

Gemma Bilsborough

Keble College

University of Oxford

**Leaf margin morphogenesis in crucifer
plants**

Trinity term, 2011

Thesis submitted for the degree of Doctor of Philosophy

Leaf margin morphogenesis in crucifer plants

Thesis submitted for the degree of Doctor of Philosophy, Trinity term 2011

Abstract

A key question in developmental biology is how form is generated. The model species *Arabidopsis thaliana* produces simple leaves with marginal outgrowths termed serrations. Serration development in *A. thaliana* requires both the transcription factor CUP-SHAPED COTYLEDON2 (*CUC2*) and the auxin efflux facilitator PIN-FORMED1 (*PIN1*), which regulates polar auxin transport by forming convergence points (Hay *et al.*, 2006; Nikovics *et al.*, 2006; Scarpella *et al.*, 2006). In Chapter 3, I investigate how *CUC2*, *PIN1* and auxin interact to control serration development. I demonstrate that *CUC2* promotes *PIN1* convergence point and auxin activity foci formation along the margin of the leaf, whilst high auxin activity represses *CUC2* expression. Furthermore, interspersed peaks of *CUC2* and auxin activity pattern serration development along the proximo-distal axis of the leaf. Thus, auxin, *PIN1* and *CUC2* form a negative feedback loop that patterns serration development.

CUC genes and *PIN1* are required for leaflet development in *Cardamine hirsuta* (Barkoulas *et al.*, 2008; Blein *et al.*, 2008), a close relative of *A. thaliana* that produces compound leaves subdivided into units termed leaflets. However, it is unclear how *CUC* and *PIN1* interact to control leaflet development. In Chapter 4, I demonstrate that similar to *A. thaliana*, *CUC* genes promote *PIN1* convergence point and auxin activity foci formation at the *C. hirsuta* leaf margin, whilst high auxin activity represses *CUC2* expression. These genetic interactions likely create interspersed peaks of *CUC2* and auxin activity that pattern leaflet development. Thus, the same negative feedback loop between *CUC*, *PIN1* and auxin patterns both leaflet development in *C. hirsuta* and serration development in *A. thaliana*.

KNOTTED1-LIKE HOMEODOMAIN (KNOX) genes are expressed in *C. hirsuta* leaves, and interact with *ChCUC* and *PIN1* in positive and negative feedback loops, respectively, to control leaflet development (Barkoulas *et al.*, 2008; Blein *et al.*, 2008). *KNOX* genes are not expressed in *A. thaliana* leaves, but deeply lobed margins reminiscent of leaflets develop in association with ectopic *KNOX* expression in leaves (Chuck *et al.*, 1996; Hay *et al.*, 2006). However, it is unclear whether regulatory interactions of *PIN1*, *CUC* and *KNOX* which occur in *C. hirsuta* leaflets are employed during *KNOX*-induced lobe development in *A. thaliana*. In Chapter 5, I demonstrate that *CUC2* and polar auxin transport are required for ectopic *KNOX* expression. Conversely, I show that *KNOX* misexpression up-regulates *CUC2* expression in *A. thaliana* leaves. Thus, interactions between *KNOX*, *CUC* and *PIN1* that occur in leaflet development in *C. hirsuta* also occur in association with *KNOX*-induced lobe development in *A. thaliana*.

In addition to investigating the regulatory interactions between known components of leaf development pathways, I sought to identify novel genes that mediate *CUC2*-dependent serration development in *A. thaliana*. In Chapter 6, I identify a suppressor of the smooth margin phenotype of *cuc2* leaves that partially restores *PIN1* localisation in the absence of functional *CUC2*. Finally, in the General Discussion I evaluate how interlinking feedback loops between *CUC*, *KNOX* and auxin pattern serration and leaflet development. I then discuss why interlinking feedback loops may have been deployed to control outgrowths in both plant and animal systems.

Acknowledgements

First, and foremost, I would like to thank my supervisor Miltos Tsiantis for accepting me as a D. Phil student in his prestigious lab at the University of Oxford. I am extremely grateful for all the exciting ideas he has suggested for my project, and for the fruitful discussions that we have shared. I am especially grateful for his guidance over my project and for his uncontainable enthusiasm for my work.

I give many thanks to Angela Hay for being my second supervisor during my D. Phil. I worked with Angela for six weeks during the summer holiday before I began my D. Phil and I enjoyed this immensely. During this period of time, Angela has trained me as a developmental scientist and provided invaluable advice and encouragement. I would also like to say “efxaristo” to Michalis Barkoulas for supervising, and working together with me, on the serration project for the first two years of my D. Phil.

It has been a privilege to work alongside everyone in the Tsiantis and Hay labs. I particularly thank Huw Jenkins and Maria Cartolano for their openness to discuss their data with me. I especially thank Huw for his help with map-based cloning and Sarah McKim for editing my thesis. I would like to make a special mention to John Baker for his photographic skills that have captured the beauty and importance of all my mutants and transgenics, and transferred this to tiff format. I also thank Ian Moore for his help with confocal microscopy.

On a final note, I would like to thank my Mum, Dad and sister for their continued support and encouragement during my D. Phil. I thank them for listening to all the trials and tribulations of my project and for always being there for me. Their advice has been fantastic.

List of abbreviations

2,4-D	2,4-Dichlorophenoxy acetic acid
35S	Cauliflower Mosaic Virus 35S promoter
ABC	ATP-binding cassette
ANOVA	Analysis of variance
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i>
ARR	ARABIDOPSIS RESONSE REGULATOR
ARR5	ARABIDOPSIS RESONSE REGULATOR5
AS	ASYMMETRIC LEAVES
ATHB-8	Arabidopsis thaliana HOMEBOX gene-8
AtML1	Arabidopsis thaliana MERISTEM LAYER1
AXR1	AUXIN RESISTANT1
BDL	BODENLOS
BELL	BEL1-LIKE HOMEODOMAIN
BLR	BELLRINGER
BOP1	BLADE-ON-PETIOLE1
BP	BREVIPEDICELLUS
<i>C. hirsuta</i>	<i>Cardamine hirsuta</i>
cDNA	Complementary DNA
ChBP	CARDAMINE HIRSUTA BREVIPEDICELLUS
CLV	CLAVATA
Col-0	Columbia
CRN	CORYNE
CUC	CUP-SHAPED COTYLEDON
CUS1	CUC2 SUPPRESSOR1
CYCB1;1	CYCLINB1;1
CZ	Central Zone

DIC	Diferential interference contrast
DMSO	Dimethyl sulfoxide
DR5	Direct Repeat 5
EDTA	Ethylenediamine tetraacetic acid
eGFP	enhanced GREEN FLUORESCENT PROTEIN
EMS	Ethane Methyl Sulfonate
FIL	FILAMENTOUS FLOWER
GAPDH	GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE
GFP	GREEN FLUORESCENT PROTEIN
GUS	β -glucuronidase
HD-ZIP III	Class III HOMEODOMAIN LEUCIN ZIPPER
HIRA	HISTONE CHAPERONE HISTONE REGULATORY PROTEIN A
IAA	Indole-3-acetic acid
IAA2	AUXIN/INDOLE-3-ACETIC ACID3
IPT	ISOPENTENYL TRANSFERASE
kn1	Knotted-1
KNAT2	KN1-LIKE IN ARABIDOPSIS THALIANA2
KNAT6	KN1-LIKE IN ARABIDOPSIS THALIANA6
KNATM	KNOX ARABIDOPSIS THALIANA MEINOX
KNOX	KN1-LIKE HOMEBOX
LB	Luria Broth
<i>Ler</i>	Landsberg <i>erecta</i>
LFY	LEAFY
LOB	LATERAL ORGAN BOUNDARIES
LOL1	LOBED LEAF1
LRR	Leucine rich repeat
MES	2-(N-Morpholino)-ethanesulphonic acid
mGFP	monster GREEN FLUORESCENT PROTEIN
MIR164A	MICRORNA164A

MP	MONOPTEROS
mRNA	messenger RNA
MS	Murishuge Skoog
NAC	NO APICAL MERISTEM/ATAF/CUP-SHAPED COTYLEDON
NPA	N-1-naphthylphthalamic acid
OC	Organising Centre
OTC	ORNITHINE TRANSCARBAMILASE
Ox	Oxford
PCR	Polymerase Chain Reaction
PHAN	PHANTASTICA
PHB	PHABULOSA
PHV	PHAVOLUTA
PID	PINOID
PIN	PIN-FORMED
PP2A	Protein phosphatase 2A
PZ	Peripheral Zone
RNA	Ribonucleic acid
RNAi	RNA interference
REV	REVOLUTA
RPK2	RECEPTOR-LIKE PROTEIN KINASE 2
RS2	ROUGH SHEATH2
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SAM	Shoot Apical Meristem
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscope
STM	SHOOT MERISTEMLESS
TALE	Three Amino Loop Extension
TIR1	TRANSPORT INHIBITOR RESPONSE1
WUS	WUSCHEL

Table of Contents

Acknowledgements.....	3
List of abbreviations	4
Table of Contents	7
Chapter 1: Introduction	17
1.1 Introduction to developmental biology	17
1.2 The shoot apical meristem: the dome of youth	18
1.2.1 Radial patterning of the SAM by the WUS/CLV feedback loop.....	21
1.2.2 Hormonal regulation of the CLV/WUS feedback loop	23
1.2.3 A TALE of <i>KNOX</i> gene regulation in the SAM	24
1.2.4 <i>KNOX</i> -mediated regulation of the SAM: orchestrating a hormonal harmony	25
1.2.5 AS1-dependent repression of <i>KNOX</i> expression	28
1.2.6 Multilevel control over <i>KNOX</i> expression and function.....	29
1.2.7 Boundary formation is required for SAM initiation	31
1.2.8 Adaxial/abaxial determinants regulate SAM initiation and maintenance.....	32
1.3 Control of leaf initiation: the only way is up	33
1.3.1 Leaf initiation.....	34
1.3.2 The regulation of auxin transport.....	37
1.3.3 The role of auxin transport in leaf primordium initiation	39

1.3.4 Auxin-mediated regulation of gene expression	41
1.3.5 The role of the cell wall and tension in the regulation of leaf initiation	42
1.4 Shaping leaf development.....	42
1.4.1 Back-to-front: the establishment of the adaxial-abaxial axis.....	47
1.4.2 The role of the margin in new growth axis formation	50
1.4.3 Patterning the leaf margin: getting the kNACk of it.....	52
1.4.4 Sizing up: the regulation of growth within the leaf	54
1.4.5 Genetic regulation of leaf growth	55
1.4.6 Positive and negative regulators in leaf growth.....	59
1.4.7 Integrating patterning and growth.....	60
1.5 Dissecting the regulation of compound leaf development.....	60
1.5.1 Leaf classifications.....	64
1.5.2 The role of <i>KNOX</i> genes in leaf dissection.....	65
1.5.3 <i>KNOX</i> genes regulate growth to control compound leaf development.....	66
1.5.4 <i>KNOX</i> -mediated hormonal regulation of leaf dissection	70
1.5.5 The role of auxin in leaf dissection.....	72
1.5.6 Establishing boundaries: a unifying mechanism for compound leaf development	73
1.5.7 The role of growth in leaf dissection	74
1.5.8 The role of TCP transcription factors in leaf dissection	75
1.5.9 The adaptive significance of leaf shape: the point of serrated and dissected leaf margins.....	77

1.5.10 Using comparative developmental genetics to elucidate the genetic basis of differences in simple and compound leaf shape	79
1.6 Aims and objectives	83
Chapter 2: Materials and methods.....	83
2.1 Materials used	83
2.1.1 Plant growth conditions	83
2.1.2 Plant material	85
2.1.3 Bacterial strains.....	85
2.1.4 Buffers and media	85
2.1.5 Oligonucleotides	89
2.2 Genetic methods	89
2.2.1 Cross-pollination of <i>A. thaliana</i> and <i>C. hirsuta</i> plants	89
2.2.2 Plant genetics	100
2.2.3 Bacterial transformations	101
2.2.4 Plant transformation of <i>A. thaliana</i> and <i>C. hirsuta</i>	101
2.2.5 Selection of transgenic plants	102
2.2.6 EMS mutagenesis and genetic screening.....	102
2.2.7 Map-based cloning.....	104
2.3 Molecular biology methods	104
2.3.1 Polymerase chain reaction (PCR)	105
2.3.2 Sequencing reactions	105

2.3.3 Genomic DNA extraction for PCR	105
2.3.4 Genomic DNA extraction for map-based cloning	106
2.3.5 Quantitative RT-PCR analysis	106
2.3.6 Plasmid construction and analysis of transgenics	107
2.4 Microscopy, histology and histochemistry	109
2.4.1 Confocal microscopy	109
2.4.2 Scanning electron microscopy (SEM)	111
2.4.3 GUS staining	111
2.4.4 Leaf clearings	112
2.4.5 Chemical treatments	112
2.5 Leaf shape analysis	113
2.5.1 Silhouettes	113
2.5.2 Quantification of leaf margin dissection index	113
2.5.3 Extended eigenshape analysis	113
Chapter 3: The role of <i>CUC2</i> and auxin in patterning serration development	116
3.1 Introduction	116
3.2 Results	118
3.2.1 <i>CUC2</i> and <i>PINI</i> activities regulate serration initiation	118
3.2.2 Interspersed peaks of auxin and <i>CUC2</i> activity pattern serration development ...	123

3.2.3 Downregulation of <i>CUC2</i> expression coincides with PIN1 convergence point and auxin activity maxima formation	123
3.2.4 <i>CUC2</i> promotes PIN1 convergence points to control auxin activity maxima	128
3.2.5 Interspersed <i>CUC2</i> activity regulates serration development	134
3.2.6 Interspersed <i>CUC2</i> activity regulates auxin distribution	134
3.2.7 Auxin represses <i>CUC2</i> expression to control serration development	139
3.2.8 Auxin signalling represses <i>CUC2</i> expression by promoting <i>MIR164A</i> transcription	144
3.2.9 <i>MP</i> does not regulate <i>CUC2</i> expression to control serration development	150
3.2.10 Epidermal PIN1 expression is sufficient to control serration and vasculature development	155
3.2.11 <i>CUC2</i> regulates serration development without affecting secondary vein number	160
3.3 Discussion	165
3.3.1 Interlinking feedback loops pattern serration development	165
3.3.2 Epidermal auxin flow patterns serration and secondary vein development	169
3.3.3 Serration development can be uncoupled from secondary vein formation	169
3.4 Conclusions	171

Chapter 4: The role of *CUC* and auxin in patterning leaflet development in

<i>C. hirsuta</i>	173
4.1 Introduction.....	173
4.2 Results	175
4.2.1 <i>ChCUC</i> and <i>PIN1</i> are required for leaflet initiation	175
4.2.2 <i>CUC2</i> maxima precede the formation of <i>PIN1</i> convergence points.....	180
4.2.3 <i>PIN1</i> convergence can be temporally uncoupled from auxin activity maxima	183
4.2.4 <i>CUC</i> genes are required for <i>PIN1</i> convergence points and auxin activity maxima to form.....	183
4.2.5 Auxin represses <i>CUC2</i> expression in the rachis of the <i>C. hirsuta</i> leaf.....	191
4.2.6 <i>CUC</i> and <i>PIN1</i> regulate secondary vein number in <i>C. hirsuta</i> leaves.....	196
4.2.7 Characterisation of wild-type vasculature development in <i>C. hirsuta</i> leaves.....	196
4.2.8 Polar auxin transport regulates secondary vein development in <i>C. hirsuta</i> leaves	201
4.2.9 <i>CUC</i> genes and <i>PIN1</i> are required for serration development in <i>A. thaliana</i> leaves	204
4.3 Discussion.....	209
4.3.1 Interlinking feedback loops pattern leaflet development in <i>C. hirsuta</i>	209
4.3.2 A conserved feedback loop between <i>CUC</i> , <i>PIN1</i> and auxin regulates leaflet and serration development.....	213
4.3.3 A change in gene expression may signal the initiation of the final leaflet pair	214
4.4 Conclusions.....	215

Chapter 5: A possible role for auxin, <i>CUC</i> and <i>KNOX</i> genes in elaborating lobed leaf morphologies	217
5.1 Introduction.....	217
5.2 Results	219
5.2.1 <i>CUC2</i> is required for <i>KNOX</i> -dependent lobe development in <i>A. thaliana</i> leaves	219
5.2.2 <i>CUC2</i> expression is upregulated and broadened in <i>A. thaliana</i> leaves misexpressing <i>KNOX</i> genes	223
5.2.3 Ectopic <i>KNOX</i> expression post-transcriptionally promotes <i>CUC2</i> expression in <i>A. thaliana</i> leaves	228
5.2.4 <i>CUC2</i> is required for <i>BP</i> misexpression at the margins of <i>A. thaliana</i> leaves	233
5.2.5 Polar auxin transport is required for <i>BP</i> misexpression at the margins of <i>A. thaliana</i> leaves	238
5.2.6 <i>ChCUC</i> genes and polar auxin transport are required for leaflet development and <i>ChBP</i> misexpression in <i>chas1</i> leaves.....	243
5.2.7 <i>CUC</i> genes do not promote <i>ChBP</i> transcription in <i>A. thaliana</i> leaves	246
5.2.8 <i>CUC</i> genes regulate cell division in <i>C. hirsuta</i> leaves but not in <i>A. thaliana</i> leaves	250
5.2.9 <i>LOLI</i> regulates leaflet development and <i>CUC2</i> expression	253
5.3 Discussion.....	257
5.3.1 Interlinking feedback loops between <i>CUC</i> , <i>KNOX</i> and auxin regulate lobe development in <i>A. thaliana</i> leaves ectopically expressing <i>KNOX</i> in the leaf.....	257

5.3.2 <i>ChCUC</i> genes regulate cell division to control leaflet development.....	263
5.3.3 <i>LOLI</i> regulates <i>CUC2</i> expression and leaflet development.....	264
5.4 Conclusions.....	265
Chapter 6: Towards identifying a novel component of the <i>CUC2</i> developmental pathway via a screen for suppressors of <i>cuc2</i>	267
6.1 Introduction.....	267
6.2 Results	269
6.2.1 Genetic screen identifies suppressors of the smooth leaf margin phenotype of <i>cuc2</i>	269
6.2.2 <i>CUS1</i> likely acts downstream of <i>CUC2</i> to control serration development	274
6.2.3 Loss-of- <i>CUS1</i> function restores serration initiation in <i>cuc2</i> leaves.....	274
6.2.4 Loss-of- <i>CUS1</i> function does not enhance serration development in <i>mir164a</i> leaves	279
6.2.5 <i>CUC2</i> and <i>CUC3</i> act in a separate pathway to <i>CUS1</i> to regulate serration development.....	282
6.2.6 Polar auxin transport is required for serration development in <i>cus1</i>	282
6.2.7 <i>CUS1</i> regulates the formation of auxin activity foci and PIN1 convergence points at the leaf margin	287
6.2.8 Loss-of- <i>CUS1</i> function does not enhance lobe development in <i>as1</i> leaves	293
6.2.9 The <i>CUS1</i> locus lies within a 380kbp region on the southern arm of chromosome II.....	298
6.3 Discussion.....	298

6.3.1 <i>CUS1</i> likely acts downstream of <i>CUC2</i> to regulate serration outgrowth	298
6.3.2 <i>CUS1</i> regulates serration initiation by repressing PIN1 convergence points	301
6.4 Conclusions.....	302
Chapter 7: General discussion.....	304
7.1 Interlinking feedback loops regulate simple and compound leaf morphology	304
7.2 Conserved genetic mechanisms regulate lobe and leaflet development.....	309
7.3 Interlinking feedback loops regulate outgrowths in plant and animal systems...	311
References	316

Chapter 1:

Introduction

Chapter 1: Introduction

1.1 Introduction to developmental biology

Development is the process of generating a complex multicellular form from a single fertilised egg, and is a critical process in the life cycle of every multicellular organism. A fundamental question that arises in developmental biology is how morphology is generated. Despite the apparent endless forms in nature (Darwin, 1859), all extant species are proposed to have evolved from a single unicellular organism, known as the universal common ancestor (Crick, 1968; Dobzhansky, 1973; Raup and Valentine, 1983; Sober and Steel, 2002). However, multicellularity arose independently in plant and animal lineages. In contrast to animal development, plant development is highly plastic as plants are sessile organisms and must develop in response to the environment.

Leaves display a wide variety of forms in nature, as was first noted in antiquity (Theophrastus, 1916), making them attractive systems in which to study morphogenesis. Over the past century, studies have attempted to determine the genetic mechanisms controlling leaf development, and I discuss these findings below. The majority of work reviewed in this chapter was performed in the model species *Arabidopsis thaliana*, however, I also examine studies performed in *Cardamine hirsuta*, tomato, maize, *Antirrhinum majus* and various other species. In this chapter, I discuss the current view of how the process of leaf initiation is regulated (Sections 1.1-1.2). I then examine the gene regulatory networks that pattern leaf development, and how these genetic mechanisms may regulate growth (Section 1.4). Finally, I review the gene regulatory pathways regulating compound leaf development (Section 1.5), before outlining the aims of this study (Section 1.6).

