., 2004)
<i>SUPERMAN (SUP)</i>	Control of the boundary between the third and fourth whorls	<i>sup-5</i>	(Gaiser et al., 1995)
<i>AG</i>	Stamen and carpel	<i>35S_{pro}:LhG4-GR/</i>	(O'Maoileidigh et

	specification	$pOp(6x)_{pro}:AG_{amiRNA}$	al., 2013)
<i>LEUNIG</i> (<i>LUG</i>)	Regulation of gynoecium and ovule development	<i>lug-8</i>	(Conner and Liu, 2000)

In all the experiments that were presented, one underlying assumption is that *NAN* and *GRA* are functionally redundant. However, this hypothesis has not been tested properly at this stage. To do so, I intend to study the phenotype of lines overexpressing *NAN* from the 35S promoter and check if they have similar phenotypes as 35S_{pro}:*GRA* lines. Furthermore, I will test if *gra* and *nan* single mutants can also rescue the *ufo* mutant phenotype by crossing the SALK and GT lines with a *ufo-1* mutant. I will also test if re-introducing the genomic regions encoding either *GRA* or *NAN* can reverse the rescue observed in the *ufo-1 nan-1 gra-1* triple mutant. This experiment would not only allow us to test if *NAN* and *GRA* are indeed functionally redundant, but will also allow us to check that the rescue of the *ufo* mutant phenotype is indeed related to the reduced activities of *NAN/GRA*.

Finally, in order to have a better understanding of *NAN* and *GRA* functions, a post-graduate student in the Wellmer laboratory (Kamila Kwaśniewska) attempted to determine the expression domain of *GRA* in wild type inflorescences using *in situ* hybridization. However, she did not observe any signal, probably because *in situs* need further optimization for the protocol to be established. Hence, I am currently generating GUS reporter constructs that will allow me to determine the expression pattern of these genes.

In summary, I have identified 2 novel regulators of flower development, which appear to antagonize *UFO* activity in the regulation of petal and stamen development and act as negative regulators of the developmental program underlying the formation of these organs. The simplest model would be that *NAN/GRA* are responsible for the degradation of a positive regulator of petal and stamen development (Figure 5.9). In plants with decreased levels of *NAN* and *GRA*, this positive regulator accumulates and may be able to overcome the absence of *UFO* activity for the activation of *AP3* and/or *PI* expression at early stages of flower development. In contrast, upon overexpression of *GRA*, this positive regulator can no longer accumulate, which results in the absence of petals. It is hence possible that this positive regulator acts downstream of *UFO*, or in parallel with this F-box protein, and is also required for the activation of *AP3* and/or *PI* at early stages of development.

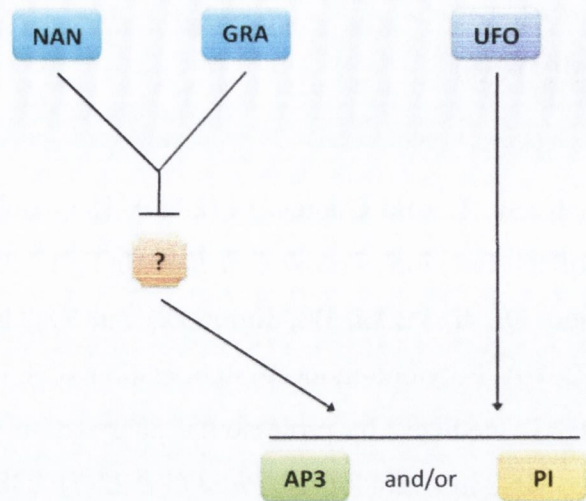


Figure 5.9 Model of the role of NAN/GRA during flower development. See full details in the main text. ‘?’ indicates a potential positive regulator of the developmental program underlying petal and stamen formation whose activity or stability is controlled by NAN/GRA.