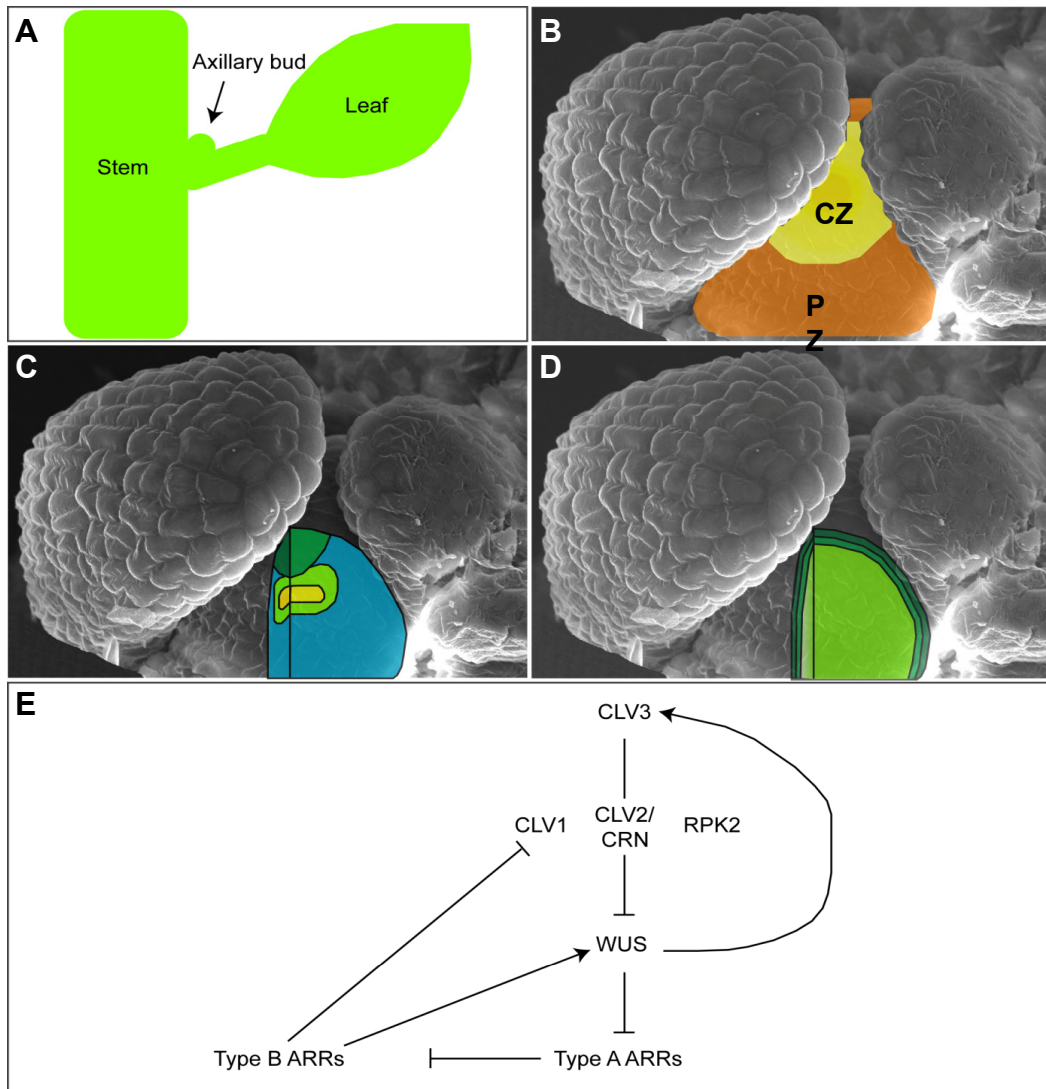
1.2 The shoot apical meristem: the dome of youth

The shoot apical meristem (SAM) is a dome-shaped structure, comprising a pool of pluripotent stem cells, that is located at the shoot apex and was first described in 1759 (Wolff, 1759). The SAM is formed during embryogenesis and generates all the epigeal organs of the plant by iteratively producing modules known as phytomers, which comprise a leaf, a stem section and an axillary bud (Figure 1.1 A). To continuously generate organs, the SAM must maintain two cell populations: firstly, cells that are competent to respond to cell fate determining factors and are recruited into initiating organs; and secondly, stem cells which divide to replenish cells recruited into lateral organs.

The SAM can be divided into two zones along the radial axis based on cell division rates, cell size and patterns of gene expression. The central zone (CZ) is located at the core of the SAM and comprises cells that divide less frequently, and are larger in size, than the cells of the peripheral zone (PZ; Figure 1.1 B; Kwiatkowska and Dumais, 2003; Reddy *et al.*, 2004; Traas and Bohn-Courseau, 2005). CZ cells are undifferentiated and comprise either stem cells or cells of the organising centre (OC; Figure 1.1 C). Clonal analysis indicates that there are between one and three stem cells per layer of the SAM, accounting for approximately nine stem cells in total (Furner and Pumfrey, 1992; Irish and Sussex, 1992; Schnittger *et al.*, 1996; Stewart and Dermen, 1970). The OC is composed of cells that specify and maintain the stem cell population, and is located beneath the stem cells. The PZ encircles the CZ and its cells are competent to respond to cell fate determining factors required for organ initiation.

Additionally, there are three tissue layers along the apical-basal axis of the SAM whose cells exhibit distinctive cell division planes (Steeves and Sussex, 1989; Figure 1.1 D). Cells within the L1 and L2 layer, collectively known as the tunica, divide anticlinally,

Figure 1.1 Regulation of the SAM. (A) Cartoon of a phytoemer (green), comprising a stem section, a leaf and an axillary bud. **(B)** Scanning electron micrograph of an *A. thaliana* SAM with the central zone (CZ; yellow) and the peripheral zone (PZ; orange) indicated. **(C)** Scanning electron micrograph of an *A. thaliana* SAM with the *CLV3* expression domain (dark green), the *CLV1* expression domain (light green) and the *WUS* expression domain (yellow) indicated. **(D)** Scanning electron micrograph of an *A. thaliana* SAM with the L1 layer (dark green), the L2 layer (mid green) and the L3 layer (light green) indicated. **(E)** Model of the CLV/WUS feedback loop. CLV3 binds to either a CLV1, a CLV2/CRN or an RPK2 receptor complex, which triggers the repression of *WUS*. *WUS* feeds back to promote the expression of *CLV3*, creating a stabilising mechanism that maintains the number of stem cells in the SAM. *WUS* also represses CK signalling by repressing *Type A-ARRs*. CK signalling promotes *WUS* expression as *Type B ARR*s directly promote *WUS* expression and repress *CLV* expression



causing these layers to be largely clonally distinct from each other, and for growth to occur radially in these layers (Steeves and Sussex, 1989). In contrast, cells in the L3 layer can divide anticlinally or periclinally, meaning that a single clonally distinct layer is not generated and, instead, a less organised region, known as the corpus, is generated (Steeves and Sussex, 1989).

Critically, positional information has a significant role in determining cell fate and patterning the axes of the SAM (Steeves and Sussex, 1989). Thus, there are several genetic pathways that pattern the SAM to ensure a supply of cells for lateral organ initiation and to maintain the SAM.

1.2.1 Radial patterning of the SAM by the WUS/CLV feedback loop

A negative feedback loop operates between stem cells and the OC to pattern the radial axis of the SAM (Brand *et al.*, 2000; Fletcher *et al.*, 1999; Schoof *et al.*, 2000). The master regulators of this negative feedback loop are the *CLAVATA (CLV)* and *WUSCHEL (WUS)* genes (Figure 1.1 E). WUS is a homeodomain transcription factor that is expressed in the OC of the SAM (Mayer *et al.*, 1998). WUS non-cell autonomously activates *CLV3* expression (Brand *et al.*, 2000; Schoof *et al.*, 2000), and is required to maintain the stem cell population (Laux *et al.*, 1996). In contrast, *CLV* genes repress *WUS* expression (Schoof *et al.*, 2000) and promote the transition of cells from the CZ to the PZ (Reddy *et al.*, 2004).

Critically, *CLV3* controls the positioning of the boundary between the CZ and the PZ independently of cell division, as inducing a twenty four hour repression of *CLV3* causes PZ cells to express CZ markers and hence revert back to stem cells (Reddy and Meyerowitz, 2005). *CLV3* is synthesised as a ninety six amino acid prepropeptide that is subsequently cleaved to form an active thirteen amino acid arabinosylated peptide that is capable of diffusing throughout the SAM and binding to three known receptor complexes (Kinoshita *et*

al., 2010; Kondo *et al.*, 2006; Nimchuk *et al.*, 2011; Ohyama *et al.*, 2009; Rojo *et al.*, 2002; Sharma *et al.*, 2003).

The first such complex to be identified was a homodimer of CLV1 proteins. CLV1 is a leucine rich receptor (LRR) kinase that binds the CLV3 ligand in its extracellular domain, and is expressed in the L3 layer of the SAM (Clark *et al.*, 1993; Lenhard and Laux, 2003; Ogawa *et al.*, 2008; Ohyama *et al.*, 2009). For almost a decade, it was thought that CLV1 prevents active forms of CLV3 from diffusing into the domain of *WUS* expression by sequestering CLV3 (Lenhard and Laux, 2003). However, overexpression of *CLV1* in the L1 and L2 layers of the SAM does not prevent the perception of CLV3 by CLV1 in the OC (Nimchuk *et al.*, 2011). Instead, binding of CLV3 to the CLV1 receptor complex causes the trafficking of CLV1 to lytic vacuoles, thus reducing CLV1 concentration at the plasma membrane (Nimchuk *et al.*, 2011).

A second complex that binds CLV3 is a tetramer comprising two CLV2 proteins, which are receptor-like kinases, and two CORYNE (CRN) proteins, which were predicted serine-threonine kinases (Bleckmann *et al.*, 2010; Kayes and Clark, 1998; Muller *et al.*, 2008) but have since been shown to lack any kinase activity (Nimchuk *et al.*, 2011). Interestingly, biochemical studies indicate that CLV1 can weakly associate with the CLV2/CRN complex, suggesting that these two complexes could interact in CLV3 signalling (Bleckmann *et al.*, 2010). However, *clv2* and *crn* mutations are additive in effect to *clv1*, suggesting that CLV1 operates independently from the CLV2-CRN complex to signal the presence of CLV3 (Kayes and Clark, 1998; Kinoshita *et al.*, 2010; Muller *et al.*, 2008). Furthermore, CLV2 is not required for CLV3-dependent targeting of CLV1 to lytic vacuoles (Nimchuk *et al.*, 2011).

More recently, a third receptor-like kinase known as RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2) was shown to form homo-dimers that bind the CLV3 peptide (Kinoshita

et al., 2010). RPK2 acts in parallel with the other complexes as *rpk2* mutations give additive phenotypes with both *clv1* and *clv2* mutations, and the RPK2 protein does not associate with CLV1 or CLV2 (Kinoshita *et al.*, 2010).

Based on these data, a negative feedback loop is thought to operate between the stem cells and OC to control the number and positioning of stem cells. *CLV3* is expressed by the stem cells and diffuses to repress *WUS* expression in the OC. *WUS* then feeds back to promote the expression of *CLV3*. However, since *WUS* acts non-cell autonomously, this has led to the hypothesis that an unknown mobile signal moves from the OC to the stem cells to promote *CLV3* expression.

1.2.2 Hormonal regulation of the CLV/WUS feedback loop

An additional level of regulation over the CLV/WUS feedback loop comes from the mitogenic hormone cytokinin, whose strongest signalling domain in the SAM correlates with the *WUS* expression domain (Gordon *et al.*, 2009). Type B ARABIDOPSIS RESPONSE REGULATORS (ARRs) are transcription factors that regulate the transcription of cytokinin response genes, whereas type A ARRs establish a negative feedback loop by repressing cytokinin signalling (reviewed by To and Kieber, 2008). Type B ARRs directly upregulate the expression of *WUS*, and repress the expression of *CLV1* and *CLV3* to indirectly upregulate *WUS* expression (Gordon *et al.*, 2009). *WUS* also establishes a positive feedback loop with cytokinin by repressing the transcription of *ARR5*, *ARR6*, *ARR7* and *ARR15* (Gordon *et al.*, 2009; Leibfried *et al.*, 2005), which are type A ARRs. *WUS* is expressed in a complementary domain to *ARR5* and binds 1kb from the transcriptional start site of the *ARR5* gene (Gordon *et al.*, 2009). Together, these data suggest that *WUS* acts to potentiate cells to respond to cytokinin and thus cell division.

1.2.3 A TALE of *KNOX* gene regulation in the SAM

Homeodomain transcription factors were first discovered in animals thirty years ago, and play key roles in metazoan development (McGinnis *et al.*, 1984; Scott and Weiner, 1984). Shortly after this, the first plant homeodomain transcription factor was discovered in maize, and was named Knotted-1 (KN1) as gain-of-function mutants produce ectopic “knots” of tissue on their leaves (Vollbrecht *et al.*, 1991). *KN1* is a member of a small family of *THREE AMINO ACID LOOP EXTENSION (TALE)* homeobox genes, known as *KNOTTED1-LIKE HOMEODOMAIN (KNOX)* genes. *KNOX* genes have been divided into three subclasses based on homeobox sequence similarity, phylogenetic analysis, expression patterns and intron positioning (Kerstetter *et al.*, 1994; Mukherjee *et al.*, 2009). Class I *KNOX* genes are present in all green plant lineages, and are expressed in overlapping domain within the SAM where they are required for SAM maintenance (Hake *et al.*, 2004; Jackson *et al.*, 1994). Class II *KNOX* genes have broad expression patterns and, at present, little is known about their function in development (Kerstetter *et al.*, 1994; Serikawa *et al.*, 1997). Finally, there is a single Class III *KNOX* gene known as *KNATM* which regulates leaf proximo-distal patterning and binds Class I *KNOX* proteins (Magnani and Hake, 2008).

There are four class I *KNOX* genes in the *A. thaliana* genome, including *SHOOTMERISTEMLESS (STM)*, *BREVIPEDICELLUS (BP)*, *KN1-LIKE IN ARABIDOPSIS THALIANA2 (KNAT2)* and *KNAT6*. *STM* is expressed throughout the SAM during early embryogenesis and loss-of-function *stm* mutants fail to initiate and maintain the SAM (Long *et al.*, 1996). This suggests that *STM* prevents the pluripotent stem cells of the SAM from differentiating. *BP* is expressed in the boundaries of the SAM, and genetic analysis revealed that *BP* acts redundantly with *STM* to regulate SAM maintenance (Byrne *et al.*, 2002). A segmental chromosomal duplication event in *A. thaliana* generated *KNAT2* and *KNAT6*, which are expressed at the base and boundaries of the SAM, respectively (Belles-Boix *et al.*,

2006; Byrne *et al.*, 2002). Although neither single mutant has a SAM phenotype, *KNAT6* acts redundantly with *STM* to regulate SAM and boundary maintenance (Belles-Boix *et al.*, 2006). Interestingly, *KNAT2* and *KNAT6* antagonise *BP* function as they are misexpressed in *bp* loss-of-function mutants, and are partly responsible for the downward-pointing silique phenotype of *bp* (Ragni *et al.*, 2008).

1.2.4 KNOX-mediated regulation of the SAM: orchestrating a hormonal harmony

KNOX proteins regulate SAM homeostasis, at least in part, by maintaining a hormonal environment of relatively high cytokinin levels and low gibberellin levels that are favourable for SAM maintenance (Figure 1.2; Hay *et al.*, 2002; Jasinski *et al.*, 2005; Yanai *et al.*, 2005). Cytokinin is an adenine-derived compound that acts as a mitogen by promoting *CYCLIN D3* expression, whereas gibberellin is a terpenoid compound that influences cell elongation by orientating microtubules (Riou-Khamlichi *et al.*, 1999; Shibaoka and Nagai, 1994). Maintaining a high cytokinin:gibberellin ratio in the SAM is important as artificially reducing cytokinin levels and increasing gibberellin levels terminates SAM activity (Jasinski *et al.*, 2005; Yanai *et al.*, 2005).

There are very few known targets of KNOX proteins, however, transcription of the gibberellin biosynthetic gene *GA20 oxidase1* is directly repressed by KNOX in many species, highlighting the importance of KNOX-mediated repression of gibberellin biosynthesis in SAM maintenance (Chen *et al.*, 2004; Hay *et al.*, 2002; Jasinski *et al.*, 2005; Ori *et al.*, 1999; Sakamoto *et al.*, 2001; Yanai *et al.*, 2005). Since gibberellins are diffusible molecules and could easily move from the leaf to the SAM, repression of biosynthesis alone would not be sufficient to regulate their levels in the SAM. However, KNOX also directly activates transcription of the catabolic gene *GA2 oxidase1* at the leaf-SAM boundaries (Bolduc and Hake, 2009; Jasinski *et al.*, 2005; Sakamoto *et al.*, 2001; Yanai *et al.*, 2005), likely preventing entry of gibberellin into the SAM.

Figure 1.2 *KNOX*-mediated regulation of hormonal balance within the SAM. Scanning electron micrograph of a SAM (blue), with two visible leaves (smallest leaf coloured yellow). An auxin activity focus (IAA; green) forms at the tip of the leaf and acts synergistically with AS1 to exclude *BP* expression from the *A. thaliana* leaf. *KNOX* genes are expressed within the SAM where they promote cytokinin activity (CK; red) whilst reducing gibberellin activity (GA; purple). *STM* up-regulates the expression of the cytokinin biosynthesis gene *IPT7*, leading to an increase in cytokinin levels in the SAM. Cytokinin activates the expression of *Type B ARR* genes that promote cytokinin signalling. However, a negative feedback loop operates within the cytokinin signalling pathway as *Type A ARR* genes are up-regulated in response to cytokinin and repress *Type B ARR* gene activity. *WUS* also acts to promote cytokinin signalling in the SAM by repressing *Type A ARR* gene activity. *STM* represses gibberellin activity in the SAM in two ways: firstly, by directly repressing the expression of the gibberellin biosynthesis enzyme GA20ox; secondly, by promoting the expression of the gibberellin catabolic enzyme GA2ox.



The significance of *KNOX*-mediated repression of gibberellin is clear as constitutive gibberellin signalling in the *spindly* mutant enhances shoot phenotypes of hypomorphic *stm* mutants (Hay *et al.*, 2002). Furthermore, exogenous application of gibberellin or constitutive gibberellin signalling suppresses *KNOX* gain-of-function phenotypes (Hay *et al.*, 2002). However, exogenous gibberellin application or constitutive gibberellin signalling does not mimic *KNOX* loss-of-function mutants (Hay *et al.*, 2002), suggesting that *KNOX* may regulate other factors to control SAM maintenance. *KNOX*-overexpressing plants have increased levels of cytokinin (Ori *et al.*, 1999; Yanai *et al.*, 2005), and *KNOX* proteins activate the cytokinin biosynthetic genes *ARABIDOPSIS THALIANA ISOPENTENYL TRANSFERASE7* (*AtIPT7*) and *AtIPT5*, and the cytokinin response gene *ARR5*. This suggests that *KNOX* can influence both cytokinin biosynthesis and response. *KNOX*-regulation of cytokinin levels is functionally important for SAM maintenance as exogenous application of cytokinin and overexpression of the cytokinin biosynthetic gene *ISOPENTENYL TRANSFERASE* (*IPT*) can partially rescue *stm* SAM defects (Yanai *et al.*, 2005). Furthermore, reduced cytokinin signalling in the *wooden leg* (*wol*) mutant causes the *stm* hypomorphic allele to become shootless (Jasinski *et al.*, 2005).

In light of these studies, it appears that *KNOX* proteins promote a high ratio of cytokinin:gibberellin, which is essential for SAM maintenance. The mechanistic output of this ratio may be to control the cellular activities within the SAM as high levels of cytokinin would promote cell division whilst low levels of gibberellin would prevent cell differentiation, thereby retaining a pool of pluripotent cells for the generation of lateral organs.

1.2.5 AS1-dependent repression of *KNOX* expression

KNOX genes are expressed within the SAM of all plant species studied to date, but their expression is down-regulated in leaf founder cells (Bharathan *et al.*, 2002; Smith *et al.*,

1992). The regulation of *KNOX* expression is crucial for leaf development as ectopic expression of *KNOX* genes causes meristems to form at the margins of leaves (Byrne *et al.*, 2000; Chuck *et al.*, 1996; Ori *et al.*, 2000; Vollbrecht *et al.*, 1991). This suggests a requirement for repressors of *KNOX* expression in the control of leaf development.

A decade ago, *ROUGH SHEATH2 (RS2)* was the first gene shown to repress *KNOX* expression in maize (Schneeberger *et al.*, 1998). *RS2* encodes a MYB domain protein whose orthologues are *ASYMMETRIC LEAVES1 (AS1)* and *PHANTASTICA (PHAN)* in *A. thaliana* and snapdragon, respectively (Byrne *et al.*, 2000; Schneeberger *et al.*, 1998; Timmermans *et al.*, 1999; Waites *et al.*, 1998). In *as1* mutants, the *KNOX* genes *BP*, *KNAT2* and *KNAT6* are ectopically expressed in leaves, correlating with ectopic shoot meristems and lobed leaf margin phenotypes (Byrne *et al.*, 2000; Ori *et al.*, 2000). Many of these aspects of the *as1* phenotype are rescued upon loss-of-function of all three *KNOX* genes, suggesting that AS1 repression of *KNOX* is functionally significant in *A. thaliana* (Ikezaki *et al.*, 2010). Although AS1 is required to repress *KNOX* gene expression in maize leaves, *KNOX* expression is downregulated in leaf founder cells of *rs2* (Schneeberger *et al.*, 1998). This suggests that ARP proteins act to maintain, and not establish, *KNOX* repression.

1.2.6 Multilevel control over *KNOX* expression and function

AS1 forms heterodimers with the LATERAL ORGAN BOUNDARIES (LOB) domain protein AS2 in the adaxial half of the leaf (Ori *et al.*, 2000; Xu *et al.*, 2003). Recently, the AS1-AS2 heterodimer was shown to directly bind to the promoter regions of *BP* and *KNAT2* (Guo *et al.*, 2008), likely via recruitment of the repressive chromatin complex HISTONE CHAPERONE HISTONE REGULATORY PROTEIN A (HIRA) (Phelps-Durr *et al.*, 2005). This suggests that AS1 may repress *KNOX* gene expression via an epigenetic mechanism. In further support of this, polycomb-group proteins with histone

methyltransferase activities repress *KNOX* expression (Katz *et al.*, 2004; Schubert *et al.*, 2006; Xu and Shen, 2008).

An additional level of regulation over KNOX protein activity has a potentially ancient origin, and involves interactions with an additional class of TALE homeodomain proteins known as the BEL1-like homeodomain family (BELL). Similar to TALE protein interactions in animals (Rieckhof *et al.*, 1997), KNOX and BELL proteins form heterodimers via interactions with their MEINOX domains, whilst the homeodomains bind the target DNA. The specific BELL partner in the interaction determines the downstream target genes and the subcellular localisation of the heterodimer (Bellaoui *et al.*, 2001; Bhatt *et al.*, 2004; Cole *et al.*, 2006; Hackbusch *et al.*, 2005; Smith *et al.*, 2002). The BELL protein BELLRINGER (BLR) forms heterodimers with both STM and BP, and has an important role in SAM maintenance (Byrne *et al.*, 2003; Roeder *et al.*, 2003). Since triple mutants of the *BELL* genes *BLR*, *POUND-FOOLISH* and *ARABIDOPSIS THALIANA HOMEODOMAIN 1* phenocopy *stm* mutants, it is likely that there are further heterodimers implicated in SAM development (Rutjens *et al.*, 2009).

In a further level of complexity, the class III KNOX protein KNATM interacts with both BP and BELL proteins (Magnani and Hake, 2008). Since the KNATM protein lacks a homeodomain, it cannot bind DNA itself (Magnani and Hake, 2008). However, it may be able to regulate transcription of KNOX targets by competing with other KNOX proteins for binding with BELLS, hence titrating the effects of KNOX proteins. Conversely, it may act as a cofactor to BP, and possibly other KNOX proteins (Magnani and Hake, 2008).