6. References

- Adams, J., Kelso, R. and Cooley, L.** (2000) 'The kelch repeat superfamily of proteins: propellers of cell function', *Trends Cell Biol* 10(1): 17-24.
- An, F., Zhao, Q., Ji, Y., Li, W., Jiang, Z., Yu, X., Zhang, C., Han, Y., He, W., Liu, Y. et al.** (2010) 'Ethylene-induced stabilization of ETHYLENE INSENSITIVE3 and EIN3-LIKE1 is mediated by proteasomal degradation of EIN3 binding F-box 1 and 2 that requires EIN2 in Arabidopsis', *Plant Cell* 22(7): 2384-401.
- Andrade, M. A., Gonzalez-Guzman, M., Serrano, R. and Rodriguez, P. L.** (2001) 'A combination of the F-box motif and kelch repeats defines a large Arabidopsis family of F-box proteins', *Plant Mol Biol* 46(5): 603-14.
- Archer, C. T., Delahodde, A., Gonzalez, F., Johnston, S. A. and Kodadek, T.** (2008) 'Activation domain-dependent monoubiquitylation of Gal4 protein is essential for promoter binding in vivo', *J Biol Chem* 283(18): 12614-23.
- Ausin, I., Alonso-Blanco, C. and Martinez-Zapater, J. M.** (2005) 'Environmental regulation of flowering', *Int J Dev Biol* 49(5-6): 689-705.
- Baek, J. H., Mahon, P. C., Oh, J., Kelly, B., Krishnamachary, B., Pearson, M., Chan, D. A., Giaccia, A. J. and Semenza, G. L.** (2005) 'OS-9 interacts with hypoxia-inducible factor 1alpha and prolyl hydroxylases to promote oxygen-dependent degradation of HIF-1alpha', *Mol Cell* 17(4): 503-12.
- Bosu, D. R. and Kipreos, E. T.** (2008) 'Cullin-RING ubiquitin ligases: global regulation and activation cycles', *Cell Div* 3: 7.
- Bowman, J. L., Smyth, D. R. and Meyerowitz, E. M.** (1989) 'Genes directing flower development in Arabidopsis', *Plant Cell* 1(1): 37-52.
- Bowman, J. L., Smyth, D. R. and Meyerowitz, E. M.** (1991) 'Genetic interactions among floral homeotic genes of Arabidopsis', *Development* 112(1): 1-20.
- Burgering, B. M. and Kops, G. J.** (2002) 'Cell cycle and death control: long live Forkheads', *Trends Biochem Sci* 27(7): 352-60.
- Causier, B., Schwarz-Sommer, Z. and Davies, B.** (2010) 'Floral organ identity: 20 years of ABCs', *Semin Cell Dev Biol* 21(1): 73-9.
- Chae, E., Tan, Q. K., Hill, T. A. and Irish, V. F.** (2008) 'An Arabidopsis F-box protein acts as a transcriptional co-factor to regulate floral development', *Development* 135(7): 1235-45.

- Chuang, C. F., Running, M. P., Williams, R. W. and Meyerowitz, E. M.** (1999) 'The PERANTHIA gene encodes a bZIP protein involved in the determination of floral organ number in *Arabidopsis thaliana*', *Genes Dev* 13(3): 334-44.
- Ciechanover, A.** (1994) 'The ubiquitin-proteasome proteolytic pathway', *Cell* 79(1): 13-21.
- Ciechanover, A., Hod, Y. and Hershko, A.** (1978) 'A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes', *Biochem Biophys Res Commun* 81(4): 1100-5.
- Clough, S. J. and Bent, A. F.** (1998) 'Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*', *Plant J* 16(6): 735-43.
- Coen, E. S. and Meyerowitz, E. M.** (1991) The war of the whorls: genetic interactions controlling flower development *Nature*, vol. 353.
- Conner, J. and Liu, Z.** (2000) 'LEUNIG, a putative transcriptional corepressor that regulates AGAMOUS expression during flower development', *Proc Natl Acad Sci U S A* 97(23): 12902-7.
- Craft, J., Samalova, M., Baroux, C., Townley, H., Martinez, A., Jepson, I., Tsiantis, M. and Moore, I.** (2005) 'New pOp/LhG4 vectors for stringent glucocorticoid-dependent transgene expression in *Arabidopsis*', *Plant J* 41(6): 899-918.
- del Pozo, J. C. and Estelle, M.** (1999) 'The *Arabidopsis* cullin AtCUL1 is modified by the ubiquitin-related protein RUB1', *Proc Natl Acad Sci U S A* 96(26): 15342-7.
- Deshaies, R. J. and Joazeiro, C. A.** (2009) 'RING domain E3 ubiquitin ligases', *Annu Rev Biochem* 78: 399-434.
- Dharmasiri, N., Dharmasiri, S. and Estelle, M.** (2005) 'The F-box protein TIR1 is an auxin receptor', *Nature* 435(7041): 441-5.
- Dohmen, R. J., Stappen, R., McGrath, J. P., Forrova, H., Kolarov, J., Goffeau, A. and Varshavsky, A.** (1995) 'An essential yeast gene encoding a homolog of ubiquitin-activating enzyme', *J Biol Chem* 270(30): 18099-109.
- Downes, B. P., Stupar, R. M., Gingerich, D. J. and Vierstra, R. D.** (2003) 'The HECT ubiquitin-protein ligase (UPL) family in *Arabidopsis*: UPL3 has a specific role in trichome development', *Plant J* 35(6): 729-42.
- Durfee, T., Roe, J. L., Sessions, R. A., Inouye, C., Serikawa, K., Feldmann, K. A., Weigel, D. and Zambryski, P. C.** (2003) 'The F-box-containing protein UFO and AGAMOUS participate in antagonistic pathways governing early petal development in *Arabidopsis*', *Proc Natl Acad Sci U S A* 100(14): 8571-6.
- 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(6): 1349.

Etlinger, J. D. and Goldberg, A. L. (1977) 'A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes', *Proc Natl Acad Sci U S A* 74(1): 54-8.

Feng, S., Martinez, C., Gusmaroli, G., Wang, Y., Zhou, J., Wang, F., Chen, L., Yu, L., Iglesias-Pedraz, J. M., Kircher, S. et al. (2008) 'Coordinated regulation of *Arabidopsis thaliana* development by light and gibberellins', *Nature* 451(7177): 475-9.

Fu, X., Richards, D. E., Ait-Ali, T., Hynes, L. W., Ougham, H., Peng, J. and Harberd, N. P. (2002) 'Gibberellin-mediated proteasome-dependent degradation of the barley DELLA protein SLN1 repressor', *Plant Cell* 14(12): 3191-200.

Gagne, J. M., Downes, B. P., Shiu, S. H., Durski, A. M. and Vierstra, R. D. (2002) 'The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in *Arabidopsis*', *Proc Natl Acad Sci U S A* 99(17): 11519-24.

Gaiser, J. C., Robinson-Beers, K. and Gasser, C. S. (1995) 'The *Arabidopsis* SUPERMAN Gene Mediates Asymmetric Growth of the Outer Integument of Ovules', *Plant Cell* 7(3): 333-345.

Geng, F., Wenzel, S. and Tansey, W. P. (2012) 'Ubiquitin and proteasomes in transcription', *Annu Rev Biochem* 81: 177-201.

Glickman, M. H. and Ciechanover, A. (2002) 'The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction', *Physiol Rev* 82(2): 373-428.

Goldenberg, S. J., Cascio, T. C., Shumway, S. D., Garbutt, K. C., Liu, J., Xiong, Y. and Zheng, N. (2004) 'Structure of the Cnd1-Cul1-Roc1 complex reveals regulatory mechanisms for the assembly of the multisubunit cullin-dependent ubiquitin ligases', *Cell* 119(4): 517-28.

Goritschnig, S., Zhang, Y. and Li, X. (2007) 'The ubiquitin pathway is required for innate immunity in *Arabidopsis*', *Plant J* 49(3): 540-51.

Gray, W. M., Hellmann, H., Dharmasiri, S. and Estelle, M. (2002) 'Role of the *Arabidopsis* RING-H2 protein RBX1 in RUB modification and SCF function', *Plant Cell* 14(9): 2137-44.

Guo, H. S., Fei, J. F., Xie, Q. and Chua, N. H. (2003) 'A chemical-regulated inducible RNAi system in plants', *Plant J* 34(3): 383-92.

Hershko, A., Ciechanover, A., Heller, H., Haas, A. L. and Rose, I. A. (1980) 'Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis', *Proc Natl Acad Sci U S A* 77(4): 1783-6.

- Hershko, A., Heller, H., Elias, S. and Ciechanover, A.** (1983) 'Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown', *J Biol Chem* 258(13): 8206-14.
- 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(1): 172-8.
- Hobbie, L., McGovern, M., Hurwitz, L. R., Pierro, A., Liu, N. Y., Bandyopadhyay, A. and Estelle, M.** (2000) 'The axr6 mutants of Arabidopsis thaliana define a gene involved in auxin response and early development', *Development* 127(1): 23-32.
- Hochstrasser, M.** (1996) 'Protein degradation or regulation: Ub the judge', *Cell* 84(6): 813-5.
- Hobe, M** (2004) 'Kontrolle der Expression des UNUSUAL FLORAL ORGANS (UFO) Gens in Arabidopsis Thaliana.' PhD Thesis.
- Hotton, S. K. and Callis, J.** (2008) 'Regulation of cullin RING ligases', *Annu Rev Plant Biol* 59: 467-89.
- Hua, Z. and Vierstra, R. D.** (2011) 'The cullin-RING ubiquitin-protein ligases', *Annu Rev Plant Biol* 62: 299-334.
- Hua, Z., Zou, C., Shiu, S. H. and Vierstra, R. D.** (2011) 'Phylogenetic comparison of F-Box (FBX) gene superfamily within the plant kingdom reveals divergent evolutionary histories indicative of genomic drift', *PLoS One* 6(1): e16219.
- Huala, E. and Sussex, I. M.** (1992) 'LEAFY Interacts with Floral Homeotic Genes to Regulate Arabidopsis Floral Development', *Plant Cell* 4(8): 901-913.
- Huibregtse, J. M., Scheffner, M., Beaudenon, S. and Howley, P. M.** (1995) 'A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase', *Proc Natl Acad Sci U S A* 92(11): 5249.
- Ikedo, M., Mitsuda, N. and Ohme-Takagi, M.** (2009) 'Arabidopsis WUSCHEL is a bifunctional transcription factor that acts as a repressor in stem cell regulation and as an activator in floral patterning', *Plant Cell* 21(11): 3493-505.
- Ingram, G. C., Goodrich, J., Wilkinson, M. D., Simon, R., Haughn, G. W. and Coen, E. S.** (1995) 'Parallels between UNUSUAL FLORAL ORGANS and FIMBRIATA, genes controlling flower development in Arabidopsis and Antirrhinum', *Plant Cell* 7(9): 1501-10.
- Inigo, S., Giraldez, A. N., Chory, J. and Cerdan, P. D.** (2012) 'Proteasome-mediated turnover of Arabidopsis MED25 is coupled to the activation of FLOWERING LOCUS