In conclusion, genetic analyses have revealed that a complex regulatory mechanism controls *KNOX* expression and action in the regulation of SAM maintenance. There are multiple pathways that regulate *KNOX* expression, including at the epigenetic level, and

through interactions with other TALE proteins, KNOX subcellular localisation and their target genes are defined. Furthermore, since KNOX proteins share homology of structure and action with animal TALE proteins, they may represent ancient regulators of cellular activities that have been recruited in plants to control SAM maintenance.

1.2.7 Boundary formation is required for SAM initiation

Development of the SAM is a classic example of how interpretation of positional information, as opposed to mosaic development, determines pattern formation. Over a decade ago, establishing a boundary between the SAM and leaf primordia was shown to be critical for SAM specification and maintenance (Souer *et al.*, 1996). NO APICAL MERISTEM/ATAF/CUP-SHAPED COTYLEDON (NAC) domain proteins are essential regulators of boundary development between the SAM and organ primordia in many species, including *A. thaliana*, tomato, petunia and *A. majus* (Aida *et al.*, 1997; Souer *et al.*, 1996; Vroemen *et al.*, 2003; Weir *et al.*, 2004). CUP-SHAPED COTYLEDON1 (*CUC1*), *CUC2* and *CUC3* in *A. thaliana*, and NO APICAL MERISTEM in petunia, are NAC domain genes that are expressed in the boundary region between organ primordia and the SAM. Their expression patterns are functionally significant as *nam* single loss-of-function mutants and *cuc1;cuc2* mutants fail to initiate a SAM, and seedlings have cotyledons fused around all sides, giving a goblet-shaped appearance (Aida *et al.*, 1997; Hibara *et al.*, 2006; Souer *et al.*, 1996; Takada *et al.*, 2001; Vroemen *et al.*, 2003).

It is thought that *CUC* genes regulate SAM formation by promoting the expression of *KNOX* genes. This is because *STM* is not expressed in *cuc1;cuc2* double mutants (Aida *et al.*, 1999), whilst ectopic *CUC1* expression induces ectopic *STM* expression and SAM formation (Takada *et al.*, 2001). This suggests that proper specification of boundaries between the cotyledons and the putative SAM is essential for a functional SAM. In turn, *KNOX* genes feed back to control the correct spatial localisation of *CUC* genes, thereby regulating SAM

maintenance via a positive feedback loop (Aida *et al.*, 1999). Recently, *STM* was shown to bind directly to the promoter of *CUC1*, but not the *CUC2* promoter, in the control of *CUC* expression (Spinelli *et al.*, 2011).

1.2.8 Adaxial/abaxial determinants regulate SAM initiation and maintenance

Genes that specify adaxial and abaxial identity in leaves also regulate the initiation and maintenance of the SAM. A group of transcription factors that specify leaf adaxial identity are the class III HOMEODOMAIN LEUCINE ZIPPER (HD-ZIP III) transcription factors, which include *REVOLUTA* (*REV*), *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*) (McConnell *et al.*, 2001; Prigge *et al.*, 2005). During embryogenesis, *HD-ZIP III* genes are expressed in the central core of the embryo and the adaxial side of the cotyledons (McConnell *et al.*, 2001; Prigge *et al.*, 2005), hence the SAM initiates within a domain of adaxial identity. This is functionally significant as *phb;phb;rev* triple mutants fail to initiate a SAM (Emery *et al.*, 2003; Prigge *et al.*, 2005). Furthermore, replacing adaxial identity with abaxial identity in mutants that misexpress the abaxial identity gene *KANADI* prevents the SAM from initiating (Kerstetter *et al.*, 2001). Although adaxial identity is required for SAM initiation, it is also sufficient to drive SAM formation as mutants that misexpress *PHABULOSA* form ectopic SAMs around the base of the leaf (McConnell and Barton, 1998). These results, therefore, suggest that correct specification of the adaxial domain during embryogenesis is both necessary and sufficient for SAM formation.

Although *HD-ZIP III* functions are required for SAM formation, tight regulation over their activity is required to control SAM maintenance. *LITTLE ZIPPER* proteins post-translationally inhibit HD-ZIP III function by competitively forming heterodimers with HD-ZIP III proteins (Kim *et al.*, 2008; Wenkel *et al.*, 2007). Establishing the correct level of repression of HD-ZIP IIIs is critical for correct SAM maintenance as *littlezipper3;littlezipper4* double mutants have expanded CZs, whereas overexpression of

LITTLEZIPPER3 inhibits the activity of the CZ, causing the SAM to terminate as a radialised leaf (Kim *et al.*, 2008; Wenkel *et al.*, 2007).

1.3 Control of leaf initiation: the only way is up

1.3.1 Leaf initiation

Over twenty years ago, clonal analyses in tobacco and cotton indicated that approximately 50-100 cells from the PZ of the SAM are recruited into an initiating primordium (Dolan and Poethig, 1998; Poethig and Sussex, 1985). Eudicot leaf primordia initially grow laterally from the SAM as rod-shaped structures before growing upwards (Long and Barton, 2001). This correlates with cells in the L2 layer of the SAM changing the direction of their division plane from anticlinal to periclinal (Poethig, 1987). Whilst the majority of cells recruited into a leaf are derived from the L2, cells from the L1 and L3 layers are also recruited into the initiating primordium (Poethig, 1987).

Leaf initiation is an iterative process, and the arrangement of leaves around the SAM is known as phyllotaxis. The most frequent phyllotactic arrangement in nature is the spiral phyllotaxis where the divergence angle between two successive organs is the golden angle of 137.5° . The patterning of phyllotaxis has long been known to be robust (Snow and Snow, 1931), and in 1868, it was suggested that lateral inhibition regulates the spacing of primordia (Hofmeister, 1868). Existing primordia were purported to produce a signal that prevents incipient primordia forming, causing incipient primordia to form in the largest gap between existing primordia (Hofmeister, 1868). In support of this, surgical removal of an existing primordium allows an incipient primordium to grow close to the excision site (Douady and Couder, 1996).

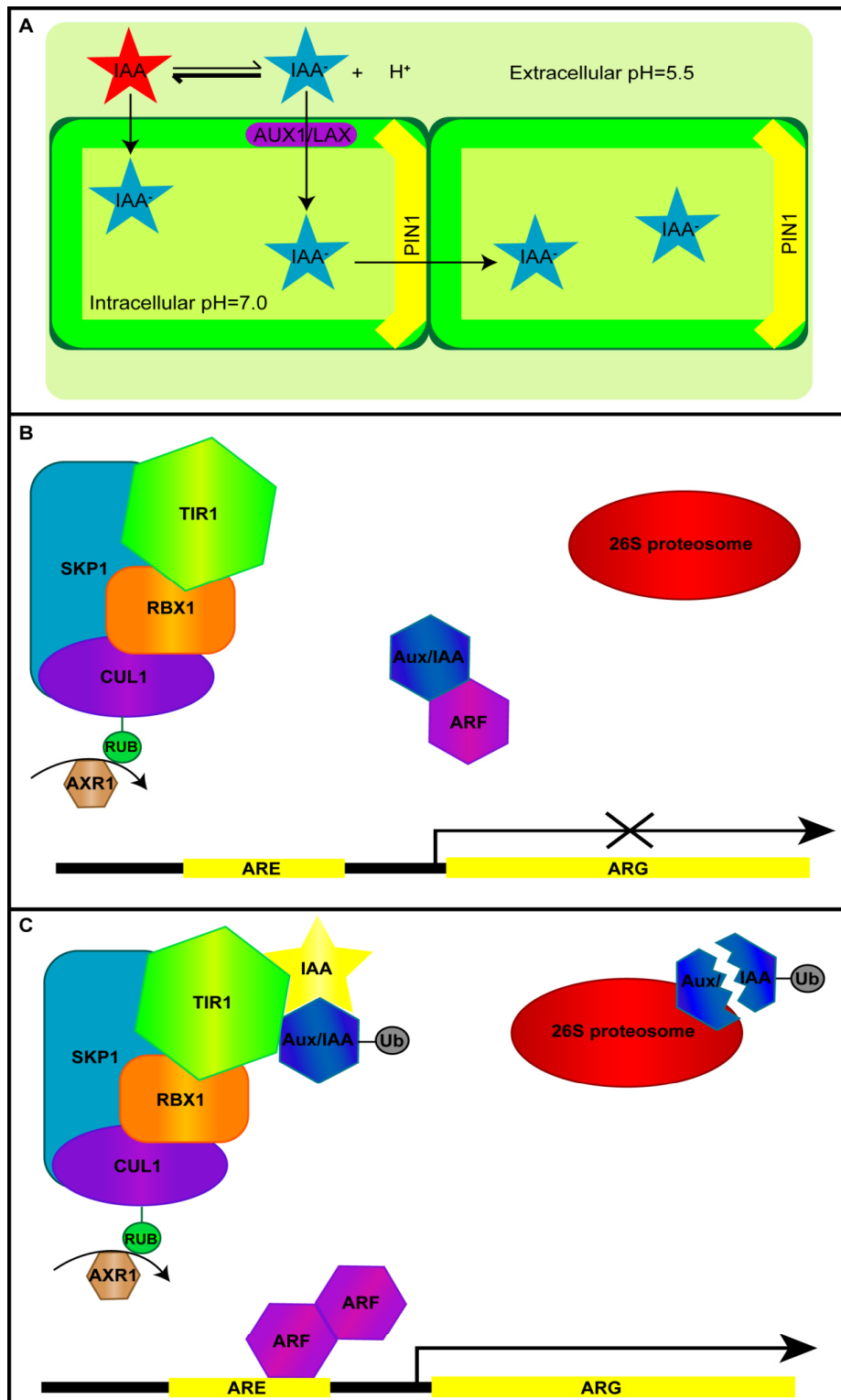
Despite knowledge of a robust patterning mechanism, the details of the mechanism remained unclear until a decade ago. Since then, the hormone auxin has been shown to play a critical role in the patterning of leaf initiation.

1.3.2 The regulation of auxin transport

Auxin is a generic term for a group of small indolic hormones whose effects on growth were first documented by Darwin in 1880, and have since been shown to have crucial roles in many aspects of plant development, including lateral organ initiation (Darwin and Darwin, 1880; Reinhardt *et al.*, 2000; Snow, 1937). Auxin is unique amongst plant hormones as it has directional transport, and in the 1970s the chemiosmotic polar diffusion model was formulated to model the basis of auxin transport (Figure 1.3 A; Goldsmith, 1977; Raven, 1975; Rubery and Sheldrake, 1974). This model postulated that since auxin is a weak acid, it is present in a protonated form in the acidic apoplast, and so is able to cross the plasma membrane by lipophilic diffusion to enter cells. Once in the comparatively more alkaline cytoplasm, auxin is deprotonated, and with an overall negative charge, it cannot diffuse back out of the cell. It, therefore, requires transport across the plasma membrane. There are two families of proteins that export auxin across the plasma membrane, and these are the PIN-FORMED (PIN) family and the ATP-binding cassette (ABC) family of transporters.

PIN proteins are auxin efflux facilitators that have a polar localisation at the plasma membrane, and the side of the cell to which they localise determines the direction of flow of auxin (Petrasek *et al.*, 2006; Vieten *et al.*, 2007; Wisniewska *et al.*, 2006; Yang and Murphy, 2009). Work over the last decade has revealed that auxin regulates PIN activity in two fundamental ways. Firstly, auxin promotes the transcription of *PIN* genes, thereby promoting PIN levels and activity (Vieten *et al.*, 2005). Secondly, PIN proteins have a dynamic subcellular localisation as they are constitutively endocytosed via a clathrin-dependent mechanism and recycled back to the plasma membrane (Dhonukshe *et al.*, 2007; Geldner *et*

Figure 1.3 Regulation of auxin transport and signalling. (A) The regulation of auxin transport. Auxin is a weak acid and is mainly present in its protonated, lipophilic form (IAA; red star) in the acidic apoplast (pale green); in its lipophilic form, auxin can freely diffuse across the cell membrane. However the hydrophilic form of auxin (IAA⁻; blue star) can be transported across the membrane by AUX1/LAX influx carriers (purple ellipse). In the more basic cytoplasm (mid green), auxin becomes deprotonated and cannot freely diffuse out of the cell. PIN1 auxin efflux carriers (yellow trapezia) are localised in the plasma membrane (bright green band) and transport auxin out of the cell. **(B)** The regulation of auxin signalling in the absence of auxin. In the absence of auxin, Aux/IAA proteins (dark blue hexagons) bind to ARF proteins (light purple hexagons), preventing AUXIN RESPONSE FACTOR (ARF) proteins binding to Auxin Response Elements (AREs; yellow bars), so repressing transcription of Auxin Response Genes (ARG; yellow bars). **(C)** The regulation of auxin signalling in the presence of auxin. The SCF complex is composed of TIR1 (green hexagon), SKP1 (blue rectangle), RBX1 (orange rectangle) and CUL1 (purple oval). Auxin binds to TIR1, leading to the ubiquitination of Aux/IAA proteins. This causes Aux/IAA proteins to be degraded by the 26S proteasome (red oval), and allows ARF proteins to dimerise, bind to AREs and promote the transcription of ARGs. The stability of the SCF complex is dependent on the addition of RUB1 molecules (green circle), catalysed by AXR1 (brown hexagon).



al., 2001). Auxin inhibits PIN endocytosis by directly binding to an endoplasmic reticulum localised protein known as AUXIN-BINDING PROTEIN1 (ABP1), which inhibits clathrin-dependent endocytosis and increases PIN activity at the plasma membrane (Effendi *et al.*, 2011; Paciorek *et al.*, 2005; Robert *et al.*, 2010).

Regulating the subcellular localisation of PIN proteins is critical for determining the flow of auxin, and there is strong evidence that the phosphorylation status of PIN proteins directly determines its polar localisation. Three membrane-associated AGC3 kinases known as PINOID (PID), WAG1 and WAG2 phosphorylate serine residues in conserved motifs within the central hydrophilic loop of PIN proteins (Dhonukshe *et al.*, 2010; Friml *et al.*, 2004; Michniewicz *et al.*, 2007). Overexpression of all three kinases causes PIN proteins to have an exclusively apical subcellular localisation (Dhonukshe *et al.*, 2010; Friml *et al.*, 2004), whereas loss-of-*PID* activity causes an apical to basal reversal in PIN polarity (Benjamins *et al.*, 2001; Christensen *et al.*, 2000; Friml *et al.*, 2004). Conversely, protein phosphatase 2A (PP2A) acts antagonistically to PID by dephosphorylating PIN proteins and promoting the basal localisation of PIN proteins (Michniewicz *et al.*, 2007). Taken together, these data strongly suggest that phosphorylation directly instructs the apical localisation of PIN proteins, whereas dephosphorylation appears to promote basal PIN localisation.

1.3.3 The role of auxin transport in leaf primordium initiation

Auxin has an instructive role in organ initiation as application of auxin to tomato SAMs stimulates primordium outgrowth from the site of application (Reinhardt *et al.*, 2000). However, only the PZ is competent to respond to auxin as application to the CZ has no effect on primordium outgrowth. Immunological data shows that PIN-FORMED1 (PIN1) proteins form convergence points in the epidermis at sites of incipient primordium formation prior to visible outgrowth (Reinhardt *et al.*, 2003). Formation of PIN1 convergence points is functionally required for lateral organ formation as *pin1* loss-of-function mutants fail to form

lateral organs on their inflorescences (Okada *et al.*, 1991). Since foci of auxin activity correlate with PIN1 convergence points, and application of auxin to the apices of *pin1* apices recovers organ initiation (Bainbridge *et al.*, 2008; Heisler *et al.*, 2005; Reinhardt *et al.*, 2003), it is inferred that PIN1 proteins direct auxin flow to promote primordium outgrowth.

For over a century, it was known that primordium initiation is an iterative process since primordium initiation repeatedly occurs in the largest space between existing primordia (Hofmeister, 1868). This suggests that if PIN1-mediated auxin flow is responsible for primordium initiation, then PIN1 localisation within the SAM would have to be dynamic and re-organise for each successive primordium initiation event. Live imaging of fluorescently-tagged PIN1 proteins in the SAM revealed that PIN1 localisation is initially directed away from adjacent primordia and then forms a convergence point defining the site of an incipient primordium (Heisler *et al.*, 2005). A concomitant increase in PIN1 levels also occurs at the site of primordium initiation as the increased auxin activity levels promote PIN1 transcription and retention of PIN1 protein in the plasma membrane (Heisler *et al.*, 2005; Paciorek *et al.*, 2005; Vieten *et al.*, 2005). Within the incipient primordium, subepidermal cells have basal PIN1 polarity which is predicted to internalise auxin flow (Heisler *et al.*, 2005; Reinhardt *et al.*, 2003). This, in conjunction with the PIN1 convergence point, creates the inverse fountain flow of auxin in leaf initiation. Once primordium outgrowth has occurred, PIN1 expression decreases and polarity changes so that a new convergence point forms in the largest space available between two existing primordia (Heisler *et al.*, 2005).

Since PIN1 localisation is critical for organ initiation, and is also highly dynamic, a key question is to determine what regulates PIN1 polarity in the control of primordium initiation. The loss-of-function phenotype of *pid* phenocopies *pin1* as *pid* mutants produces naked inflorescences (Bennett *et al.*, 1995). Furthermore, local application of auxin to the *pid*

inflorescence rescues primordium outgrowth, suggesting that *PID* functions to promote primordium initiation by correctly positioning auxin maxima (Reinhardt *et al.*, 2003).

Although auxin export from cells appears to have a critical role in leaf primordium formation, it is thought that auxin influx may also have a role to play. Loss-of-function of four *AUX* influx carriers causes phyllotaxis to be aberrant and, correlating with this, discrete foci of auxin activity are lost in these SAMs (Bainbridge *et al.*, 2008). Thus, despite the passive entry of auxin into cells, it appears that auxin transport into cells is required for correct patterning of leaf initiation in the SAM.

1.3.4 Auxin-mediated regulation of gene expression

The first experiments investigating how auxin rapidly induces changes in gene expression were performed over four decades ago (Key *et al.*, 1967; Theologis *et al.*, 1985; Theologis and Ray, 1982). A decade later, selective protein degradation was implicated in auxin response as many auxin-resistant mutants were found to be defective in components of the ubiquitin-proteasome pathway (del Pozo and Estelle, 1999; Del Pozo *et al.*, 1998; Leyser *et al.*, 1993). Subsequently, auxin signalling was shown to be controlled by an SCF^{TIR1/AFB} E3 ubiquitin ligase complex that has four major components, including the F-box protein TRANSPORT INHIBITOR RESPONSE1 (TIR1). TIR1 is an auxin receptor that directly binds auxin, allowing substrates to bind (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005; Tan *et al.*, 2007). Binding of auxin to TIR1 causes the ubiquitination of target proteins within degron sequences, causing their degradation by the 26S proteasome (Figure 1.3 B-C; Dharmasiri and Estelle, 2004; Vierstra, 2003).

Target proteins of TIR1 include repressors of auxin signalling known as Aux/IAAs (Pufky *et al.*, 2003; Remington *et al.*, 2004). There are twenty nine *Aux/IAA* genes in the *A. thaliana* genome, many of which act redundantly (Overvoorde *et al.*, 2005; Remington *et al.*,

2004). In the absence of auxin, Aux/IAAs form heterodimers with, and repress the activity of, AUXIN RESPONSE FACTOR (ARF) proteins to prevent auxin signalling (Figure 1.3 B; Ulmasov *et al.*, 1997). ARFs are transcription factors with a family of twenty three members that bind to a conserved hexanucleotide auxin responsive element (ARE) in the presence of auxin, regulating the transcription of auxin-inducible genes (Figure 1.3 C; Ballas *et al.*, 1993; Ulmasov *et al.*, 1999).

Auxin signalling has a critical role in the control of organ initiation as the loss-of-function ARF mutant *monopteros* mutants fails to initiate organ primordia from the inflorescence (Hardtke and Berleth, 1998), phenocopying *pin1* mutants. Crucially, application of auxin to *mp* inflorescences does not rescue primordium initiation, which is in contrast to *pin1* (Reinhardt *et al.*, 2003). This finding suggests that *MP* is involved in signalling the presence of auxin to promote the outgrowth of a primordium. Interestingly, *mp;pin1* double mutants do not form leaves, suggesting that combined auxin transport and auxin signalling is required for leaf initiation (Schuetz *et al.*, 2008). Auxin signalling is also required for normal leaf development as *auxin resistant1* (*axr1*) mutants, in which the activity of the SCF^{TIR1/AFB} E3 ubiquitin ligase complex is reduced, produce leaves with more deeply serrated margins compared to wild type (Leyser *et al.*, 1993; Pozo *et al.*, 1998).

Although little is known about the downstream targets of auxin signalling that regulate leaf initiation, correlative expression patterns during leaf initiation may help identify targets. As the leaf primordium emerges from the SAM, PIN1 and auxin activity levels increase whereas *STM* levels are drastically reduced (Heisler *et al.*, 2005). Furthermore, loss-of-*STM* function in the *pin1* mutant background strongly enhances the *pin1* phenotype (Aida *et al.*, 2002; Hay *et al.*, 2006). Loss-of-*BP* function also suppresses leaf number defects and organ initiation in *pin1* mutants (Hay *et al.*, 2006). These data suggest that auxin transport and signalling could regulate *KNOX* expression. Additionally, *LEAFY* (*LFY*) gene expression

increases concomitantly with PIN1 expression as the leaf primordium is initiated (Heisler *et al.*, 2005), suggesting that PIN1 and *LFY* may interact to promote leaf initiation. However, stronger evidence is required to determine the downstream targets of auxin signalling to unravel the mechanistic basis of how auxin patterns leaf initiation.

1.3.5 The role of the cell wall and tension in the regulation of leaf initiation

Computational modelling studies have shown that auxin dynamics can account for the patterning of organ initiation (Jonsson *et al.*, 2006; Smith *et al.*, 2006). However, alternative theories have been proposed to describe the positioning of organ initiation based on physical forces rather than chemical signals. The biophysical theory was proposed over a decade ago and suggests that the positioning of organs arise at sites of tissue buckling caused by differences in tension between the L1 layer and the tissue beneath (Green *et al.*, 1996). A recent study suggested that subcellular PIN1 localisation and microtubule organisation is determined by the mechanical force experienced by the individual cell within the epidermis (Heisler *et al.*, 2010). Auxin accumulation within a cell promotes growth by expansion (Reinhardt *et al.*, 2000) which exerts a force on an adjacent cell; this causes PIN1 proteins to localise to the side of the cell experiencing the force and microtubules to orient parallel to the direction of the stress (Heisler *et al.*, 2010). This would create a positive feedback loop between auxin transport and tension within the SAM.