T transcription', *Plant Physiol* 160(3): 1662-73.

Jack, T. (2004) 'Molecular and genetic mechanisms of floral control', *Plant Cell* 16 Suppl: S1-17.

Jack, T., Brockman, L. L. and Meyerowitz, E. M. (1992) 'The homeotic gene APETALA3 of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens', *Cell* 68(4): 683-97.

Jones, J. M. and Gellert, M. (2003) 'Autoubiquitylation of the V(D)J recombinase protein RAG1', *Proc Natl Acad Sci U S A* 100(26): 15446-51.

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(5974): 85-9.

Kawakami, T., Chiba, T., Suzuki, T., Iwai, K., Yamanaka, K., Minato, N., Suzuki, H., Shimbara, N., Hidaka, Y., Osaka, F. et al. (2001) 'NEDD8 recruits E2-ubiquitin to SCF E3 ligase', *EMBO J* 20(15): 4003-12.

Kepinski, S. and Leyser, O. (2005) 'The *Arabidopsis* F-box protein TIR1 is an auxin receptor', *Nature* 435(7041): 446-51.

Krizek, B. A. and Meyerowitz, E. M. (1996) 'The *Arabidopsis* homeotic genes APETALA3 and PISTILLATA are sufficient to provide the B class organ identity function', *Development* 122(1): 11-22.

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(22): 4180-90.

Kuroda, H., Yanagawa, Y., Takahashi, N., Horii, Y. and Matsui, M. (2012) 'A comprehensive analysis of interaction and localization of *Arabidopsis* SKP1-like (ASK) and F-box (FBX) proteins', *PLoS One* 7(11): e50009.

Lahav-Baratz, S., Sudakin, V., Ruderman, J. V. and Hershko, A. (1995) 'Reversible phosphorylation controls the activity of cyclosome-associated cyclin-ubiquitin ligase', *Proc Natl Acad Sci U S A* 92(20): 9303-7.

Lai, Z., Ma, W., Han, B., Liang, L., Zhang, Y., Hong, G. and Xue, Y. (2002) 'An F-box gene linked to the self-incompatibility (S) locus of *Antirrhinum* is expressed specifically in pollen and tapetum', *Plant Mol Biol* 50(1): 29-42.

Laufs, P., Coen, E., Kronenberger, J., Traas, J. and Doonan, J. (2003) 'Separable roles of UFO during floral development revealed by conditional restoration of gene function', *Development* 130(4): 785-96.

Lechner, E., Achard, P., Vansiri, A., Potuschak, T. and Genschik, P. (2006) 'F-box

proteins everywhere', *Curr Opin Plant Biol* 9(6): 631-8.

Lechner, E., Xie, D., Grava, S., Pigaglio, E., Planchais, S., Murray, J. A., Parmentier, Y., Mutterer, J., Dubreucq, B., Shen, W. H. et al. (2002) 'The AtRbx1 protein is part of plant SCF complexes, and its down-regulation causes severe growth and developmental defects', *J Biol Chem* 277(51): 50069-80.

Lee, I., Wolfe, D. S., Nilsson, O. and Weigel, D. (1997) 'A LEAFY co-regulator encoded by UNUSUAL FLORAL ORGANS', *Curr Biol* 7(2): 95-104.

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(6): 805-14.

Levin, J. Z. and Meyerowitz, E. M. (1995) 'UFO: an Arabidopsis gene involved in both floral meristem and floral organ development', *Plant Cell* 7(5): 529-48.

Li, J. F., Park, E., von Arnim, A. G. and Nebenfuhr, A. (2009) 'The FAST technique: a simplified Agrobacterium-based transformation method for transient gene expression analysis in seedlings of Arabidopsis and other plant species', *Plant Methods* 5: 6.