It is well established that auxin stimulates leaf initiation, however, it has also been shown that application of expansin and pectin methyl esterase, which loosen the cell wall, can phenocopy auxin application by stimulating leaf initiation at the site of application (Fleming *et al.*, 1997; Hamant *et al.*, 2008). A later study showed that the presence of the cell wall is required for PIN polarity as removing the cell wall causes PIN polarity to become apolar (Feraru *et al.*, 2011). In addition, CELLULOSE SYNTHASE CATALYTIC SUBUNIT 3 (CESA3), an enzyme involved in synthesising cellulose, is also required for PIN polarity as

loss-of-function *cesa3* mutants have symmetrically localised PIN proteins (Feraru *et al.*, 2011). However, as yet, no functional significance has been ascribed to cell wall-mediated regulation of PIN polarity in the SAM.

1.4 Shaping leaf development

Leaf primordia initiate as radially symmetrical structures and comprise cells that are initially histologically and transcriptomically indistinguishable from each other (Bowman *et al.*, 2002). However, to generate diversity in leaf shape, leaf primordia must be patterned to generate three axes of polarity: the adaxial-abaxial axis, the proximo-distal axis and the medio-lateral axis. Patterning the adaxial-abaxial axis is critical to specify an upper surface for photosynthesis and a lower surface for gas exchange, whereas patterning along the proximo-distal axis sculpts leaf margin shape.

1.4.1 Back-to-front: the establishment of the adaxial-abaxial axis

Surgical experiments performed sixty years ago in potato first hinted at the critical role of positional information, as opposed to cell lineage, in the patterning of the adaxial-abaxial axis (Sussex, 1951) as a mobile signal from the SAM was proposed to regulate adaxial-abaxial polarity. More recently, laser ablation and microsurgery showed that a signal emanating from the L1 layer of the SAM promotes adaxial cell fate, and that sustained exposure to the signal is required for correct adaxial specification in the leaf (Reinhardt *et al.*, 2003). Although the identity of the signal is not currently known, a complex, multilevel pathway has emerged that patterns the adaxial-abaxial axis of the leaf.

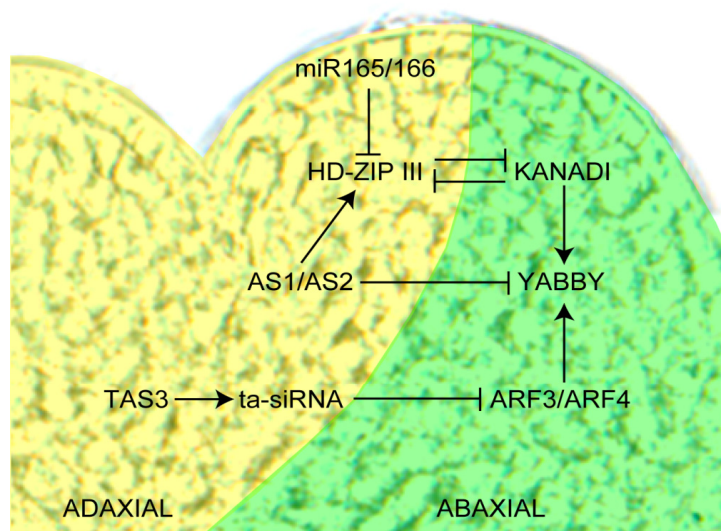
The first gene implicated in adaxial-abaxial patterning was *PHAN*, which encodes a MYB-domain transcription factor in *A. majus* (Waites *et al.*, 1998). Loss-of-function *phan* mutants produce abaxialised leaves with no blade, suggesting that *PHAN* functions to promote adaxial leaf identity and leaf blade outgrowth (Waites *et al.*, 1998). Although *PHAN*

orthologues in tomato and tobacco also control adaxial leaf identity (Kim *et al.*, 2003; McHale and Koning, 2004), loss-of-function of the *PHAN* orthologues *AS1* and *RS2* in *A. thaliana* and maize, respectively, do not have severe leaf polarity defects (Byrne *et al.*, 2000; Timmermans *et al.*, 1999; Tsiantis *et al.*, 1999). This indicates that other pathways are likely to act redundantly in regulating adaxial–abaxial identity.

Antagonistic interactions between *HD-ZIP III* genes and *KANADI* genes play a critical role in regulating adaxial-abaxial leaf polarity in *A. thaliana* (Figure 1.4). The *HD-ZIP III* genes *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*) are expressed in the adaxial side of the leaf where they promote adaxial cell fate (Emery *et al.*, 2003; McConnell *et al.*, 2001; Otsuga *et al.*, 2001; Prigge *et al.*, 2005). In contrast, the *KANADI* genes *KAN1*, *KAN2* and *KAN3* are expressed in the abaxial side of leaves (Eshed *et al.*, 2001; Eshed *et al.*, 2004; Kerstetter *et al.*, 2001), and loss-of-function *kan* mutants produce radial, adaxialised leaves with ectopic expression of *HD-ZIP III*s (Eshed *et al.*, 2004; Kerstetter *et al.*, 2001). Thus, the mutual repression between *HD-ZIP III* and *KANADI* proteins generates their complementary expression domains and provides a fundamental mechanism for the patterning of the adaxial-abaxial axis of the leaf.

A complex regulatory network also controls the expression of *HD-ZIP III* and *KANADI* genes. In *A. thaliana*, loss-of-*ARF3* and *ARF4* functions suppress *KAN1* misexpression phenotypes, despite *ARF3* and *ARF4* expression not being dependent on *KANADI* activity, suggesting that *ARF3* and *ARF4* act in parallel to *KANADI* to promote abaxial leaf fate (Pekker *et al.*, 2005). In contrast, *AS1* and *AS2* act antagonistically to *ARF3* and *ARF4* by repressing downstream polarity targets (Garcia *et al.*, 2006; Li *et al.*, 2005; Xu *et al.*, 2006). Although *as1* and *as2* mutants do not have leaf polarity defects, constitutive overexpression of *AS2* causes leaf adaxialisation, suggesting that the *AS1-AS2* complex could regulate polarity redundantly with other factors in *A. thaliana* (Lin *et al.*, 2003).

Figure 1.4 Gene regulatory networks controlling adaxial-abaxial polarity. A mutual antagonism exists between *HD-ZIP III* genes, which promote adaxial leaf fate, and *KANADI* genes, which promote abaxial leaf fate. *HD-ZIP III* expression is repressed by *miR165/166* and promoted by *AS1* and *AS2*. *YABBY* genes promote abaxial leaf fate and are activated by *KANADI* and *ARF3/4*, but are repressed by *AS1/2*. In turn, *ARF3/4* expression is regulated by a small RNA pathway involving *TAS3* which promotes the expression of *ta-siRNAs*, leading to the repression of *ARF3/4*. This complex regulatory network creates a sharp boundary along the adaxial-abaxial axis of the leaf.



Pathways regulating translation are likely involved. *PIGGYBACK* (*PGY*) genes encode cytoplasmic large subunit ribosomal proteins, and loss-of-function *pgy* mutations are enhancers of *as1*, causing severe leaf polarity defects (Pinon *et al.*, 2008). *PGY* also interact with *HD-ZIP III* and *KAN* genes to control leaf adaxial-abaxial development (Pinon *et al.*, 2008).

YABBY transcription factors are a hub through which many adaxial-abaxial specificity determinants act. Mutants with reduced *YABBY* activities produce narrow leaves that are partially adaxialised, whereas overexpression of *YABBYs* causes partial abaxialisation of leaves (Eshed *et al.*, 2001; Golz *et al.*, 2004; Sawa *et al.*, 1999; Siegfried *et al.*, 1999). *KAN* and *ARF3/ARF4* promote the expression of the *YABBY* gene *FILAMENTOUS FLOWER1* in the abaxial leaf domain, whilst *AS1* and *AS2* repress *FIL* expression in the adaxial side of the leaf (Eshed *et al.*, 2004; Garcia *et al.*, 2006; Li *et al.*, 2005; Lin *et al.*, 2003; Xu *et al.*, 2006).

Small RNAs (sRNAs) have also been implicated as important regulators of a precise boundary between the adaxial and abaxial domain of the leaf (Emery *et al.*, 2003; Juarez *et al.*, 2004; Kidner and Martienssen, 2004; Mallory *et al.*, 2004). *miR165/miR166* are expressed in the abaxial leaf domain where they prevent the ectopic expression of *PHB*, *PHV* and *REV* (Juarez *et al.*, 2004; Kidner and Martienssen, 2004). Restriction of *HD-ZIP III* transcripts to the adaxial domain is functionally relevant because miRNA-insensitive *HD-ZIP III* alleles produce adaxialised leaves, whereas overexpression of *MIR165B* causes abaxialisation of leaves (Emery *et al.*, 2003; Juarez *et al.*, 2004; Kidner and Martienssen, 2004; Mallory *et al.*, 2004; McConnell *et al.*, 2001). In contrast, *ARF3* and *ARF4* are targeted by *TAS3*-derived *trans*-acting short-interfering RNAs (ta-siRNAs) (Allen *et al.*, 2005; Williams *et al.*, 2005). *A. thaliana* ta-siRNA mutants do not have leaf polarity defects, suggesting that they act redundantly with other components to control adaxial/abaxial

polarity (Adenot *et al.*, 2006; Fahlgren *et al.*, 2006; Hunter *et al.*, 2006; Peragine *et al.*, 2004). However, in maize *LEAFBLADELESS* (*LBL*) encodes a key component of the ta-siRNA biogenesis pathway that prevents the ectopic accumulation of *miR166* in the adaxial leaf domain (Juarez *et al.*, 2004). Loss-of-function *lbl* mutants produce abaxialised leaves due to the ectopic expression of *miR166* in the adaxial domain of the leaf (Juarez *et al.*, 2004), suggesting that the ta-siRNA pathway has a critical role in adaxial/abaxial polarity in maize leaves.

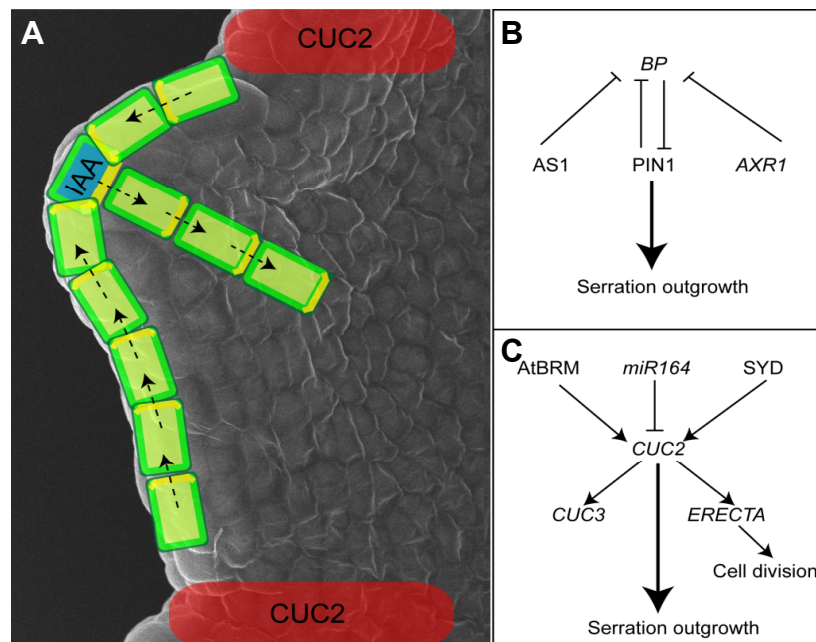
1.4.2 The role of the margin in new growth axis formation

Since antiquity, it has been noted that there is variation in leaf margin morphology (Theophrastus, 1916), whilst the genetic basis of leaf margin morphology began to be studied over a century ago (Correns, 1909). Despite these early observations, the patterning mechanisms that operate along the proximo-distal axis to sculpt leaf margin morphology have not been studied in depth. Recent studies showed that regulatory networks including hormones, transcription factors, miRNAs and growth components are implicated in shaping leaf margin morphology, although the degree to which these factors interact is not clear.

PIN1-mediated polar auxin transport has a fundamental role in shaping the margins of leaves as *pin1* leaves do not produce serrations (Hay *et al.*, 2006). PIN1 forms convergence points at the margins of leaves and, correlating with this, foci of auxin activity form at sites of

serration development (Figure 1.5 A; Hay *et al.*, 2006). The first PIN1 convergence point occurs midway along the leaf margin during early leaf development (Scarpella *et al.*, 2006), and subsequent PIN1 convergence points form basipetally along the proximo-distal axis of the leaf, correlating the iterative formation of serrations (Hay *et al.*, 2006). Abolishing auxin transport prevents the formation of auxin activity maxima and serration development (Hay *et al.*, 2006), suggesting that PIN1 patterning along the margin of the leaf is critical for leaf shape determination by controlling the spatial distribution of the growth regulator auxin.

Figure 1.5 Regulation of serration development by auxin transport and *CUC2* pathways. (A) Scanning electron micrograph close-up of a serration. PIN1 (yellow trapezia) has a polar subcellular localisation and forms convergence points at sites of serration development, driving the flow of auxin (broken arrows) to create a focus of auxin activity (IAA; blue cell) at the tip of the serration. PIN1 adopts a basal subcellular localisation in the cells beneath the convergence point, causing the internalisation of auxin flow. CUC2 (red rectangles) is expressed in the sinus regions between serrations. (B) Auxin transport mediated by PIN1 represses *BP* expression in the *A. thaliana* leaf to promote serration outgrowth. Auxin signalling, mediated by *AXR1*, converges with AS1 function to repress *BP* expression in the *A. thaliana* leaf. (C) *CUC2* transcription is repressed at the epigenetic level by *AtBRM* and *SYD*, and at the post-transcriptional level by *miR164*. *CUC2* promotes *CUC3* and *ERECTA* transcription to control serration outgrowth.



Recent studies have shown that serration development is associated with vasculature development, and polar auxin transport is thought to be intimately involved in both processes (Scarpella *et al.*, 2006). PIN1 expression is the earliest known marker of vein development, and PIN1 convergence point formation at the leaf margin correlates with the formation of secondary veins that connect the midvein to the leaf margin (Scarpella *et al.*, 2006). Crucially, reducing polar auxin transport both prevents serration development and disrupts vasculature development by causing increased numbers of secondary veins to form (Hay *et al.*, 2006; Scarpella *et al.*, 2006). Thus, it will be interesting to determine the extent to which serration and vasculature development are coupled to control leaf margin morphology.

Auxin is further implicated in leaf shape patterning as the auxin signalling pathway acts synergistically with AS1 to repress *BP* expression in *A. thaliana* leaves (Hay *et al.*, 2006). Loss-of-function *axr1;asl* double mutants produce leaves that are deeply lobed, and misexpress *BP* in addition to producing ectopic stipules in the sinuses (Hay *et al.*, 2006). In *A. thaliana* leaves misexpressing *BP*, PIN1 expression is repressed in the distal part of the leaf but persists for longer in the developing lobes, correlating with more deeply lobed phenotypes (Hay *et al.*, 2006). This suggests that *BP* may regulate PIN1 expression to promote prolonged localised growth and therefore pattern leaf margin morphology (Figure 1.5 B).

1.4.3 Patterning the leaf margin: getting the kNACk of it

The transcription factor CUC2 plays a critical role in shaping the *A. thaliana* leaf margin by regulating both serration initiation and depth (Hasson *et al.*, 2011; Nikovics *et al.*, 2006). *CUC2* is expressed along the leaf margin and is downregulated at sites of serration development (Figure 1.5 A). This is critical for serration development as *cuc2* loss-of-function mutants fail to produce serrations (Nikovics *et al.*, 2006). *CUC2* transcripts are also targeted for degradation by *miR164*, thereby restricting the *CUC2* expression pattern (Guo *et*

al., 2005; Laufs *et al.*, 2004; Mallory *et al.*, 2004; Nikovics *et al.*, 2006; Sieber *et al.*, 2007). This miRNA-dependent regulation of *CUC2* expression is functionally relevant for leaf margin development as *mir164a* mutants and transgenic plants expressing a miRNA-resistant *CUC2* gene produce leaves with more deeply serrated margins (Mallory *et al.*, 2004; Nikovics *et al.*, 2006). On the basis of these data, a model was proposed in which the relative levels of *miR164* and *CUC2* determine the depth of the outgrowth formed at the leaf margin, as higher *CUC2:miR164* ratios produce smoother margins (Nikovics *et al.*, 2006). Interestingly, *miR164* is not thought to initially downregulate *CUC2* at sites of serration formation since *mir164a* mutants produce leaves with similar numbers of serrations as wild type, and *MIR164A* is not expressed at the serration tip during serration initiation (Nikovics *et al.*, 2006). These data suggest that additional factors regulate *CUC2* expression.

CUC2 has also been shown to genetically interact with *CUC3* to control serration development by positively regulating *CUC3* transcription and its own (Figure 1.5 C; Hasson *et al.*, 2011). *CUC3* is a NAC domain gene that is not regulated by *miR164* but is repressed by TCP3 (Koyama *et al.*, 2007). *CUC3* is expressed in the sinuses of serrations and is thought to regulate serration depth as loss-of-function mutants produce leaves with shallower serrations than wild type (Hasson *et al.*, 2011). Expressing *CUC3* under the *CUC2* promoter cannot rescue serration initiation, suggesting that *CUC3* function cannot compensate for *CUC2* in the initiation of serrations (Hasson *et al.*, 2011). In contrast, *CUC1* is not thought to function in serration development as *CUC1* is not transcribed in *A. thaliana* leaves and *cuc1* mutants produce leaves with serrated margins (Mallory *et al.*, 2004). However, ectopic expression of *CUC1* under the *CUC2* promoter can rescue serration development in *A. thaliana* (Hasson *et al.*, 2011).

It has also been shown that there is an epigenetic component in the control of *CUC2* expression (Kwon *et al.*, 2006). AtBRM is a SWI/SNF chromatin remodelling ATPase that

was identified in a genetic screen as an enhancer of the *cuc2* phenotype (Kwon *et al.*, 2006). AtBRM and the closely-related protein SYD promote the transcription of *CUC2* in the boundary between the SAM and organ primordia (Kwon *et al.*, 2006). Although *CUC2* expression is maintained by an epigenetic mechanism, it is not known whether AtBRM and SYD act directly on the *CUC2* locus and whether they regulate *CUC2* expression at the leaf margin.

Despite the important role that *CUC2* plays in serration development, very little is known about its mode of action. Phenotypic analysis of *cuc2-ID*, a semi-dominant mutant of *cuc2*, suggests that *CUC2* promotes leaf growth by prolonging cell division and preventing cell expansion (Larue *et al.*, 2009). Furthermore, a genetic screen for suppressors of the *cuc2-ID* phenotype indicate that the receptor kinase *ERECTA* acts downstream of *CUC2* to promote cell division (Larue *et al.*, 2009). Thus, *CUC2* may regulate the development of serrations via a cell division-dependent patterning mechanism (Figure 1.5 C).

1.4.4 Sizing up: the regulation of growth within the leaf

Leaf organ size is a defining characteristic of many species and is extremely predictable under constant environmental conditions, suggesting that regulation of growth has a genetic basis. Furthermore, periclinal chloroplast chimeras suggest that positional information is required for coordinating growth within leaves (Marcotrigiano, 2001). There are three main stages of growth: cell division, cell differentiation and cell expansion, although these stages are not mutually exclusive as they can overlap within a single leaf.

Clonal analyses in tobacco and cotton indicate that approximately 50-100 cells from each of three cell layers of the SAM are recruited into an initiating primordium (Dolan and Poethig, 1998; Poethig and Sussex, 1985). Cells within the centre of the primordium give rise to the middle and tip of the leaf, whereas peripheral cells generate the lateral parts of the leaf

(Poethig and Sussex, 1985). In tobacco, submarginal cells divide parallel to the margin and are not thought to contribute to lamina expansion (Poethig and Sussex, 1985); cell division also ceases at the same time in marginal and intercalary regions, suggesting that cell growth at the margin does not regulate tobacco leaf shape (Poethig and Sussex, 1985). Although submarginal cells in cotton leaves divide perpendicular to the margin, growth is not polarised and is not thought to control leaf margin morphogenesis (Dolan and Poethig, 1998). Significantly, clonal analyses in tobacco and cotton, along with quantitative analyses of mitotic indices in many species such as *Jasminum*, *Tropaeolum*, *Lupinus* and *Xanthium*, demonstrate that marginal meristems are unlikely to exist in leaves (Dolan and Poethig, 1998; Poethig and Sussex, 1985). This is in contrast with the work of Avery who proposed that marginal meristems exist at the leaf margin where cell divisions occur perpendicular to the leaf margin and contribute greatly to leaf shape (Avery, 1933).

Recently, clonal analysis and molecular studies in *A. thaliana* demonstrated that a proliferative region at the junction between the leaf blade and petiole contributes to leaf organogenesis (Ichihashi *et al.*, 2011). Cell division is more active in the blade/petiole junction compared with the more distal regions of the leaf blade and petiole, and the smallest cells of the leaf are located in the blade/petiole junction during leaf primordium development (Ichihashi *et al.*, 2011). In addition, clonal analysis revealed that cells of the blade and petiole likely arise from the blade/petiole junction (Ichihashi *et al.*, 2011), suggesting that cell divisions at the blade/petiole junction in *A. thaliana* leaves may have important roles in controlling leaf growth.

Over twenty years ago, clonal analyses in tobacco also showed that cell division becomes progressively restricted to the more basal parts of the leaf (Poethig and Sussex, 1985). Since then, the arrest of cell division was shown to occur basipetally along the proximo-distal axis of the *A. thaliana* leaf (Donnelly *et al.*, 1999), and the boundary between

cell division and expansion was called the cell cycle arrest front (Donnelly *et al.*, 1999; Nath *et al.*, 2003; White, 2006). Quantitative studies in *A. thaliana* revealed that the cell cycle arrest front remains at a constant distance of approximately 100µm from the base of the leaf (Kazama *et al.*, 2010). It is thought that the cell proliferation regulator KLUH/CYP78A5 (KLU) generates a gradient of an as yet unknown mobile growth factor along the proximo-distal axis of the leaf to position the cell cycle arrest front (Kazama *et al.*, 2010).

1.4.5 Genetic regulation of leaf growth

Class II TCP genes also regulate the shape and progression of the cell cycle arrest front in leaves (Nath *et al.*, 2003; Palatnik *et al.*, 2003). CINCINNATA (CIN) is a class II TCP transcription factor in *A. majus*, whereas five *TCP* genes are downregulated in *jaw1-D* *A. thaliana* dominant mutants. Loss-of-function *cin* mutants and *jaw1-D* dominant mutants produce leaves with crinkled surfaces and serrated leaf margins (Nath *et al.*, 2003). This is because delayed progression of the cell cycle arrest front causes the margin to grow for longer compared to the medial regions (Nath *et al.*, 2003). Once the cell cycle arrest front has passed, the redundantly acting *PEAPOD* (*PPD*) genes *PPD1* and *PPD2* promote differentiation of stomatal and procambial cells in *A. thaliana* leaves (White, 2006).

Genetic analyses have shown that growth regulation along the medio-lateral and proximo-distal axes can be uncoupled, and that cell number can be uncoupled from cell expansion (Kim *et al.*, 1999; Narita *et al.*, 2004). The ROTUNDIFOLIA4 (ROT4) peptide represses cell division along the proximo-distal axis to control cell number (Narita *et al.*, 2004). In contrast, *ROT3* promotes cell expansion along the proximo-distal leaf axis (Kim *et al.*, 1999). Since ROT3 catalyses the activation step of brassinosteroid biosynthesis (Kim *et al.*, 2005), this strongly indicates that brassinosteroids have a key role in the regulation of leaf growth. In contrast, *ANGUSTIFOLIA3* (*AN3*) promotes cell division along the medio-lateral axis of the leaf to positively regulate leaf width growth (Horiguchi *et al.*, 2005). Furthermore,

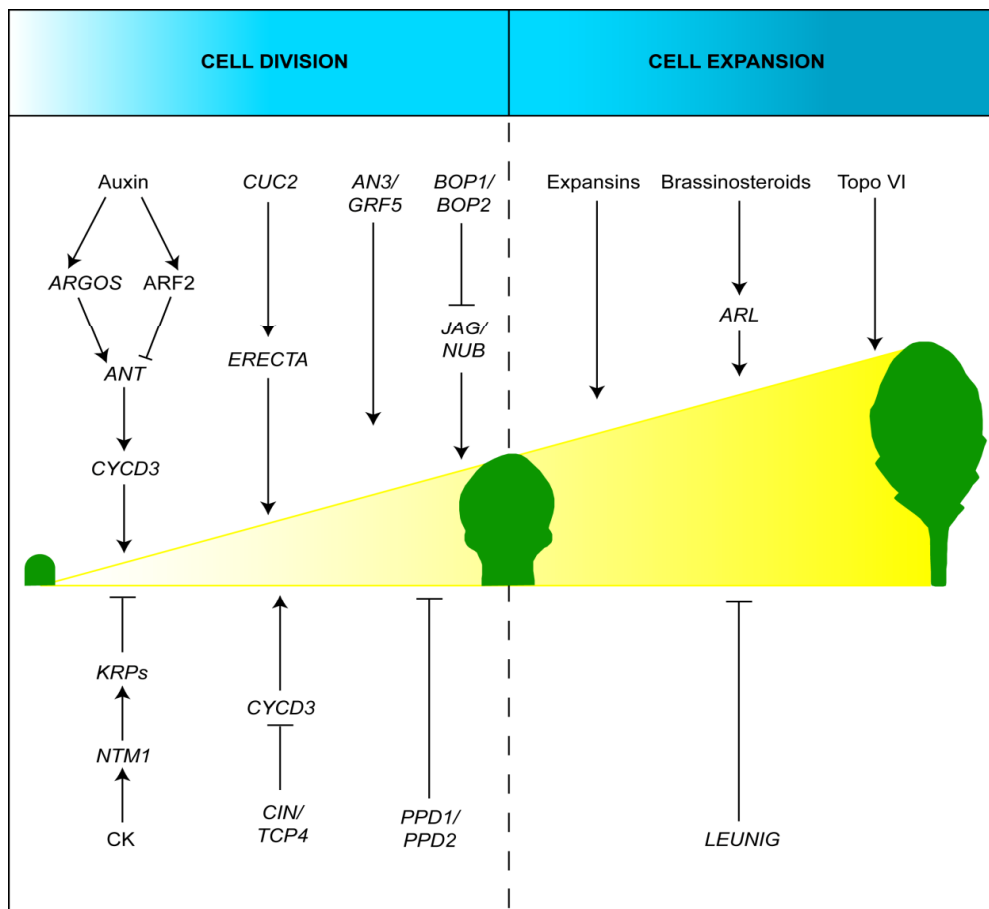
yeast two-hybrid assays have shown that AN3 interacts with AtGRF5 to promote cell division at the margins of the leaf (Horiguchi *et al.*, 2005).

1.4.6 Positive and negative regulators in leaf growth

Leaf growth is controlled by a complex gene regulatory network (Figure 1.6). AINTEGUMENTA (*ANT*) is a transcription factor that is expressed in the incipient leaf primordium where it promotes *CYCD3;1* expression and extends the period of cell division within the leaf (Elliott *et al.*, 1996; Klucher *et al.*, 1996; Krizek, 1999; Mizukami and Fischer, 2000). Auxin both positively and negatively regulates *ANT* expression since the auxin-inducible gene *ARGOS* acts as an upstream activator of *ANT* (Hu *et al.*, 2003), whilst *ARF2* represses *ANT* expression (Schruff *et al.*, 2006). However, it is not currently known whether *ARGOS* and *ARF2* have differences in auxin sensitivity that influence the direction of *ANT* expression. Additional factors that regulate cell proliferation in leaf development include *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2* (Hepworth *et al.*, 2005), which repress the expression of the C₂H₂ zinc finger transcription factors JAGGED (*JAG*) and NUBBIN (*NUB*) (Dinneny *et al.*, 2006; Dinneny *et al.*, 2004; Ohno *et al.*, 2004). Interestingly, *jag;nub* double mutants produce smaller leaves with fewer cell divisions that are more deeply serrated than wild type. This suggests that the duration of cell division may regulate the depth of marginal outgrowth produced.

In both plants and animals, cyclin-dependent kinases regulate cell cycle progression by selectively regulating the activity of proteins known as cyclins that promote cell cycle transitions (Dunphy, 1994; Pines, 1994). Transgenic plants overexpressing cyclin-dependent kinase *KIP-RELATED PROTEIN2* (*KRP2*) produce leaves with decreased rates of cell division and larger cell volumes, correlating with small leaves and deeply serrated margins (De Veylder *et al.*, 2001). The NAC domain transcription factor *NTM1* positively regulates the transcription of *KRP2*, and *NTM1* expression is thought to be under hormonal control as

Figure 1.6 A complex gene regulatory network controls cell division and cell expansion during simple leaf development. Auxin promotes cell division in leaves by promoting ARGOS and ARF2 expression, leading to the activation of *CYCD3* expression. *CUC2* regulates cell division by promoting the expression of *ERECTA*, whereas *BOP1/BOP2* promote cell division by activating *JAG/NUB* expression. *AN3/GRF* promote cell division, although the mechanism by which they regulate cell division is not known. Cell division is repressed by KRPs, which are negatively regulated by the cytokinin-dependent control of NTM1. CIN/TCP4 act to repress cell division by repressing *CYCD3* expression. PPD1/PPD2 also act to repress cell division. Conversely, expansins, brassinosteroids and TOPO IV promote cell expansion, whereas LEUNIG represses cell expansion.



cytokinin treatment increases levels of *NTM1* (Kim *et al.*, 2006). This shows that cytokinin may play a negative role in regulating cell division in leaves, and is in contrast to other studies that have shown cytokinin to promote cell division by upregulating *CYCD3* expression (Riou-Khamlichi *et al.*, 1999).

Although perturbing cell division has striking effects on leaf growth and final morphology, the regulation of cell expansion is also important in controlling leaf growth. Reducing the activity of a group of *EXPANSIN* genes in *A. thaliana*, petunia and rice reduces cell expansion, leading to the production of smaller leaves (Cho and Cosgrove, 2000; Choi *et al.*, 2003; Zenoni *et al.*, 2004). Cell expansion is also positively regulated by the *ARGOS-LIKE* (*ARL*) gene that acts downstream of brassinosteroids (Hu *et al.*, 2006), and the DNA topoisomerase VI complex (topo VI) which promotes endoreduplication and increases in ploidy levels (Sugimoto-Shirasu *et al.*, 2005; Sugimoto-Shirasu *et al.*, 2002). Comparatively little is known about the genetic pathways involved in the repression of cell expansion during leaf development. *rotunda* alleles of the transcriptional corepressor *LEUNIG* produce leaves with wide laminae due to reduced repression of cell expansion late during leaf growth (Cnops *et al.*, 2004). This suggests that *LEUNIG* represses cell expansion to control leaf shape.

A tight relationship exists between cell proliferation and expansion which is explained by the compensation theory. This theory posits that a decrease in cell proliferation feeds back to stimulate an increase in cell expansion. Analysis of chimeric systems suggests that two independent pathways operate to control compensation (Kawade *et al.*, 2010). Firstly, *AN3*-dependent compensation is a non-cell autonomous process in which a mobile signal, which is restricted to half of the leaf, promotes cell expansion (Kawade *et al.*, 2010). Secondly, a *KRP2*-dependent pathway acts in a cell-autonomous manner to repress cell division (Kawade *et al.*, 2010). Since loss-of-*AN3* function and overexpression of *KRP2* decreases cell division rates and increases cell size, whereas overexpressing *AN3* increases cell number without

altering cell size, this suggests that the AN3- and KRP2-dependent pathways act specifically in response to decreased cell division.

1.4.7 Integrating patterning and growth

In many biological organs, growth is not uniform in all regions. Such anisotropic growth causes parts of organs to be displaced relative to each other, thereby determining organ morphology. It is thought that genes can regulate morphology by determining the local rates and orientations of growth (Coen *et al.*, 2004). The genetic mechanisms regulating leaf growth have largely been studied in *A. thaliana* and *A. majus*.

Over a decade ago, studies in *A. majus* indicated that the juxtapositioning of adaxial and abaxial identities is required for lamina outgrowth (Waites *et al.*, 1998). However, a key question remains as to how the patterning of the adaxial-abaxial boundary regulates the outgrowth of leaf lamina. In *A. thaliana*, the stimulator of cell division *ANT* acts with abaxial determinants *FIL* and *YAB3* to promote abaxial identity as *ant;fil;yab3* triple mutants have reduced expression of the adaxial determinant *PHB* and produce radialised leaves (Nole-Wilson and Krizek, 2006). Thus, correct specification of the adaxial/abaxial boundary may be required to stimulate cell division and blade outgrowth.

Differential growth along the proximo-distal axis of the leaf is also important for controlling leaf shape as this creates the blade and petiole. An interaction between leaf patterning genes *BOP1* and *BOP2* and growth regulators *JAG* and *NUB* is thought to be crucial for generating *A. thaliana* leaf morphology (Hepworth *et al.*, 2005). Together, *BOP1* and *BOP2* act to repress the outgrowth of lamina as *bop1;bop2* double mutants produce leaves with lamina outgrowths along the petiole (Hepworth *et al.*, 2005). *BOP1* and *BOP2* are proposed to act as upstream repressors of *JAG* and *NUB* expression as *bop1;bop2* double mutants have ectopic expression of *JAG* and *NUB* in petiole regions (Hepworth *et al.*, 2005).

Since JAG is a positive regulator cell division, regulation of its expression by patterning genes demonstrates how establishing patterns of polarity genes can regulate growth to generate complex leaf shapes.

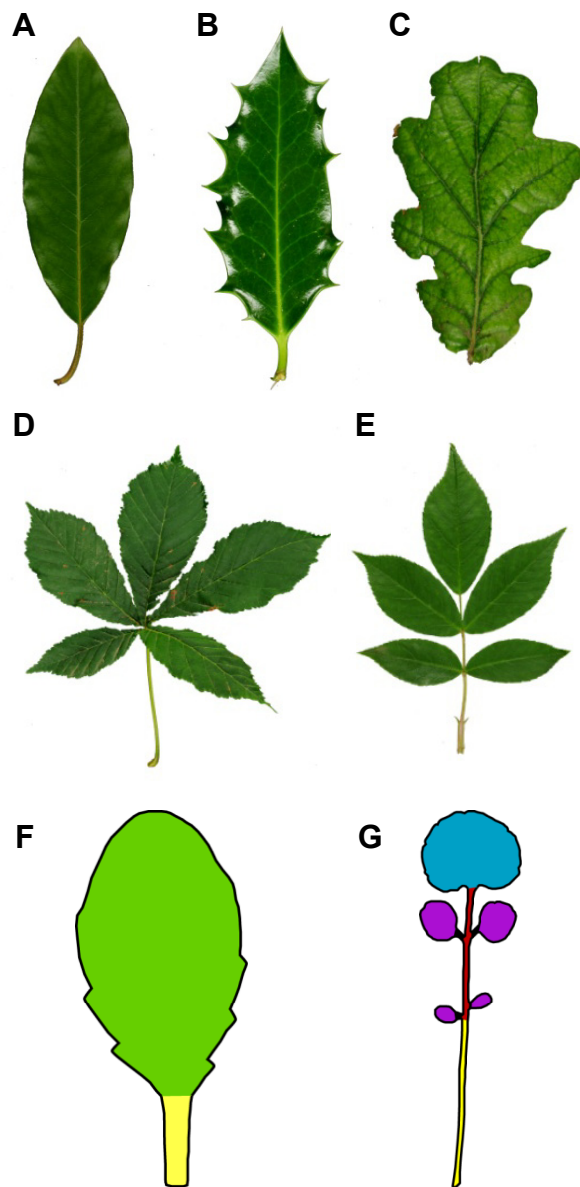
BOP genes are also thought to regulate leaf axial patterning and growth by regulating *AS2* and *KNOX* gene expression (Jun *et al.*, 2010; Semiarti *et al.*, 2001). BOP1 and BOP2 are transcriptional activators of *AS2*, with BOP1 binding directly to the promoter of *AS2* (Jun *et al.*, 2010). *AS2* is required for adaxial-abaxial leaf patterning and repression of *KNOX* gene expression (Semiarti *et al.*, 2001). Both *bop1;bop2* and *as2* mutants ectopically express *KNOX* genes and produce ectopic lamina outgrowths (Hepworth *et al.*, 2005; Semiarti *et al.*, 2001). However, since *KNOX* genes are excluded from *A. thaliana* leaves (Hay *et al.*, 2006), it is likely that *KNOX*-independent functions of *AS2* regulate wild type leaf growth.

1.5 Dissecting the regulation of compound leaf development

1.5.1 Leaf classifications

There is wide diversity in leaf morphology across the plant kingdom, which has been documented since ancient times (Theophrastus, 1916). Thus, leaves provide an excellent system in which to study the evolution of shape changes. Diversity in leaf shape has also meant that leaf morphology has long been used in the classification of species (Linnaeus, 1751). One basic classification of leaves divides them into two categories: simple leaves, which produce an entire blade (Figure 1.7 A-C); and compound leaves, which are divided into subunits termed leaflets (Figure 1.7 D-E). Simple leaves can be further classified by the shapes of their margins, which are described as: smooth when no outgrowths develop at the margin; serrated, when small teeth are generate at the margin; and lobed, when larger outgrowths are produced at the margin (Figure 1.7 A-C). Compound leaves can be described as pinnate, when the leaflets form from many spatially separate parts of the rachis, or

Figure 1.7 Morphological diversity of leaves. (A-C) Simple leaves of *Ficus benjamina* with smooth margins (A) *Ilex aquilifolium* with serrated margins (B) and *Quercus robur* with lobed margins (C). (D-E) Compound leaves of *Aesculus hippocastanum* with palmate structure (D) and *Fraxinus excelsior* with pinnate structure (E). (F) Cartoon of a simple *A. thaliana* rosette leaf comprising a blade (green) and a petiole (yellow). (G) Cartoon of a compound *C. hirsuta* rosette leaf comprising a terminal leaflet (blue), lateral leaflets (purple), a rachis (red) and a petiole (yellow).



palmate, when the distances between leaflets are highly compressed so that they arise from the same position along the rachis (Figure 1.7 D-E).

In compound leaves, leaflets are generated as new axes of lateral growth, however, the marginal outgrowths of simple leaves are not new lateral growth axes as the lamina remains entire. Two opposing hypotheses describe how new lateral growth axes are generated in leaves. In the first hypothesis, lateral blastozones are described as parts of the leaf margin that are competent to produce lateral growth axes (Hagemann and Gleissberg, 1996). Thus, only a proportion of the total number of proliferating leaf cells generates leaflets. In contrast, an alternative hypothesis is that marginal meristems generate lateral outgrowths through stereotypical patterns of cell divisions that take place perpendicular to the margin (Avery, 1933). This suggests that the cells of the leaf margin have properties of stem cells. However, clonal analysis in tobacco and cotton demonstrate that marginal meristems are unlikely to exist (Dolan and Poethig, 1998; Poethig and Sussex, 1985), and so the former hypothesis is more likely to describe leaflet formation in compound leafed species.

Analysis of paleobotanical data suggests that leaves of the first seed plants evolved from an indeterminate, radial shoot branching system (Zimmermann, 1952). Ancestral angiosperms produced simple and entire leaves, whilst compound leafed species evolved from simple leafed species on several independent occasions (Bharathan *et al.*, 2002; Doyle and Endress, 2000). There are two opposing theories that describe the homology between simple and compound leaves. One hypothesis posits that compound leaves are partially indeterminate, and that an individual leaflet is homologous to a single simple leaf (Sattler and Rutishauser, 1992). An alternative hypothesis proposes that a whole compound leaf is homologous to a whole simple leaf, and that leaflets are generated from the blade (Kaplan, 1975). Comparative studies of the gene regulatory networks involved in generating simple

and compound leaf forms have been critical in highlighting patterning mechanisms that have been differentially deployed for leaf morphogenesis.

1.5.2 The role of *KNOX* genes in leaf dissection

KNOX genes were indirectly implicated in leaf dissection over two decades ago since misexpression of *KNI* in simple maize leaves generates ectopic tissue due to prolonged indeterminacy of cells (Sinha *et al.*, 1993; Vollbrecht *et al.*, 1991). Subsequently, ectopic expression of *KNOX* genes in the simple leaves of *A. thaliana* was shown to produce deeply lobed leaf margins reminiscent of leaflets, associated with ectopic meristems (Chuck *et al.*, 1996). Downregulation of *KNOX* gene expression in the SAM is correlated with leaf initiation in all species investigated to date (Bharathan *et al.*, 2002), however, unlike in simple leafed species, *KNOX* gene expression is thought to be reactivated in the leaves of most compound leafed species (Bharathan *et al.*, 2002). Furthermore, *KNOX* gene expression in leaves has been recruited on several independent occasions in compound leafed species (Bharathan *et al.*, 2002).

Over the past five years, genetic analyses in the compound leafed species *C. hirsuta* have provided great insight into the role of *KNOX* genes in compound leaf development (Barkoulas *et al.*, 2008; Hay and Tsiantis, 2006). Promoter-swap experiments with *STM* and *BP* promoters revealed that *cis*-regulatory changes are responsible for the expression of *KNOX* genes within the *C. hirsuta* leaf (Hay and Tsiantis, 2006). *KNOX* gene expression is necessary and sufficient for leaflet formation in *C. hirsuta* as reducing *STM* levels significantly reduces leaflet numbers (Hay and Tsiantis, 2006), whereas overexpression of *STM* stimulates the production of increased numbers of higher order leaflets (Hay and Tsiantis, 2006). Interestingly however, *KNOX* overexpression does not affect leaflet formation along the primary rachis of the *C. hirsuta* and tomato leaf (Hay and Tsiantis, 2006; Hareven *et al.*, 1996). This, therefore, raises the question of how *KNOX* genes regulate leaflet

development within compound leaves, and whether there are additional factors acting along the proximo-distal axis of the leaf that restrict *KNOX* action.

AS1 was already known to negatively regulate the expression of *KNOX* genes in simple leafed species such as *A. thaliana* and maize (Byrne *et al.*, 2000; Guo *et al.*, 2008; Ori *et al.*, 2000; Timmermans *et al.*, 1999; Tsiantis *et al.*, 1999), however, it was not known whether AS1-mediated repression of *KNOX* genes was altered in compound leafed species, thereby accounting for differences in leaf morphology. Expression analyses in *C. hirsuta* showed that *ChAS1* is expressed within the leaf, and that *ChAS1* has conserved the ability to repress *KNOX* genes (Hay and Tsiantis, 2006). Furthermore, genetic analyses revealed that *ChAS1* is required for correct placement of leaflets along the proximo-distal axis of the rachis (Hay and Tsiantis, 2006). *chas1* mutants produce leaflets over a compressed proximo-distal axis, which is associated with *ChBP* misexpression at the base of the leaf and increased *H4* expression (Hay and Tsiantis, 2006). This strongly suggests that AS1 acts to pattern leaflet formation via repression of *BP*, as well as regulate growth in order to control leaf morphogenesis.

1.5.3 *KNOX* genes regulate growth to control compound leaf development

Recent studies have begun to elucidate how and when *KNOX* genes are required to promote leaf dissection (Shani *et al.*, 2009; Hay and Tsiantis, 2006). Stage-specific overexpression of *KNOX* genes in tomato and *A. thaliana* suggests that there are specific spatio-temporal domains within leaves that are competent to respond to *KNOX* gene activity (Shani *et al.*, 2009). Overexpression of the tomato *KNOX* gene *Tkn2* during primary morphogenesis significantly increases higher order leaflet formation, whereas *Tkn2* overexpression during secondary morphogenesis has no effect on leaf shape (Shani *et al.*, 2009). Furthermore, overexpression of *STM* in *A. thaliana* generates leaflet-like marginal structures when expressed during primary morphogenesis (Shani *et al.*, 2009). This strongly

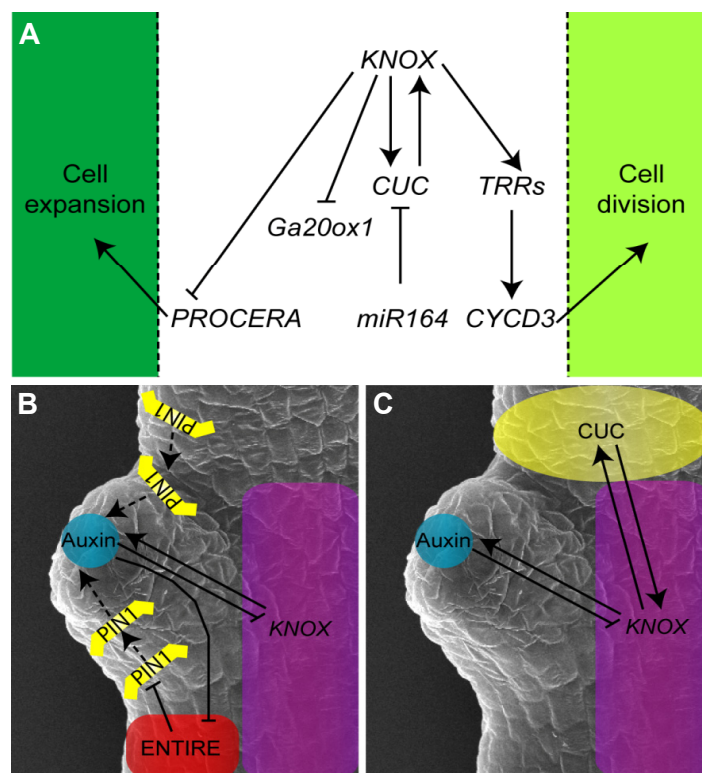
suggests that *KNOX* genes regulate marginal outgrowths during primary morphogenesis in both compound and simple leafed species.