Lin, H. K., Wang, G., Chen, Z., Teruya-Feldstein, J., Liu, Y., Chan, C. H., Yang, W. L., Erdjument-Bromage, H., Nakayama, K. I., Nimer, S. et al. (2009) 'Phosphorylation-dependent regulation of cytosolic localization and oncogenic function of Skp2 by Akt/PKB', *Nat Cell Biol* 11(4): 420-32.

Lippman, Z. B., Cohen, O., Alvarez, J. P., Abu-Abied, M., Pekker, I., Paran, I., Eshed, Y. and Zamir, D. (2008) 'The making of a compound inflorescence in tomato and related nightshades', *PLoS Biol* 6(11): e288.

Liu, C. W., Li, X., Thompson, D., Wooding, K., Chang, T. L., Tang, Z., Yu, H., Thomas, P. J. and DeMartino, G. N. (2006) 'ATP binding and ATP hydrolysis play distinct roles in the function of 26S proteasome', *Mol Cell* 24(1): 39-50.

Liu, F., Ni, W., Griffith, M. E., Huang, Z., Chang, C., Peng, W., Ma, H. and Xie, D. (2004) 'The ASK1 and ASK2 genes are essential for Arabidopsis early development', *Plant Cell* 16(1): 5-20.

Ma, H. and dePamphilis, C. (2000) 'The ABCs of floral evolution', *Cell* 101(1): 5-8.

Maere, S., Heymans, K. and Kuiper, M. (2005) 'BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks', *Bioinformatics* 21(16): 3448-9.

Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R. and Ratcliffe, P. J. (1999) 'The tumour suppressor protein VHL targets hypoxia-inducible factors for

oxygen-dependent proteolysis', *Nature* 399(6733): 271-5.

McBride, K. E. and Summerfelt, K. R. (1990) 'Improved binary vectors for Agrobacterium-mediated plant transformation', *Plant Mol Biol* 14(2): 269-76.

McGrath, J. P., Jentsch, S. and Varshavsky, A. (1991) 'UBA 1: an essential yeast gene encoding ubiquitin-activating enzyme', *EMBO J* 10(1): 227-36.

Miao, Y. and Zentgraf, U. (2010) 'A HECT E3 ubiquitin ligase negatively regulates Arabidopsis leaf senescence through degradation of the transcription factor WRKY53', *Plant J* 63(2): 179-88.

Moore, I., Galweiler, L., Grosskopf, D., Schell, J. and Palme, K. (1998) 'A transcription activation system for regulated gene expression in transgenic plants', *Proc Natl Acad Sci U S A* 95(1): 376-81.

Moyroud, E., Minguet, E. G., Ott, F., Yant, L., Pose, D., Monniaux, M., Blanchet, S., Bastien, O., Thevenon, E., Weigel, D. et al. (2011) 'Prediction of regulatory interactions from genome sequences using a biophysical model for the Arabidopsis LEAFY transcription factor', *Plant Cell* 23(4): 1293-306.

Nussbaum, A. K., Dick, T. P., Keilholz, W., Schirle, M., Stevanovic, S., Dietz, K., Heinemeyer, W., Groll, M., Wolf, D. H., Huber, R. et al. (1998) 'Cleavage motifs of the yeast 20S proteasome beta subunits deduced from digests of enolase 1', *Proc Natl Acad Sci U S A* 95(21): 12504-9.

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', *Plant Cell*.

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(6955): 257-63.

Parcy, F. (2005) 'Flowering: a time for integration', *Int J Dev Biol* 49(5-6): 585-93.

Parcy, F., Nilsson, O., Busch, M. A., Lee, I. and Weigel, D. (1998) 'A genetic framework for floral patterning', *Nature* 395(6702): 561-6.

Petroski, M. D. and Deshaies, R. J. (2005) 'Function and regulation of cullin-RING ubiquitin ligases', *Nat Rev Mol Cell Biol* 6(1): 9-20.

Picard, D. (1993) 'Steroid-binding domains for regulating the functions of heterologous proteins in cis', *Trends Cell Biol* 3(8): 278-80.

Ramos, J. A., Zenser, N., Leyser, O. and Callis, J. (2001) 'Rapid degradation of auxin/indoleacetic acid proteins requires conserved amino acids of domain II and is

proteasome dependent', *Plant Cell* 13(10): 2349-60.

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(20): 2700-7.

Samach, A., Klenz, J. E., Kohalmi, S. E., Risseuw, E., Haughn, G. W. and Crosby, W. L. (1999) 'The UNUSUAL FLORAL ORGANS gene of *Arabidopsis thaliana* is an F-box protein required for normal patterning and growth in the floral meristem', *Plant J* 20(4): 433-45.