Whilst it is clear that *KNOX* genes are required for leaflet formation in compound leafed species, a critical question is how *KNOX* genes regulate growth during leaflet formation. Transgenic plants with reduced *ChSTM* levels have reduced levels of *H4* expression, and leaves produce larger cells (Hay and Tsiantis, 2006). Furthermore, there are increased levels of cell division and reduced cell elongation in *chas1* mutants, which correlates with increased levels of the *KNOX* gene *ChBP* (Hay and Tsiantis, 2006). This suggests that *KNOX* genes promote cell division and delay the transition to cell elongation to control leaflet formation. It will, therefore, be interesting to determine the downstream targets of *KNOX* genes in the control of growth.

1.5.4 *KNOX*-mediated hormonal regulation of leaf dissection

As discussed in section 1.2.4, *KNOX* genes regulate the balance between cytokinin and gibberellin levels in SAM homeostasis, however, recent studies in tomato have shown that *KNOX* genes regulate cytokinin and gibberellin activities to control compound leaf development (Jasinski *et al.*, 2008; Shani *et al.*, 2010). Overexpression of the cytokinin catabolic enzyme *AtCKX3* suppresses leaflet development in *KNOX*-overexpressing lines, whilst overexpressing the cytokinin biosynthesis enzyme *AtIPT7* restores leaflet development in backgrounds with repressed *KNOX* activity (Shani *et al.*, 2010). This suggests that cytokinin can compensate for *KNOX* activity in the control of leaflet development. *KNOX* overexpression also causes a significant upregulation of cytokinin response genes *TOMATO RESPONSE REGULATORS* (*TRRs*), providing direct evidence that *KNOX* genes promote cytokinin response (Figure 1.8 A; Shani *et al.*, 2010). Cytokinins promote cell division in the compound leaf of tomato as overexpression of *AtIPT7* significantly increases levels of the cell cycle gene *SlCycD3;3* (Shani *et al.*, 2010). Since cytokinin response is greatest at the leaf

Figure 1.8 Auxin, *CUC* and *KNOX* genes control the growth of leaflets. (A) In compound leaf development, *CUC* genes are repressed at the post-transcriptional level by *miR164* and are involved in a positive feedback loop with *KNOX* genes. *KNOX* genes promote the expression of cytokinin response regulators (TRRs), which promote *CycD3* expression and cell division. *KNOX* genes also repress gibberellin levels by repressing *Ga20ox* and *PROCERA* expression, thereby repressing cell expansion. **(B)** PIN1 (yellow trapezia) forms convergence points at the sites of leaflet development, causing auxin to be transported (broken arrows) to create an auxin activity maximum (IAA; blue circle). Auxin represses the expression of the Aux/IAA protein ENTIRE (red rectangle), which feeds back to repress the formation of PIN1 convergence points. Auxin also represses the transcription of *KNOX* genes in the compound leaf, whilst *KNOX* genes promote the formation of PIN1 convergence points. **(C)** Scanning electron micrograph close-up of a leaflet. Auxin activity foci form (blue circle) at the tips of leaflets; *CUC* genes (yellow oval) are expressed in the distal sinuses of leaflets; and *KNOX* genes (purple rectangle) are expressed in the rachis and are downregulated at sites of leaflet development.



margin and leaflets, the net effect of increased cell division is an enhanced morphogenetic capacity at the leaf margin and the production of super-compound leaves (Shani *et al.*, 2010).

Genetic and pharmacological evidence suggest that *KNOX* genes regulate gibberellin activity in compound leaves (Hay *et al.*, 2002; Jasinski *et al.*, 2008). The recessive loss-of-function tomato mutant *clausa* (*clau*) has increased levels of *KNOX* gene expression and increased numbers of leaflets (Avivi *et al.*, 2000; Jasinski *et al.*, 2007). Significantly, there is reduced expression of the gibberellin biosynthetic gene *SIGA20ox1* in *clau* leaves, and exogenous application of gibberellin suppresses the *clau* phenotype (Jasinski *et al.*, 2008). This suggests that *KNOX* genes act to repress gibberellin levels to control leaflet number. Double mutant analysis suggests it is likely that *KNOX* controls growth via the activity of *PROCERA* (*PRO*), which encodes a DELLA-type growth repressor involved in repressing gibberellin signalling in tomato (Jasinski *et al.*, 2008). *pro* mutants produce leaves with fewer leaflets and larger cells, suggesting that *PRO* restricts growth by repressing cell expansion (Jasinski *et al.*, 2008). Thus, *KNOX*-mediated control of gibberellin activity influences leaflet numbers via repression of cell expansion (Figure 1.8 A).

Although *KNOX* genes appear to play a critical role in leaflet formation in some species such as *C. hirsuta* and tomato, it is important to note that they are not universal markers of compound leafed species. For example, *KNOX* genes are expressed in the simple leaves of *Lepidium oleraceum* (Bharathan *et al.*, 2002). During early leaf development, *L. oleraceum* produces marginal outgrowths, however, these are lost during secondary morphogenesis (Bharathan *et al.*, 2002). Furthermore, it is well known that *KNOX* genes are not expressed in the compound leafed species of pea (Gourlay *et al.*, 2000; Hofer *et al.*, 2001). Instead, genes active in the floral meristems of *A. thaliana* such as *UNIFOLIATA* (*UNI*), an orthologue of *FLORICAULA* (*FLO*)/*LEAFY* (*LFY*), acts synergistically with *STAMINA PISTILLOIDA* (*STP*), an orthologue of *UNUSUAL FLORAL ORGANS* (*UFO*), to

promote leaflet development in pea (DeMason and Schmidt, 2001; Hofer *et al.*, 1997; Taylor *et al.*, 2001). Thus, whilst *KNOX* genes may have been deployed in the genetic toolkit of many compound leafed species for the generation of leaflets, other factors likely play a role.

1.5.5 The role of auxin in leaf dissection

Recent studies in *C. hirsuta*, tomato and pea have revealed a central role for auxin in the initiation of leaflets in compound leafed species (Barkoulas *et al.*, 2008; DeMason and Chawla, 2004; Koenig *et al.*, 2009). This suggests that a common mechanism for the formation of lateral axes has been deployed across compound leafed species. Genetic screens for regulators of leaflet initiation in *C. hirsuta* first indicated that auxin transport is required for leaflet initiation as *chpin1* mutants fail to form leaflets (Barkoulas *et al.*, 2008). In further support of this, application of chemical inhibitors of auxin transport to *C. hirsuta* and tomato also prevents leaflet formation (Barkoulas *et al.*, 2008; Koenig *et al.*, 2009). Studies of PIN1 expression have revealed that PIN1 forms epidermal convergence points along the rachis, directing the flow of auxin towards the tip of the initiating leaflet (Barkoulas *et al.*, 2008; Koenig *et al.*, 2009). PIN1 then adopts a basal localisation in subepidermal cells, thereby internalising auxin flow away from the leaflet (Barkoulas *et al.*, 2008; Koenig *et al.*, 2009). These PIN1 expression patterns are reminiscent of the patterns in the shoot which polarise the outgrowth of leaf primordia (Heisler *et al.*, 2005; Reinhardt *et al.*, 2003).

PIN1 convergence points correlate with a single subepidermal cell with high auxin activity marking the earliest step in leaflet initiation (Figure 1.8 B; Barkoulas *et al.*, 2008). PIN1-mediated auxin transport has been shown to generate foci of auxin activity along the rachis as *pin1* mutants and plants treated with chemical inhibitors of auxin transport do not form foci of auxin activity along the rachis of the leaf (Barkoulas *et al.*, 2008). Since reduced auxin transport prevents leaflet formation, this suggests that auxin activity foci are necessary for leaflet initiation. Broad application of auxin to compound leaves promotes the ectopic

outgrowth of lamina along the rachis, and application of discrete spots of auxin to the rachis of tomato leaves stimulates the outgrowth of leaflets (Barkoulas *et al.*, 2008; Koenig *et al.*, 2009). These data suggest that auxin is necessary and sufficient for the initiation of leaflets along the rachises of compound leafed species.

Auxin has also been implicated in the regulation of growth in intercalary regions between leaflets during secondary morphogenesis (Koenig *et al.*, 2009). *ENTIRE* encodes an Aux/IAA protein in tomato, and loss-of-function mutants produce large, simple leaves (Wang *et al.*, 2005; Zhang *et al.*, 2007). *entire* mutant leaves initiate leaflets but growth in intercalary regions, associated with PIN1 expression, leads to ectopic blade outgrowth and fusion of leaflets (Koenig *et al.*, 2009). Thus, it appears that Aux/IAA function in the regions between leaflets acts to suppress auxin activity and growth, and therefore delimit leaflets. However, this raises the question of whether high auxin activity at the tip of the leaflet promoting growth as opposed to low auxin activity in the sinus regions repressing growth is more important for regulating leaflet development (Figure 1.8 B).

Genetic analyses have suggested a possible interaction between auxin and *KNOX* expression as *PIN1* function is required for increased leaflets in *KNOX*-overexpressing lines, and ectopic expression of *KNOX* genes causes new PIN1 convergence points to form (Barkoulas *et al.*, 2008). Furthermore, auxin application reduces *STM* expression in the rachis of the *C. hirsuta* leaf (Barkoulas *et al.*, 2008). This suggests that a negative feedback loop exists where *KNOX* gene expression is required for the formation of PIN1 convergence points in the epidermis of the rachis (Figure 1.8 B). PIN1-mediated auxin flow creates foci of auxin activity that lead to the repression of *KNOX* expression at sites of leaflet initiation. Thus, a feedback loop between auxin and *KNOX* genes present in the SAM may have been redeployed in the leaves of compound leafed species to generate lateral growth axes.

Furthermore, auxin may influence growth of leaflets by stimulating foci of cell division as *pin1* mutants have reduced levels of cell division (Barkoulas *et al.*, 2008).

1.5.6 Establishing boundaries: a unifying mechanism for compound leaf development

Creating a boundary of slowly dividing cells between lateral organs and the SAM is critical for normal SAM maintenance and phyllotaxis, and *CUC* genes are intimately involved in this process. Recently, studies into the role of *CUC* genes in leaflet formation were made in several compound leafed species (Berger *et al.*, 2009; Blein *et al.*, 2008). Expression analyses showed that *CUC* genes are expressed in discrete strips in the distal boundary above leaflet primordia, and their expression precedes leaflet outgrowth (Berger *et al.*, 2009; Blein *et al.*, 2008). Transgenic reduction of *CUC* genes causes fewer leaflets to form and increased levels of leaflet fusion in four eudicot species with compound leaves, including *C. hirsuta*, tomato and pea (Blein *et al.*, 2008). Thus, boundary regulation by *CUC* genes may represent a unifying mechanism deployed in the genetic toolkits of all compound leafed species for leaflet development.

Genetic analyses of *goblet* mutants in tomato have elaborated the role that *CUC* genes play in regulating leaflet development. *GOBLET* encodes the only *CUC* gene present in the tomato genome, and both loss- and gain-of-function mutants produce simplified leaves with a reduced level of leaflet reiteration (Berger *et al.*, 2009). Loss-of-function mutants fail to initiate higher order leaflets, whereas gain-of-function mutants initiate higher order leaflets which fuse together, generating lobes (Berger *et al.*, 2009). These results suggest that *CUC* genes are required to initiate leaflets and maintain leaflet separation.

In *A. thaliana*, *CUC1* and *CUC2* transcripts are regulated by *miR164* (Mallory *et al.*, 2004; Nikovics *et al.*, 2006). Studies in tomato showed that *GOBLET* is also under *miR164*

regulation, and this is critical for leaflet development as mutations in the *miR164* binding site of *GOBLET* lead to expansion of the *GOBLET* expression domain and leaflet fusions (Berger *et al.*, 2009). *miR164* is expressed in the initiating leaflet primordium and is downregulated in the boundaries between leaflets and, therefore, has a largely complementary expression pattern to *GOBLET* (Berger *et al.*, 2009). Together, these data suggest that a sharp boundary between *miR164* and *GOBLET* expression is required for correct leaflet development.

CUC genes have been shown to interact with *KNOX* genes in a positive feedback loop in the *A. thaliana* SAM, and genetic analysis in *C. hirsuta* suggests that this feedback loop operates within compound leaves (Figure 1.8 C; Blein *et al.*, 2008). *ChCUC* genes are required for the reiterative formation of supernumary leaflets in *KNOX* overexpressing lines, and inducible overexpression of *KN1* in *C. hirsuta* increases *ChCUC1*, *ChCUC2* and *ChCUC3* levels (Blein *et al.*, 2008). Furthermore, levels of *STM* are decreased in transgenic lines with reduced *CUC* activity (Blein *et al.*, 2008). Thus, it appears that the positive feedback loop between *CUC* and *KNOX* genes, in addition to the negative feedback loop between auxin and *KNOX* that are present in the SAM, have been recruited into compound leaf development. It is likely that *CUC* genes exert their control over leaflet development by regulating growth. This is because *CUC* genes have complementary patterns of expression to cell division markers and reducing *CUC* levels narrows the marginal blastozone (Berger *et al.*, 2009; Blein *et al.*, 2008). Thus, it is likely that *CUC* genes repress growth at a local level, and in so doing, promote leaflet formation at the global level.

1.5.7 The role of growth in leaf dissection

To date, genetic analyses have focused on identifying the gene regulatory networks that pattern the three main axes of the compound leaf, whilst comparatively less is known about the regulation of growth in the control of leaf shape. For leaflet development to occur, a new axis of growth must be specified. Over a decade ago, it was suggested that leaflets

develop from cells that are actively dividing at the leaf margin and, more recently, analysis of *H4* expression patterns in *C. hirsuta* leaves confirmed that leaflets are associated with active cell division (Barkoulas *et al.*, 2008; Hagemann and Gleissberg, 1996). Clonal analysis using a transposon excision system in *C. hirsuta* revealed that between two-four cells are recruited from the margin of the rachis to generate a leaflet, therefore implying that the leaflet founder cells must actively divide to produce all the cell layers of the leaflet (Barkoulas *et al.*, 2008).

1.5.8 The role of TCP transcription factors in leaf dissection

Studies in compound leafed species have revealed a link between proliferative growth and leaflet development. *LANCEOLATE (LA)* encodes a type II TCP transcription factor in tomato that is negatively regulated by *miR319* (Ori *et al.*, 2007). *LA* has a dynamic spatio-temporal expression pattern during leaf development which is thought to be responsible for generating leaf shape in tomato (Ori *et al.*, 2007; Shleizer-Burko *et al.*, 2011). *LA* expression is low during early leaf development and a sharp increase in expression occurs prior to, and is thought to promote the transition to, secondary morphogenesis (Shleizer-Burko *et al.*, 2011). *LA* is transcribed throughout the early leaf primordium and gradually becomes restricted to the margins, however, it is thought that high levels of *SlmiR319* during early leaf development keeps *LA* transcript levels low during early leaf development (Shleizer-Burko *et al.*, 2011).

Misexpression of *LA* causes leaf simplification due to the precocious leaf maturation, whereas downregulation of *LA* causes prolonged, indeterminate growth and the production of a highly complex leaf form (Shleizer-Burko *et al.*, 2011). This strongly suggests that elevated *LA* expression is responsible for the transition from cell division to cell differentiation, and therefore has a crucial role in regulating final leaf shape. Although *LA* function is clearly in the regulation of growth, recent studies have begun to unravel the role that *LA* plays in patterning leaf development. Gain-of-function *La* mutations, which abolish repression by

MIR319, completely suppress leaflet development in the supercompound leaves of *KNOX* overexpressing lines, suggesting that *KNOX* genes may regulate *LA* expression in their control leaf shape (Ori *et al.*, 2007). In addition, *CUC* gene expression was shown to be dramatically reduced in *La* mutant leaves (Blein *et al.*, 2008), suggesting that *TCP* genes may regulate growth via repression of *CUC* gene expression.

Interestingly, correlations have been made between *LA* expression and heteroblasty, and also between *LA* expression and differences in leaf shape in several Solanaceae species (Shleizer-Burko *et al.*, 2011). This raises important questions as to the role that *TCPs* have played in the evolution of the compound leaf form over a broad range of taxa.

1.5.9 The adaptive significance of leaf shape: the point of serrated and dissected leaf margins

The wide diversity in leaf margin morphology amongst extant plant species raises the question of what selective advantages different leaf margin shapes confer to individuals. Over the past century, strong correlations between climate and serrated leaf margins have been reported as the proportion of species in a given flora with serrated leaf margins inversely correlates with mean annual temperature (Bailey and Sinnott, 1915; Bailey and Sinnott, 1916; Greenwood, 2005; Greenwood *et al.*, 2004; Wilf, 1997; Wolfe, 1979; Wolfe, 1993). Furthermore, the number and size of teeth, but also the degree of leaf dissection, is inversely correlated with temperature (Huff *et al.*, 2003; Royer *et al.*, 2005).

A century after the correlation between leaf margin complexity and mean annual temperature was made, the selective mechanisms that drive leaf margins to be more complex in colder environments remain poorly understood. However, there are several potential explanations for why high leaf complexity may be beneficial for plants growing in cold environments. For example, serrated margins have greater rates of photosynthesis and

transpiration compared with smooth margins, and physiological activity at leaf margins has been shown to be greatest at the beginning of the growing season (Baker-Brosh and Peet, 1997; Parkhurst and Louks, 1972; Roth-Nebelsick, 2001; Royer and Wilf, 2006). Additionally, serrations have been shown to enhance gas exchange in the early growing season (Royer and Wilf, 2006), thereby allowing greater rates of photosynthesis. These factors may be beneficial when ambient temperatures are low and growing seasons are short because an increase in transpirational and solute flow to the young leaves may allow increased photosynthesis and growth rates.

An additional advantage that serrated margins confer is that the hydathodes that develop at the tips of serrations allow the process of guttation, whereby the release of root pressure drives the flow of water from the root to the shoot when soil water content and humidity levels are high. This, therefore, prevents the mesophyll tissue from flooding and freeze-thaw embolisms from occurring (Feild *et al.*, 2005). For this reason, plants with serrated leaf margins may be prevalent in wet environments (Bailey and Sinnott, 1916; Burnham *et al.*, 2001; Greenwood, 2005; Kowalski and Dilcher, 2003; MacGinitie, 1953; Wolfe, 1993).

Evidence for natural selection acting on a leaf shape polymorphism was demonstrated in *Ipomoea hederacea*. Leaf shape variants exist in natural populations of *I. hederacea* due to segregation of two alleles at a single locus (Elmore, 1986). The dominant allele present in the heterozygous and homozygous form gives rise to lobed and deeply lobed leaves, respectively, whereas homozygous recessive individuals produces entire leaves (Bright and Rausher, 2008; Elmore, 1986). Natural selection was shown to operate at all populations studied, with either homozygotes for the dominant allele or heterozygotes having the highest fitness levels (Bright and Rausher, 2008). Despite demonstrating that natural selection operates on a leaf

shape polymorphism, disappointingly, the gene regulating leaf shape and the environmental conditions responsible for the selection could not be identified (Bright and Rausher, 2008).

In light of these ecological studies, it is likely that high leaf complexity may increase the fitness of individuals growing in cold, wet environments. However, there are huge differences in shape between leaves with serrated, lobed or dissected margins. Thus, it would be interesting to uncover the selective advantages that serrated as opposed to lobed or compound leaves confer upon individuals.

1.5.10 Using comparative developmental genetics to elucidate the genetic basis of differences in simple and compound leaf shape

Over the past century, several simple and compound leafed species have been utilised in isolation to determine how leaf shape arises. However, by studying the gene regulatory networks that control leaf morphology in closely-related species with divergent morphologies, the mechanisms underlying the evolution of leaf morphology can begin to be understood. *A. thaliana* is a model species that produces simple leaves comprising a petiole with a blade attached to the distal end, and outgrowths termed serrations are produced along the margin of the blade (Figure 1.7 F). *C. hirsuta* is a closely-related crucifer that produces compound leaves consisting of a petiole attached to a rachis which supports the terminal leaflet at the distal end, and produces lateral leaflets along its margins (Figure 1.7 G).

C. hirsuta is an attractive species to be used in comparison to *A. thaliana* to determine the genetic basis of differences in leaf morphology as it is diploid with a small genome, self-compatible and can be transformed (Hay and Tsiantis, 2006). Furthermore, *C. hirsuta* diverged from *A. thaliana* relatively recently, approximately fourteen million years ago (Koch *et al.*, 2001). Interestingly, a phylogenetic analysis of the broader Brassicaceae group that includes *A. thaliana* and *C. hirsuta*, combined with an optimisation of leaf margin

character states onto the most parsimonious tree, suggests that the most recent common ancestor of *A. thaliana* and *C. hirsuta* is likely to have produced compound leaves. Thus, it is likely that the simple leaf form of *A. thaliana* is a derived trait (Piazza *et al.*, 2010).

Comparative studies using *C. hirsuta* have already highlighted key genes involved in regulating compound leaf development. An initial study showed that *KNOX* genes are both necessary and sufficient for leaflet development, and act by repressing the transition from cell division to cell differentiation (Hay and Tsiantis, 2006). Furthermore, *cis*-regulatory changes were identified as being responsible for the expression of *KNOX* genes in *C. hirsuta* leaves and exclusion from *A. thaliana* leaves (Hay and Tsiantis, 2006). Within two years, a negative feedback loop between *KNOX* genes and auxin was demonstrated as *KNOX* genes were shown to be repressed by auxin and required for PIN1 convergence points to form (Barkoulas *et al.*, 2008). Furthermore, a positive feedback loop was also demonstrated between *ChSTM* and *ChCUC* genes (Blein *et al.*, 2008). Thus, the power of utilising *C. hirsuta* in addition to *A. thaliana* in comparative developmental biology studies is clear. It will be important for future research to determine the gene regulatory networks that exist in both species to control serration and leaflet development to establish the mechanisms underlying the evolution of leaf morphology.