Scheffner, M., Huibregtse, J. M., Vierstra, R. D. and Howley, P. M. (1993) 'The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53', *Cell* 75(3): 495-505.

Schmid, M., Davison, T. S., Henz, S. R., Pape, U. J., Demar, M., Vingron, M., Scholkopf, B., Weigel, D. and Lohmann, J. U. (2005) 'A gene expression map of *Arabidopsis thaliana* development', *Nat Genet* 37(5): 501-6.

Schulman, B. A., Carrano, A. C., Jeffrey, P. D., Bowen, Z., Kinnucan, E. R., Finnin, M. S., Elledge, S. J., Harper, J. W., Pagano, M. and Pavletich, N. P. (2000) 'Insights into SCF ubiquitin ligases from the structure of the Skp1-Skp2 complex', *Nature* 408(6810): 381-6.

Schultz, E. A. and Haughn, G. W. (1991) 'LEAFY, a Homeotic Gene That Regulates Inflorescence Development in *Arabidopsis*', *Plant Cell* 3(8): 771-781.

Schumann, N., Navarro-Quezada, A., Ullrich, K., Kuhl, C. and Quint, M. (2011) 'Molecular evolution and selection patterns of plant F-box proteins with C-terminal kelch repeats', *Plant Physiol* 155(2): 835-50.

Silver, J. D., Ritchie, M. E. and Smyth, G. K. (2009) 'Microarray background correction: maximum likelihood estimation for the normal-exponential convolution', *Biostatistics* 10(2): 352-63.

Skaar, J. R., Pagan, J. K. and Pagano, M. (2009) 'SnapShot: F box proteins I', *Cell* 137(6): 1160-1160 e1.

Smalle, J. and Vierstra, R. D. (2004) 'The ubiquitin 26S proteasome proteolytic pathway', *Annu Rev Plant Biol* 55: 555-90.

Smith, D. M., Kafri, G., Cheng, Y., Ng, D., Walz, T. and Goldberg, A. L. (2005) 'ATP binding to PAN or the 26S ATPases causes association with the 20S proteasome, gate opening, and translocation of unfolded proteins', *Mol Cell* 20(5): 687-98.

Smyth, D. R., Bowman, J. L. and Meyerowitz, E. M. (1990) 'Early flower development in *Arabidopsis*', *Plant Cell* 2(8): 755-67.

- 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(4): 265-73.
- Smyth, G. K.** (2005) Individual channel analysis of two-colour microarrays. In: 55th Session of the International Statistics Institute, 5-12 April 2005, Sydney Convention & Exhibition Centre, Sydney, Australia (CD), 116.
- Souer, E., Rebocho, A. B., Blik, M., Kusters, E., de Bruin, R. A. and Koes, R.** (2008) 'Patterning of inflorescences and flowers by the F-box protein DOUBLE TOP and the LEAFY homolog ABERRANT LEAF AND FLOWER of Petunia', *Plant Cell* 20(8): 2033-48.
- 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(2): 425-34.
- Tanaka, K.** (2009) 'The proteasome: overview of structure and functions', *Proc Jpn Acad Ser B Phys Biol Sci* 85(1): 12-36.
- Thrower, J. S., Hoffman, L., Rechsteiner, M. and Pickart, C. M.** (2000) 'Recognition of the polyubiquitin proteolytic signal', *EMBO J* 19(1): 94-102.
- Tiwari, S. B., Hagen, G. and Guilfoyle, T. J.** (2004) 'Aux/IAA proteins contain a potent transcriptional repression domain', *Plant Cell* 16(2): 533-43.
- Tiwari, S. B., Wang, X. J., Hagen, G. and Guilfoyle, T. J.** (2001) 'AUX/IAA proteins are active repressors, and their stability and activity are modulated by auxin', *Plant Cell* 13(12): 2809-22.
- Toufighi, K., Brady, S. M., Austin, R., Ly, E. and Provart, N. J.** (2005) 'The Botany Array Resource: e-Northern, Expression Angling, and promoter analyses', *Plant J* 43(1): 153-63.
- Turner, G. C., Du, F. and Varshavsky, A.** (2000) 'Peptides accelerate their uptake by activating a ubiquitin-dependent proteolytic pathway', *Nature* 405(6786): 579-83.
- Ulmasov, T., Hagen, G. and Guilfoyle, T. J.** (1997) 'ARF1, a transcription factor that binds to auxin response elements', *Science* 276(5320): 1865-8.
- van der Horst, A., de Vries-Smits, A. M., Brenkman, A. B., van Triest, M. H., van den Broek, N., Colland, F., Maurice, M. M. and Burgering, B. M.** (2006) 'FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP', *Nat Cell Biol* 8(10): 1064-73.
- van Wijk, S. J. and Timmers, H. T.** (2010) 'The family of ubiquitin-conjugating

enzymes (E2s): deciding between life and death of proteins', *FASEB J* 24(4): 981-93.

Vierstra, R. D. (2009) 'The ubiquitin-26S proteasome system at the nexus of plant biology', *Nat Rev Mol Cell Biol* 10(6): 385-97.

Wang, F., Zhu, D., Huang, X., Li, S., Gong, Y., Yao, Q., Fu, X., Fan, L. M. and Deng, X. W. (2009) 'Biochemical insights on degradation of Arabidopsis DELLA proteins gained from a cell-free assay system', *Plant Cell* 21(8): 2378-90.

Wang, Y. and Yang, M. (2006) 'The ARABIDOPSIS SKP1-LIKE1 (ASK1) protein acts predominately from leptotene to pachytene and represses homologous recombination in male meiosis', *Planta* 223(3): 613-7.

Weigel, D., Alvarez, J., Smyth, D. R., Yanofsky, M. F. and Meyerowitz, E. M. (1992) 'LEAFY controls floral meristem identity in Arabidopsis', *Cell* 69(5): 843-59.

Weigel, D. and Meyerowitz, E. M. (1994) 'The ABCs of floral homeotic genes', *Cell* 78(2): 203-9.

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(7): e117.

Wesley, S. V., Helliwell, C. A., Smith, N. A., Wang, M. B., Rouse, D. T., Liu, Q., Gooding, P. S., Singh, S. P., Abbott, D., Stoutjesdijk, P. A. et al. (2001) 'Construct design for efficient, effective and high-throughput gene silencing in plants', *Plant J* 27(6): 581-90.

Wielopolska, A., Townley, H., Moore, I., Waterhouse, P. and Helliwell, C. (2005) 'A high-throughput inducible RNAi vector for plants', *Plant Biotechnol J* 3(6): 583-90.

Wilkinson, M. D. and Haughn, G. W. (1995) 'UNUSUAL FLORAL ORGANS Controls Meristem Identity and Organ Primordia Fate in Arabidopsis', *Plant Cell* 7(9): 1485-1499.

Winter, C. M., Austin, R. S., Blanvillain-Baufume, S., Reback, M. A., Monniaux, M., Wu, M. F., Sang, Y., Yamaguchi, A., Yamaguchi, N., Parker, J. E. et al. (2011) 'LEAFY target genes reveal floral regulatory logic, cis motifs, and a link to biotic stimulus response', *Dev Cell* 20(4): 430-43.

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', *Proc Natl Acad Sci U S A* 109(33): 13452-7.

Xu, G., Ma, H., Nei, M. and Kong, H. (2009) 'Evolution of F-box genes in plants: different modes of sequence divergence and their relationships with functional

diversification', *Proc Natl Acad Sci U S A* 106(3): 835-40.

Yang, M., Hu, Y., Lodhi, M., McCombie, W. R. and Ma, H. (1999) 'The Arabidopsis SKP1-LIKE1 gene is essential for male meiosis and may control homologue separation', *Proc Natl Acad Sci U S A* 96(20): 11416-21.

Yang, X., Kalluri, U. C., Jawdy, S., Gunter, L. E., Yin, T., Tschaplinski, T. J., Weston, D. J., Ranjan, P. and Tuskan, G. A. (2008) 'The F-box gene family is expanded in herbaceous annual plants relative to woody perennial plants', *Plant Physiol* 148(3): 1189-200.

Zachariae, W., Schwab, M., Nasmyth, K. and Seufert, W. (1998) 'Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex', *Science* 282(5394): 1721-4.

Zhai, Q., Yan, L., Tan, D., Chen, R., Sun, J., Gao, L., Dong, M. Q., Wang, Y. and Li, C. (2013) 'Phosphorylation-Coupled Proteolysis of the Transcription Factor MYC2 Is Important for Jasmonate-Signaled Plant Immunity', *PLoS Genet* 9(4): e1003422.

Zhao, D., Han, T., Risseuw, E., Crosby, W. L. and Ma, H. (2003a) 'Conservation and divergence of ASK1 and ASK2 gene functions during male meiosis in Arabidopsis thaliana', *Plant Mol Biol* 53(1-2): 163-73.

Zhao, D., Ni, W., Feng, B., Han, T., Petrasek, M. G. and Ma, H. (2003b) 'Members of the Arabidopsis-SKP1-like gene family exhibit a variety of expression patterns and may play diverse roles in Arabidopsis', *Plant Physiol* 133(1): 203-17.