1.6 Aims and objectives

The aim of this study is to investigate the fundamental question in developmental biology of how morphology is generated. To address this question, I study the gene regulatory networks controlling serration and leaflet development in *A. thaliana* and *C. hirsuta*, respectively.

Objectives

- 1) To determine the interactions between *CUC2*, auxin and *PIN1* in the control of serration development in *A. thaliana* (Chapter 3).
- 2) To determine the interactions between *CUC2*, auxin and *PIN1* in the control of leaflet development in *C. hirsuta* (Chapter 4).
- 3) To determine the role of auxin, *CUC2* and *KNOX* in elaborating lobed leaf morphologies (Chapter 5).
- 4) To identify new components that mediate the effects of *CUC2* in controlling serration development (Chapter 6).

In Chapter 3, I investigate the regulatory interactions between *CUC2*, auxin and *PIN1* in the control of serration development in *A. thaliana* leaves. Previous studies showed that the transcription factor *CUC2* and auxin transport, mediated by the auxin efflux facilitator PIN1, regulate serration development (Hay *et al.*, 2006; Nikovics *et al.*, 2006). I initially monitor the spatio-temporal dynamics between PIN1 localisation and auxin activity foci with respect to *CUC2* expression. Consequently, I test the genetic interactions between *CUC2* and PIN1, and whether alternating peaks of auxin and *CUC2* activity pattern serration development. Finally, I use a transgenic approach to understand whether epidermal auxin transport is sufficient to pattern serration development.

In Chapter 4, I extend my analysis by investigating the regulatory interactions between *CUC2*, auxin and PIN1 in leaflet development in *C. hirsuta*. I analyse the spatio-temporal dynamics of *CUC2* expression, PIN1 localisation and auxin activity foci using colocalised fluorescent reporters. Subsequently, I use a combination of genetic and molecular techniques to determine the genetic interactions between *CUC2* and PIN1-mediate auxin transport in the control of leaflet development. Finally, I assess the relationship between leaflet development and vasculature development.

In Chapter 5, I investigate the regulatory interactions between PIN1, *CUC2* and *KNOX* genes in lobe development associated with ectopic *KNOX* expression in *A. thaliana* leaves. Previous studies showed that *KNOX* genes are expressed in *C. hirsuta* leaves whereas they are excluded from *A. thaliana* leaves (Hay and Tsiantis, 2006). However, ectopic *KNOX* expression in *A. thaliana* leaves causes the production of deeply lobed margins reminiscent of leaflets (Chuck *et al.*, 1996; Hay *et al.*, 2006). I employ molecular genetics and pharmacological techniques to determine the regulatory interactions of *CUC2* and auxin transport with *KNOX* genes in leaflet-like lobe development in *A. thaliana* leaves. I subsequently test whether *CUC* genes regulate *ChBP* transcription in *A. thaliana* leaves. Finally, I investigate how *CUC* genes regulate cell division to control leaf margin morphogenesis in both *A. thaliana* and *C. hirsuta*.

In addition to investigating the regulatory interactions between known components of leaf development pathways, in Chapter 6 I investigate new components that mediate the effects of *CUC2* in regulating serration development. I perform an EMS-mutagenesis screen to identify mutations that suppress the smooth margin phenotype of *cuc2*. I characterise one of these mutants and study its interaction with gene regulatory pathways known to control serration development. I also take a map-based cloning approach to delimit the location of this mutation within the *A. thaliana* genome.

Finally, in the General Discussion, I compare the interlinking feedback loops controlling leaflet development in *C. hirsuta* with serration development in *A. thaliana* to infer how morphology is generated. I also compare the genetic mechanisms regulating leaflet-like lobe development associated with ectopic *KNOX* expression in *A. thaliana* with leaflet development in *C. hirsuta* to elucidate how morphological divergence arises. I then assess the significance of interlinking feedback loops in the control of outgrowths in both plants and animals by comparing leaflet development in *C. hirsuta* to limb bud development in mice and chick.

Chapter 2:

Materials and Methods

Chapter 2: Materials and methods

2.1 Materials used

2.1.1 Plant growth conditions

For plants grown on soil, seeds were sown on a medium of soil (Erin multipurpose compost, autoclaved) and vermiculite (Sinclair vermiculite medium, 2.0-5.0mm) in a 1:1 ratio. The soil medium was treated with 2% Intercept 70WG (Scotts). Seeds were sown at a density of 5 seeds per 6x6cm pot (Pöppelmann), and were stratified in the dark at 4°C for 4d. Plants were grown in a greenhouse with supplemented lighting at a light intensity of 100 μ mol photons m⁻²s⁻¹ under a long-day photoperiod of 18h light at 20°C and 6h dark at 16°C.

For plants grown under axenic conditions, seeds were sterilised in 10% bleach (Parazone) for 10mins, followed by incubation in 70% ethanol for 10mins, and then washed 6 times in ddH₂O. Sterilised seeds were sown on a medium of Murishuge Skoog (MS)-agar (0.8% agar (Melford); 0.43% MS (Melford)) in 12x12x1.5cm plates (Greiner bio-one) at a density of 30 seeds per plate. Seeds were stratified in the dark at 4°C for 4d. Plants were grown in a growth cabinet under a long-day photoperiod of 18h light at 20°C and 6h dark at 16°C with a light intensity of 100 μ mol photons m⁻²s⁻¹.

2.1.2 Plant material

All *A. thaliana* alleles and transgenic lines used in this study are listed in Table 2.1; only Columbia (Col-0) and Landsberg *erecta* (Ler) ecotypes were used in this study.

Table 2.1 *A. thaliana* genetic stocks used in this study

Allele/transgenic line	Ecotype	Reference
<i>axr1-3</i>	Col-0	CS3374, ABRC
<i>as1-1</i>	Col-0	CS3075, ABRC
<i>bd1/BDL</i>	Col-0	Hamann <i>et al.</i> , 1999
<i>cuc2-1</i>	<i>Ler</i>	Aida <i>et al.</i> , 1997
<i>cuc2-3</i>	Col-0	Hibara <i>et al.</i> , 2006
<i>cuc3-105</i>	Col-0	N176536, NASC
<i>cus1</i>	Col-0	EMS-mutagenesis screen of <i>cuc2-3</i> in this study
<i>mir164a-4</i>	Col-0	Nikovics <i>et al.</i> , 2006
<i>mp-319</i>	Col-0	Donner <i>et al.</i> , 2009
<i>pin1-1</i>	Col-0	Blilou <i>et al.</i> , 2005; backcrossed to Col-0 5 times
<i>pin1-7</i>	Col-0	Smith <i>et al.</i> , 2006
<i>35S::MIR164A</i>	Col-0	Nikovics <i>et al.</i> , 2006
<i>ATHB-8::GUS</i>	Col-0	Baima <i>et al.</i> , 1995
<i>AtML1::CUC2::VENUS</i>	Col-0	Constructed and transformed in Col-0 in this study
<i>AtML1::PIN1::GFP</i>	Col-0	Constructed and transformed in Col-0 in this study
<i>BP::GUS</i>	Col-0	Ori <i>et al.</i> , 2000
<i>ChBP::GUS</i>	Col-0	Hay and Tsiantis, 2006
<i>CUC2::CUC2::VENUS</i>	Col-0	Heisler <i>et al.</i> , 2005; transformed in Col-0.
<i>CUC2::GUS</i>	Col-0	Hibara <i>et al.</i> , 2006
<i>CYCB1;1::GUS</i>	Col-0	Renaudin <i>et al.</i> , 1996
<i>DR5::GFP</i>	Col-0	Benkova <i>et al.</i> , 2003
<i>DR5::VENUS</i>	Col-0	Transformed in Col-0 in this study
<i>FIL>>BP</i>	<i>Ler</i>	Hay <i>et al.</i> , 2006
<i>IAA2::GUS</i>	Col-0	Luschnig <i>et al.</i> , 1998
<i>LFY>>STM</i>	Col-0	D. Weigel, unpublished
<i>MIR164A::GUS</i>	Col-0	Nikovics <i>et al.</i> , 2006
<i>PIN1::PIN1::GFP</i>	Col-0	Benkova <i>et al.</i> , 2003

All *C. hirsuta* alleles and transgenic lines used in this study are listed in Table 2.2; the Oxford (Ox) ecotype was used in this study.

Table 2.2 *C. hirsuta* genetic stocks used in this study

Allele/transgenic line	Ecotype	Reference
<i>chas1-1</i>	Ox	Hay and Tsiantis, 2006
<i>lol1</i>	Ox	P. Sarchet, unpublished
<i>chpin1</i>	Ox	Barkoulas <i>et al.</i> , 2008
<i>35S::MIR164B</i>	Ox	Blein <i>et al.</i> , 2008
<i>ATHB-8::GUS</i>	Ox	Constructed and transformed in Ox in this study
<i>ChBP>>GUS</i>	Ox	Hay and Tsiantis, 2006
<i>CUC2::CUC2:VENUS</i>	Ox	Heisler <i>et al.</i> , 2005; transformed in Ox
<i>CUC3RNAi</i>	Ox	Blein <i>et al.</i> , 2008
<i>CYCB1;1::GUS</i>	Ox	E. Kougioumoutzi, unpublished
<i>DR5::VENUS</i>	Ox	Barkoulas <i>et al.</i> , 2008
<i>PIN1::PIN1:GFP</i>	Ox	Barkoulas <i>et al.</i> , 2008

2.1.3 Bacterial strains

The DH5α strain of *Escherichia coli* was used for plasmid amplification. The GV3101 strain of *Agrobacterium tumifaciens* was used for transforming *A. thaliana* and *C. hirsuta*.

2.1.4 Buffers and media

Media, solutions and buffers were prepared following the methods described in (Sambrook and Russell, 2001).

2.1.5 Oligonucleotides

Oligonucleotides used for standard PCR, quantitative RT-PCR, sequencing and genotyping are listed in Table 2.3. Oligonucleotides used for rough positioning and fine positioning in map-based cloning are listed in Table 2.4 and Table 2.5, respectively.

Table 2.3 Primers used for PCR, quantitative RT-PCR, sequencing and genotyping

Primer name	Primer sequence	Use
AtML1-HindIII-F	AAGCTTATCAAAGAAAAACAAGAA	Amplification of the <i>AtML</i> promoter
AtML1-KpnI-R	CTGGTACCGGATTCAGGGAGTTTCTTTAA	Amplification of the <i>AtML</i> promoter
PIN1-F	GGGATCCCCAAAAGAGGAAACACGAATG	Amplification of <i>PIN1:GFP</i>
PIN1-R	GCGGTACCTCCCTCTTCACCACTTCTCTC	Amplification of <i>PIN1:GFP</i>
CUC2-F	ATGGACATTCCGTATTACCAC	Amplification of <i>CUC2:VENUS</i>
VENUS-R	TTACTTGTACAGCTCGTCCATGC	Amplification of <i>CUC2:VENUS</i>
CUC2-3F	TACCACGCCCCTTACTCAAG	Genotyping <i>cuc2-3</i>
CUC2-3R	CATTTTCCTTGCATATTACCTCCT	Genotyping <i>cuc2-3</i>
CUC3-105-F	TTGCGGTGGAAGATGTGTTA	Genotyping <i>cuc3-105</i>
CUC3-105R	CCAAGGGCCAAGATTCTACA	Genotyping <i>cuc3-105</i>
MIR164A-F	TTTCCTATGTTTTATCATTTTGCC	Genotyping <i>mir164a-4</i>
MIR164A-R	GAAATTTAGGGCAGACAAGCC	Genotyping <i>mir164a-4</i>
MP-319-F	CCTGGAACTGATGAGCTGAC	Genotyping <i>mp-319</i>
MP-319-R	CCTTCTTCACTCATCTGCTGG	Genotyping <i>mp-319</i>
PIN1-7-F	CCAACACTCTAGTCATGGGGATA	Genotyping <i>pin1-7</i> allele
PIN1-7-R	CTGAGAGTATGGAGATAGAC	Genotyping <i>pin1-7</i> allele
SAIL-LB	GCCTTTTCAGAAATGGATAAATAGCCTTG CTTCC	Genotyping <i>cuc2-3</i>
SALK-LB	TGGTTCACGTAGTGGGCCATCG	Genotyping SALK lines
cBPqRT-F	ACTCTTCCCATCAGGATTGTTGA	qRT-PCR of <i>ChBP</i> transcript level
cBPqRT-R	CCATTCAAGAAGCAATGGAGTT	qRT-PCR of <i>ChBP</i> transcript level
CUC2qRT-F	CAGCCGTAGCACCAACACAA	qRT-PCR of <i>CUC2</i> transcript level
CUC2qRT-R	GTCTAAGCCCAAGGCCCGTAGTA	qRT-PCR of <i>CUC2</i> transcript level
GAPDHqRT-F	TGACCACCGTCCACTCCATCAC	qRT-PCR of <i>cGAPDH</i> transcript
GAPDHqRT-R	GCTCTTCCACCTCTCCAGTCCTTC	qRT-PCR of <i>cGAPDH</i> transcript
MIR164AqRT-F	CCCTCATGTGCTTGGAATG	qRT-PCR of <i>MIR164A</i> transcript
MIR164AqRT-R	GCAAATGAGACGGATTTCTGTG	qRT-PCR of <i>MIR164A</i> transcript
OTCqRT-F	TGAAGGGACAAAGGTTGTGTATGTT	qRT-PCR of <i>OTC</i> transcript level
OTCqRT-R	CGCAGACAAAGTGGAATGGA	qRT-PCR of <i>OTC</i> transcript level
M13-F	TGTAAAACGACGGCCAGT	Sequencing inserts
M13-R	CAGGAAACAGCTATGACC	Sequencing inserts

Table 2.4 List of primers used in rough positioning in map-based cloning

Chr	Marker	BAC	PCR product size (bp)		Primer sequences (forward/reverse)
			Col-0	Ler	
I	1-AC0011661-1259	T23J18	182	150	GTAGGACAAAACCAAGTACA/ TAATAAAGTTCCAGCTTTGA
I	1-AC003979-2653	T22J18	171	156	CACTGCAACAAAGTGGAAT/ ATCCGTTTCAATATCCACAA
I	1-AC006423-5434	F28H19	148	102	TGCGGGAGTGTGATAGAATA/ TCCTCGAAAGATTCATTGAT
I	1-AC00375-7539	F19K23	200	<200	GAATTCTGTAACATCCCATTTC/ GGTCTAATTGCCGTTGTTGC
I	1-AC010675-8645	T17F3	130	114	GGACCGACGGTTACGAGAG/ TAACGGGCCGTTGCAAGA
II	2-AC007069-0197	T23K3	133	118	CGTGTTTACCGGGTCGGA/ AAAACCCTTGAAGAATACG
II	2-AC005917-4269	F3P11	210	150	ATGTATTTGTTGCAAAATAA/ TGCACAGAAGAAAAAACTA
II	2-AC005623-5887	T20P8	124	112	TCCGATTCGATTAACTC/ TTATTTCTATTTC AAGACT
II	2-AC004697-8295	T16B24	143	129	ATGAACGGAGTAGCTATC/ CGCGTAGAACATAATCTGTA
III	3-AB022217-2402	MGL6	218	165	ACCTGTTTCAGTCTATGTTAC/ GGGAATTATTAACATTATCA
III	3-AB024028-4332	K1G2	171	157	ATGAGCTTTAGGAGTGTGTA/ AATTTTGTCCCAAAGAATA
III	3-AB002534-6967	T32N15	143	124	CAAAAGAAATGCAACGAGAC/ TTTGATCATGAATGGTAGTG
III	3-AL132954-8728	T26I12	181	165	GAGCAACATTAAGGATAGAA/ ATCTCATACTCATAATATGTAG
IV	4-AF069442-0706	T4I9	145	101	GCATTAGCGTGCAATTAAGC/ ATCAACCCCAAAAATGCATA
IV	4-AL049482-3304	F17A8	137	117	CTGGACCCTAGTGGATGT/ GACGGTTCTCCATTAATTAT
IV	4-Z97343-5268	FCA8	151	126	TTCGGAGAAAGAAACGACAT/

					ATGGAAGTATTCAGGCATTA
IV	4-AL023094-8912	T4L20	128	118	ACCCTAAAACAATGTCTCTT/ TGCTAACATGGAAATTTGTC
V	5-AB010070-0918	MBK20	120	146	CTCTGTTGGGGCAAAACC/ GATGCTGGAGAGTAGCTTAG
V	5-AB006708-2862	MYJ24	112	100	TTCATGAGAGCGGCATTC/ GCAAAATGTTTGGACAATTA
V	5-AF058826-5037	T26D22	181	138	CACAGGCCATTGGATGTA/ TGTTAGAACCCACCATTG
V	5-AB023033-7443	K6M13	141	108	CCTGTTCCAATGAATATG/ TGTAGCTGCTGAGTTGTC
V	5-AB009053-9339	MQB2	135	115	AAAAGGCGACTACTAGCA/ GCCATTTATTTGGTCAAC

Table 2.5 List of primers used in fine positioning in map-based cloning

Marker	BAC	PCR product size (bp)		Primer sequences (forward/reverse)
		Col-0	Ler	
2-AC006234-4519	F5H14	175	>175	TAGTCGTTTCTCTGGTTGTAG/ TTGCCTGTCGATGTAGATTTGT
2-AC006841-4621	F3K23	128	>128	GATGCCTTTCTCCTGGTTG/ AATATAGCCGTCGCTTCATCA
2-AC007168-4814	T16B14	191	155	ATGGCGGCTTGTTCCCTTG/ CCGCAGCATTAGCTAGTG
2-AC006592-4866	F14M13	123	106	CGTTCTATCGGTTTCTG/ TAAAAGGGAGTTTTTCCTA
2-AC002391-4997	T20D16	156	>156	CACAATGATCGCCTTCT/ TACGGACATGCACTATCAG
2-AC004482-5111	F27L4	113	129	TTATGTTGTGTTGGAGTTGG/ TTGGTATGAATACGTTCTAA
2-AC006403-5271	T28I24	99	93	ATAGGCATTATAGTTATGAG/ TTTCGACATTGGCTTGAA
2-AC007266-5339	F27A10	114	103	GGGTCTGAATGGAAGAAC/ GTAGGACAAAACCAAGTACA

2.2 Genetic methods

2.2.1 Cross-pollination of *A. thaliana* and *C. hirsuta* plants

To cross-pollinate between different genotypes, the sepals, petals and stamens of unopened flower buds of female acceptor plants were removed, revealing the carpel. Mature pollen grains from the flowers of male donor plants were then applied to the stigma of the acceptor plant carpels. Hybrid F1 seeds were then harvested from individual dried siliques.

2.2.2 Plant genetics

Multiple mutant/transgenic backgrounds generated in this study are outlined below. Homozygosity of mutant alleles was determined by a combination of scoring Mendelian ratios of F2 progeny, genotyping (see Table 2.3 for primers) and phenotyping. Homozygosity of fluorescent reporters was determined by all F3 progeny expressing the fluorophore.

A. thaliana

GFP/VENUS-positive plants from the F2 generation of a cross between *DR5::GFP* and *CUC2::CUC2:VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *DR5::GFP;CUC2::CUC2:VENUS* plants. M. Barkoulas generated F2 families.

GFP/VENUS-positive plants from the F2 generation of a cross between *PIN1::PIN1:GFP* and *CUC2::CUC2:VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *PIN1::PIN1:GFP;CUC2::CUC2:VENUS* plants. M. Barkoulas generated F2 families.

GFP-positive *cuc2-3* homozygotes from the F2 generation of a cross between *cuc2-3* and *DR5::GFP* homozygotes were self-pollinated to generate F3 families homozygous for *cuc2-3* and *DR5::GFP*. M. Barkoulas generated F2 families.

GFP-positive *cuc2-3* homozygotes from the F2 generation of a cross between *cuc2-3* and *PIN1::PIN1:GFP* homozygotes were self-pollinated to generate F3 families homozygous for *cuc2-3* and *PIN1::PIN1:GFP*.

GFP/VENUS-positive plants from the F2 generation of a cross between *AtML1::CUC2:VENUS* and *DR5::GFP* homozygotes were self-pollinated to generate F3 families homozygous for *AtML1::CUC2:VENUS* and *DR5::GFP*.

GFP/VENUS-positive plants from the F2 generation of a cross between *AtML1::CUC2:VENUS* and *PIN1::PIN1:GFP* homozygotes were self-pollinated to generate F3 families homozygous for *AtML1::CUC2:VENUS* and *PIN1::PIN1:GFP*.

VENUS-positive *pin1-7/PIN1* heterozygotes from the F2 generation of a cross between *pin1-7* heterozygotes and *DR5::VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *DR5::VENUS* and heterozygous for *pin1-7/PIN1*. Plants were genotyped for the *pin1-7* allele (see Table 2.3 for primers).

GUS-positive *pin1-1/PIN1* heterozygotes from the F2 generation of a cross between *pin1-1* heterozygotes and *CUC2::GUS* homozygotes were self-pollinated to generate F3 families homozygous for *CUC2::GUS* and heterozygous for *pin1-1/PIN1*. M. Barkoulas generated F2 families.

VENUS-positive *pin1-7/PIN1* heterozygotes from the F2 generation of a cross between *pin1-7* heterozygotes and *CUC2::CUC2:VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *CUC2::CUC2:VENUS* and heterozygous for *pin1-7/PIN1*. M. Barkoulas generated F2 families.

axr1-3 homozygotes from the F2 generation of a cross between *axr1-3* and *cuc2-3* homozygotes were self-pollinated to generate F3 families that segregated *axr1-3;cuc2-3* double mutants in a Mendelian 1:3 ratio. A. Hay generated F2 families.

bdl/BDL heterozygotes from the F2 generation of a cross between *bdl/BDL* heterozygotes and *cuc2-3* homozygotes were self-pollinated to generate F3 families that segregated *bdl/BDL;cuc2-3* mutants in a Mendelian 3:13 ratio. A. Hay generated F2 families.

VENUS-positive *axr1-3* homozygotes from the F2 generation of a cross between *axr1-3* and *CUC2::CUC2:VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *axr1-3* and *CUC2::CUC2:VENUS*. M. Barkoulas generated F2 families.