Zhao, D., Yang, M., Solava, J. and Ma, H. (1999) 'The ASK1 gene regulates development and interacts with the UFO gene to control floral organ identity in Arabidopsis', *Dev Genet* 25(3): 209-23.

Zheng, N., Schulman, B. A., Song, L., Miller, J. J., Jeffrey, P. D., Wang, P., Chu, C., Koepp, D. M., Elledge, S. J., Pagano, M. et al. (2002) 'Structure of the Cull1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex', *Nature* 416(6882): 703-9.

Zhu, H. and Bunn, H. F. (2001) 'Signal transduction. How do cells sense oxygen?', *Science* 292(5516): 449-51.

Appendix 1

Buffers used in this work

Name	Composition
2x SDS loading buffer	25% (v/v) 4x stacking buffer, 20% (v/v) glycerol, 4% SDS (v/v), 2% (v/v) β -mercaptoethanol, 1% (w/v) bromphenol blue
ChIP Dilution Buffer	16.7 mM Tris-HCl pH8.1, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS, 1x Complete Protease Inhibitor Cocktail
ChIP Elution buffer	0.1 M glycine, 0.5 M NaCl, 0.05% Tween-20
ChIP Lysis Buffer	16.7 mM Tris-HCl pH8.1, 167 mM NaCl, 1.2 mM EDTA, 1.1 % Triton X-100, 0.01% SDS, 1x Complete Protease Inhibitor Cocktail
Co-cultivation solution	0.25 x MS, 1% sucrose, 100 μ M acetosyringone, 0.005% Silwett L-77
Column buffer	20 mM Tris-HCl pH7.5, 200 mM NaCl, 1 mM EDTA
CTAB	100 mM Tris HCl (pH8.0), 20 mM EDTA, 1.4 M NaCl, 2% (w/v) cetyltrimethyl ammonium bromide Facultative 1% polyvinyl pyrrolidone 40, 000
Dialysis buffer	25 mM Tris pH7.5, 10 mM NaCl, 10 mM $MgCl_2$, 10% (v/v) glycerol
Edward's extraction buffer	200 mM Tris-HCl pH7.5-8.0, 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS
Elution buffer	20 mM Tris-HCl pH7.5, 200 mM NaCl, 1 mM EDTA, 10 mM maltose
Fixation solution	1% (w/v) formaldehyde, 10 mM NaPi pH7.0, 50 mM NaCl, 0.1 M sucrose
IP buffer	0.05 M HEPES pH7.5, 0.15 M NaCl, $ZnSO_4$, 5 mM $MgCl_2$, 1.1% Triton-X, 0.01% SDS
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
M1 buffer	10 mM NaH ₂ PO ₄ , 10 mM Na ₂ HPO ₄ , 100 mM NaCl, 1 M hexylene glycol, 10 mM β-mercaptoethanol, 1x Complete Protease Inhibitor Cocktail
M2 buffer	10 mM NaH ₂ PO ₄ , 10 mM Na ₂ HPO ₄ , 100 mM NaCl, 10 mM MgCl ₂ , 0.5% Triton X100, 1 M hexylene glycol, 10 mM β-mercaptoethanol, 1x Complete Protease Inhibitor Cocktail
M3 buffer	10 mM NaH ₂ PO ₄ , 10 mM Na ₂ HPO ₄ , 100 mM NaCl, 10 mM β-mercaptoethanol, 1x Complete Protease Inhibitor Cocktail
MC buffer	10 mM NaH ₂ PO ₄ , 10 mM Na ₂ HPO ₄ , 50 mM NaCl, 0.1 M sucrose
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
Ponceasu solution	0.2% (w/v) Ponceau, 10% (v/v) acetic acid
Resuspension solution	20 mM Tris-HCl pH7.5, 200 mM NaCl, 1 mM EDTA, 1 x protease inhibitor (Roche Complete Mini), 1 mM phenylmethanesulfonylfluoride
Separating buffer	1.5 M Tris pH8.8, 0.4% (w/v) SDS
SOB	2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 10 mM MgSO ₄ , 10 mM MgCl ₂
Stacking buffer	0.5 M Tris pH 6.8 0.4% (w/v) SDS
TB buffer	10 mM PIPES/KOH pH6.7, 15 mM CaCl ₂ , 250 mM KCl, 55 mM MnCl ₂
Transfer buffer	10 mM Tris base, 0.1 M glycine, 10% methanol
Tris-glycine buffer	0.025 M Tris pH8.3, 0.192 M glycine, 0.1% SDS
Washing solution	10 mM MgCl ₂ , 100 μM acetosyringone
YEB agar	5 g/L beef extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, 0.5 g/L MgCl ₂ , 4.5% (w/v) agar