GUS-positive *axr1-3* homozygotes from the F2 generation of a cross between *axr1-3* and *MIR164A::GUS* homozygotes were self-pollinated to generate F3 families homozygous for *axr1-3* and *MIR164A::GUS*. A. Hay generated F2 families.

GUS-positive *bdl/BDL* heterozygotes from the F2 generation of a cross between *bdl/BDL* heterozygotes and *MIR164A::GUS* homozygotes were self-pollinated to generate F3 families homozygous for *MIR164A::GUS* and heterozygous for *bdl/BDL*. A. Hay generated F2 families.

axr1-3;35S::MIR164A plants from the F2 generation of a cross between *axr1-3* and *35S::MIR164A* homozygotes were self-pollinated to generate F3 families homozygous for *axr1-3* and *35S::MIR164A*. A. Hay generated F2 families.

bdl/BDL;35S::MIR164A plants from the F2 generation of a cross between *bdl/BDL* heterozygotes and *35S::MIR164A* homozygotes were self-pollinated to generate F3 families homozygous for *35S::MIR164A* and heterozygous for *bdl/BDL*. A. Hay generated F2 families.

cuc2-3 homozygotes from the F2 generation of a cross between *mp-319/MP* heterozygotes and *cuc2-3* homozygotes were self-pollinated to generate F3 families homozygous for *cuc2-3* and heterozygous for *mp-319/MP*. Plants were genotyped for the *cuc2-3* allele and the *mp-319* allele (see Table 2.3 for primers).

mir164a-4 homozygotes from the F2 generation of a cross between *mp-319/MP* heterozygotes and *mir164a-4* homozygotes were self-pollinated to generate F3 families homozygous for *mir164a-4* and heterozygous for *mp-319/MP*. Plants were genotyped for the *mp-319* allele and the *mir164a-4* allele (see Table 2.3 for primers).

GUS-positive *mp-319/MP* heterozygotes from the F2 generation of a cross between *mp-319/MP* heterozygotes and *CUC2::GUS* homozygotes were self-pollinated to generate F3 families homozygous for *CUC2::GUS* and heterozygous for *mp-319/MP*. Plants were genotyped for the *mp-319* allele (see Table 2.3 for primers).

VENUS-positive *mp-319/MP* heterozygotes from the F2 generation of a cross between *mp-319/MP* heterozygotes and *CUC2::CUC2:VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *CUC2::CUC2:VENUS* and heterozygous for *mp-319/MP*. Plants were genotyped for the *mp-319* allele (see Table 2.3 for primers).

GFP-positive *AtML1::PIN1:GFP* homozygotes from the F2 generation of a cross between *pin1-7/PIN1;AtML1::PIN1:GFP* and Col-0 were self-pollinated to generate F3 families homozygous for *AtML1::PIN1:GFP* in a wild-type background. Plants were genotyped for the presence of the *pin1-7* allele (see Table 2.3 for primers).

VENUS/GFP-positive *AtML1::PIN1:GFP;DR5::VENUS* homozygotes from the F2 generation of a cross between *AtML1::PIN1:GFP* and *DR5::VENUS* were self-pollinated to generate F3 families homozygous for *AtML1::PIN1:GFP* and *DR5::VENUS*.

VENUS/GFP-positive *pin1-7/PIN1* heterozygotes from the F2 generation of a cross between *pin1-7/PIN1;AtML1::PIN1:GFP* and *DR5::VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *DR5::VENUS* and *AtML1::PIN1:GFP* and heterozygous for *pin1-7/PIN1*. Plants were genotyped for the *pin1-7* allele (see Table 2.3 for primers)

GUS-positive *cuc2-3* homozygotes from the F2 generation of a cross between *cuc2-3* and *IAA2::GUS* homozygotes were self-pollinated to generate F3 families homozygous for *cuc2-3* and *IAA2::GUS*. M. Barkoulas generated F2 families.

GUS-positive *cuc2-3* homozygotes from the F2 generation of a cross between *cuc2-3* and *ATHB-8::GUS* homozygotes were self-pollinated to generate F3 families homozygous for *cuc2-3* and *ATHB-8::GUS*.

cus1 homozygotes from the F2 generation of a cross between *cus1;cuc2-3* and Col-0 were self-pollinated to generate F3 families homozygous for *cus1*. Plants were genotyped for the presence of the *cuc2-3* allele (see Table 2.3 for primers).

GFP-positive *cus1* homozygotes from the F2 generation of a cross between *cus1* and *DR5::GFP* homozygotes were self-pollinated to generate F3 families homozygous for *cus1* and *DR5::GFP*.

GFP-positive *cus1* homozygotes from the F2 generation of a cross between *cus1* and *PIN1::PIN1:GFP* homozygotes were self-pollinated to generate F3 families homozygous for *cus1* and *PIN1::PIN1:GFP*.

GFP-positive *cus1;cuc2-3* homozygotes from the F2 generation of a cross between *cus1;cuc2-3* and *DR5::GFP* homozygotes were self-pollinated to generate F3 families

homozygous for *cus1;cuc2-3* and *DR5::GFP*. Plants were genotyped for the presence of the *cuc2-3* allele (see Table 2.3 for primers).

GFP-positive *cus1;cuc2-3* homozygotes from the F2 generation of a cross between *cus1;cuc2-3* and *PIN1::PIN1:GFP* homozygotes were self-pollinated to generate F3 families homozygous for *cus1;cuc2-3* and *PIN1::PIN1:GFP*. Plants were genotyped for the presence of the *cuc2-3* allele (see Table 2.3 for primers).

cus1 homozygotes from the F2 generation of a cross between *pin1-7/PIN1* heterozygotes and *cus1* homozygotes were self-pollinated to generate F3 families homozygous for *cus1* and heterozygous for *pin1-7/PIN1*. Plants were genotyped for the presence of the *pin1-7* allele (see Table 2.3 for primers).

mir164a-4 homozygotes from the F2 generation of a cross between *mir164a-4* and *cus1* homozygotes were self-pollinated to generate F3 families homozygous for *mir164a-4* and *cus1*. Plants were genotyped for the *mir164a-4* allele (see Table 2.3 for primers).

axr1-3 homozygotes from the F2 generation of a cross between *axr1-3* and *cus1* homozygotes were self-pollinated to generate F3 families homozygous for *axr1-3* and *cus1*.

asl-1 homozygotes from the F2 generation of a cross between *asl-1* and *cus1* homozygotes were self-pollinated to generate F3 families homozygous for *asl-1* and *cus1*.

asl-1 homozygotes from the F2 generation of a cross between *asl-1* and *cuc2-3* homozygotes were self-pollinated to generate F3 families homozygous for *asl-1* and *cuc2-3*.

axr1-3;asl-1 homozygotes from the F2 generation of a cross between *axr1-3;asl-1* and *axr1-3;cuc2-3* homozygotes were self-pollinated to generate F3 families homozygous for *axr1-3*, *asl-1* and *cuc2-3*.

asl-1 homozygotes from the generation of a cross between *asl-1* and *mir164a-4* homozygotes were self-pollinated to generate F3 families homozygous for *asl-1* and *mir164a-4*. Plants were genotyped for the *mir164a-4* allele (see Table 2.3 for primers).

mir164a-4 homozygotes from the F2 generation of a cross between *mir164a-4* and *cuc2-3* homozygotes were self-pollinated to generate F3 families homozygous for *mir164a-4* and *cuc2-3*. Plants were genotyped for the *cuc2-3* allele and the *mir164a-4* allele (see Table 2.3 for primers).

asl-1;mir164a-4 homozygotes from the F2 generation of a cross between *asl-1;mir164a-4* and *asl-1;cuc2-3* homozygotes were self-pollinated to generate F3 families homozygous for *asl-1*, *mir164a-4* and *cuc2-3*. Plants were genotyped for the *cuc2-3* allele and the *mir164a-4* allele (see Table 2.3 for primers).

FIL>>BP homozygotes from the F2 generation of a cross between *FIL>>BP* and *cuc2-3* homozygotes were self-pollinated to generate F3 families homozygous for *FIL>>BP* and *cuc2-3*.

LFY>>STM homozygotes from the F2 generation of a cross between *LFY>>STM* and *cuc2-3* homozygotes were self-pollinated to generate F3 families homozygous for *LFY>>STM* and *cuc2-3*. P. Piazza generated F2 families.

VENUS-positive *FIL>>BP* homozygotes from the F2 generation of a cross between *FIL>>BP* and *CUC2::CUC2:VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *FIL>>BP* and *CUC2::CUC2:VENUS*.

VENUS-positive *LFY>>STM* homozygotes from the F2 generation of a cross between *LFY>>STM* and *CUC2::CUC2:VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *LFY>>STM* and *CUC2::CUC2:VENUS*.

VENUS-positive *as1-1* homozygotes from the F2 generation of a cross between *as1-1* and *CUC2::CUC2:VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *as1-1* and *CUC2::CUC2:VENUS*. M. Barkoulas generated F2 families.

VENUS-positive *axr1-3;as1-1* homozygotes from the F2 generation of a cross between *axr1-3;as1-1* and *CUC2::CUC2:VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *axr1-3*, *as1-1* and *CUC2::CUC2:VENUS*.

VENUS-positive *mir164a-4* homozygotes from the F2 generation of a cross between *mir164a-4* and *CUC2::CUC2:VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *mir164a-4* and *CUC2::CUC2:VENUS*. M. Barkoulas generated F2 families.

VENUS-positive *as1-1;mir164a-4* homozygotes from the F2 generation of a cross between *as1-1;mir164a-4* and *CUC2::CUC2:VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *as1-1*, *mir164a-4* and *CUC2::CUC2:VENUS*.

GUS-positive *axr1-3* homozygotes from the F2 generation of a cross between *axr1-3* and *CUC2::GUS* homozygotes were self-pollinated to generate F3 families homozygous for *axr1-3* and *CUC2::GUS*.

as1-1;CUC2::GUS: GUS-positive *as1-1* homozygotes from the F2 generation of a cross between *as1-1* and *CUC2::GUS* homozygotes were self-pollinated to generate F3 families homozygous for *as1-1* and *CUC2::GUS*. A. Hay generated F2 families.

GUS-positive *axr1-3;as1-1* homozygotes from the F2 generation of a cross between *axr1-3;as1-1* and *CUC2::GUS* homozygotes were self-pollinated to generate F3 families homozygous for *axr1-3*, *as1-1* and *CUC2::GUS*.

35S::*MIR164A* homozygotes from the F2 generation of a cross between *axr1-3* and 35S::*MIR164A* homozygotes were self-pollinated to generate F3 families homozygous *axr1-3* and 35S::*MIR164A*. A. Hay generated F2 families.

35S::*MIR164A* homozygotes from the F2 generation of a cross between *as1-1* and 35S::*MIR164A* homozygotes were self-pollinated to generate F3 families homozygous for *as1-1*;35S::*MIR164A*. A. Hay generated F2 families.

35S::*MIR164A* homozygotes from the F2 generation of a cross between *axr1-3*; *as1-1* and 35S::*MIR164A* homozygotes were self-pollinated to generate F3 families homozygous for *axr1-3*, *as1-1* and 35S::*MIR164A*.

GUS-positive *cuc2-3* homozygotes from the F2 generation of a cross between *cuc2-3* and *BP::GUS* homozygotes were self-pollinated to generate F3 families homozygous for *cuc2-3* and *BP::GUS*.

GUS-positive *mir164a-4* homozygotes from the F2 generation of a cross between *mir164a-4* and *BP::GUS* homozygotes were self-pollinated to generate F3 families homozygous for *mir164a-4* and *BP::GUS*.

GUS-positive *axr1-3* homozygotes from the F2 generation of a cross between *axr1-3*; *BP::GUS* and *cuc2-3* homozygotes were self-pollinated to generate F3 families homozygous for *axr1-3*, *cuc2-3* and *BP::GUS*.

GUS-positive *as1-1* homozygotes from the F2 generation of a cross between *as1-1*; *BP::GUS* and *cuc2-3* homozygotes were self-pollinated to generate F3 families homozygous for *as1-1*, *cuc2-3*, and *BP::GUS*.

GUS-positive *axr1-3;asl-1* homozygotes from the F2 generation of a cross between *axr1-3;asl-1;BP::GUS* and *cuc2-3* homozygotes were self-pollinated to generate F3 families homozygous for *axr1-3*, *asl-1* and *cuc2-3;BP::GUS*.

GUS-positive *asl-1;mir164a-4* homozygotes from the F2 generation of a cross between *asl-1;mir164a-4* and *BP::GUS* homozygotes were self-pollinated to generate F3 families homozygous for *asl-1*, *mir164a-4* and *BP::GUS*.

GUS-positive *mir164a-4* homozygotes from the F2 generation of a cross between *mir164a-4;BP::GUS* and *cuc2-3* homozygotes were self-pollinated to generate F3 families homozygous for *mir164a-4*, *cuc2-3* and *BP::GUS*.

GUS-positive *asl-1;mir164a-4* homozygotes from the F2 generation of a cross between *asl-1;mir164a-4;BP::GUS* and *cuc2-3* homozygotes were self-pollinated to generate F3 families homozygous for *asl-1*, *mir164a-4*, *cuc2-3* and *BP::GUS*.

GUS-positive *cuc2-3* homozygotes from the F2 generation of a cross between *cuc2-3* and *ChBP::GUS* homozygotes were self-pollinated to generate F3 families homozygous for *cuc2-3* and *ChBP::GUS*.

GUS-positive *35S::MIR164A;cuc3-105* homozygotes from the F2 generation of a cross between *35S::MIR164A;cuc3-105* and *ChBP::GUS* homozygotes were self-pollinated to generate F3 families homozygous for *35S::MIR164A*, *cuc3-105* and *ChBP::GUS*.

GUS-positive *cuc2-3* homozygotes from the F2 generation of a cross between *cuc2-3* and *CYCBI;1::GUS* homozygotes were self-pollinated to generate F3 families homozygous for *cuc2-3* and *CYCBI;1::GUS*. F2 families were generated by M. Barkoulas.

GUS-positive *35S::MIR164A;cuc3-105* homozygotes from the F2 generation of a cross between *35S::MIR164A;cuc3-105* and *CYCBI;1::GUS* homozygotes were self-

pollinated to generate F3 families homozygous for *35S::MIR164A*, *cuc3-105* and *CYCB1;1::GUS*.

C. hirsuta

GFP/VENUS-positive plants from the F2 generation of a cross between *PIN1::PIN1:GFP* and *CUC2::CUC2:VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *PIN1::PIN1:GFP* and *CUC2::CUC2:VENUS*. F2 families were generated by M. Barkoulas.

GFP/VENUS-positive plants from the F2 generation of a cross between *PIN1::PIN1:GFP* and *DR5::VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *PIN1::PIN1:GFP* and *DR5::VENUS*. F2 families were generated by M. Barkoulas.

VENUS-positive *35S::MIR164B;CUC3RNAi* plants from the F2 generation of a cross between *35S::MIR164B;CUC3RNAi* and *DR5::VENUS* homozygotes were self-pollinated to generate F3 families of *35S::MIR164B*, *CUC3RNAi* and *DR5::VENUS*. F2 families were generated by M. Barkoulas.

GFP-positive *35S::MIR164B;CUC3RNAi* plants from the F2 generation of a cross between *PIN1::PIN1:GFP* and *35S::MIR164B;CUC3RNAi* homozygotes were self-pollinated to generate F3 families of *PIN1::PIN1:GFP*, *35S::MIR164B* and *CUC3RNAi*. M. Barkoulas generated F2 families.

VENUS-positive *chpin1-1/PIN1* heterozygotes from the F2 generation of a cross between *chpin1-1/PIN1* heterozygotes and *CUC2::CUC2:VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *CUC2::CUC2:VENUS* and heterozygous for *chpin1-1/PIN1*. M. Barkoulas generated F2 families.

35S::*MIR164B*;CUC3RNAi plants from the F2 generation of a cross between *chas1-1* and 35S::*MIR164B*;CUC3RNAi homozygotes were self-pollinated to generate F3 families homozygous for *chas1-1* and segregating 35S::*MIR164B* and CUC3RNAi.

GUS-positive 35S::*MIR164B*;CUC3RNAi plants from the F2 generation of a cross between 35S::*MIR164B*;CUC3RNAi and *ChBP*>>*GUS* homozygotes were self-pollinated to generate F3 families of 35S::*MIR164B*, CUC3RNAi and *ChBP*>>*GUS*.

GUS-positive 35S::*MIR164B*;CUC3RNAi plants from the F2 generation of a cross between 35S::*MIR164B*;CUC3RNAi and *CYCB1;1::GUS* homozygotes were self-pollinated to generate F3 families of 35S::*MIR164B*, CUC3RNAi and *CYCB1;1::GUS*

VENUS-positive *lol1* homozygotes from the F2 generation of a cross between *lol1* and CUC2::*CUC2:VENUS* homozygotes were self-pollinated to generate F3 families homozygous for *lol1* and CUC2::*CUC2:VENUS*.

2.2.3 Bacterial transformations

For transformations of chemically competent DH5α *E. coli* cells, 100ng plasmid was added to 100μl defrosted cells, and cells were incubated at 4°C for 30mins. Cells were then heat-shocked at 42°C for 30s, before being incubated at 4°C for 2mins. Cells were then added to 200μl LB liquid medium and incubated at 37°C at 225rpm for 1h. Cells were then plated on LB agar plates with appropriate antibiotics and incubated at 37°C overnight. For plasmids with kanamycin resistance, cells were grown on 100μg/ml kanamycin (Sigma) LB agar plates; for plasmids with ampicillin resistance, cells were grown on 100μg/ml ampicillin (Sigma) LB agar plates; for plasmids with spectinomycin resistance, cells were grown on 70μg/ml spectinomycin (Sigma) LB agar plates.

For transformations of electrocompetent GV301 *A. tumefaciens*, 100ng plasmid was added to 100µl defrosted cells and cells were electroporated at 2kV, 200Ω and 25µF. 500µl LB liquid medium was then added to the cells, which were incubated at 28°C at 225rpm for 2h. Cells were then plated on 25µg/ml gentamycin LB agar plates with appropriate additional antibiotics for the transformed plasmid, and incubated at 28°C for 2d. For plasmids with kanamycin resistance, cells were grown on 100µg/ml kanamycin (Sigma) LB agar plates; for plasmids with ampicillin resistance, cells were grown on 100µg/ml ampicillin (Sigma) LB agar plates; for plasmids with spectinomycin resistance, cells were grown on 70µg/ml spectinomycin (Sigma) LB agar plates.

2.2.4 Plant transformation of *A. thaliana* and *C. hirsuta*

Vectors containing T-DNA, including the cassette-of-interest, were introduced into the GV3101 strain of *A. tumefaciens* by electroporation, and colonies were grown on LB agar plates with appropriate antibiotics at 28°C for 2d. A single positive colony was used to inoculate 5ml LB liquid medium with appropriate antibiotics, and was cultured at 28°C for 2d. This small culture was used to inoculate 1L YEB with appropriate antibiotics, and was cultured at 28°C until an optical density of $OD_{600nm}=0.7-1$ was reached. Cells were pelleted and re-suspended in 500ml infiltration medium (5% sucrose (Fisher), 10mM $MgCl_2$ (Sigma), 1x Gamborg's vitamins (Sigma), 100ng/ml 6-benzylaminopurine (Sigma), 0.03% silwet-77 (GE Silicones)). Inflorescences with unopened buds were submerged in the infiltration medium for 12mins, and then wrapped in cling film and stored at 4°C overnight. Plants were subsequently transferred to the greenhouse and the resulting seeds were harvested once dried.

2.2.5 Selection of transgenic plants

To select for transgenic plants resistant to BASTA (commercial name “Kaspar”; Aventis), plants were grown on soil in a greenhouse and sprayed with 400µM BASTA when the cotyledons were expanded.

To select for transgenic plants resistant to hygromycin (Duchefa), seeds were sterilised with 10% bleach for 10mins, followed by 70% ethanol for 10mins, before being washed with ddH₂O six times. Seeds were then plated on MS agar plates with 25µg/ml hygromycin (Duchefa) for *A. thaliana* or 50µg/ml for *C. hirsuta*. Seeds were stratified at 4°C for 3 days in the dark before being transferred to growth cabinets.

2.2.6 EMS mutagenesis and genetic screening

cuc2-3 (Col-0 ecotype) seed was mutagenised by agitation with 0.2% ethane methyl sulfonate (EMS; Sigma) for 10h, washed extensively in ddH₂O, then sown and their seeds harvested in pools of five M1 plants. Approximately 845 M2 plants were screened for individuals suppressing the smooth margin phenotype of *cuc2-3* by producing leaves with serrated margins. From this screen, fifteen suppressors were identified. Suppressors were verified as having the *cuc2-3* T-DNA insertion using *cuc2-3* genotyping primers CUC2-3F, CUC2-3R and SAIL-LB. At least three backcrosses to *cuc2-3* were performed before mutant phenotypes were characterised.

2.2.7 Map-based cloning

Map-based cloning is a technique that identifies the genetic location responsible for a mutant phenotype by determining linkage to genetic markers whose known physical locations in the genome are known. In this study, map-based cloning was performed to attempt to identify the genetic locus of *cus1*, a suppressor of *cuc2*. *cus1* mutants were identified in the *cuc2-3* background (Col-0 ecotype) as suppressing the smooth margin phenotype of *cuc2* by producing leaves with serrated margins. *cus1* mutants were backcrossed three times to *cuc2-3* before mapping was performed to remove additional mutations in the genome that were not responsible for the mutant phenotype. Map-based cloning was then performed as described below.

The recessive *cus1* mutant in the *cuc2-3* background (Col-0 ecotype) was out-crossed to *cuc2-1* (*Ler* ecotype). F1 hybrids were verified as having the expected smooth margin phenotype of *cuc2*, and genomic DNA was extracted from rosette leaves of the F1 hybrids to be used as control heterozygous DNA during mapping. F1 plants were self-fertilised, and F2 seed was harvested. In the F2 population, the *cus1* phenotype segregated in a Mendelian ratio of 3:1, and genomic DNA was extracted from the rosette leaves of 45 homozygous F2 individuals displaying the serrated leaf phenotype of *cus1*. 50ng of genomic DNA was taken from each of the 45 individual samples, and was pooled together for use in Bulk Segregant Analysis.

Bulk Segregant Analysis is a technique that rapidly determines linkage to genetic markers using pooled DNA samples from individuals with the mutant phenotype that were segregating in a F2 population derived from a single out-cross. Col-0, *Ler*, F1 heterozygous and pooled F2 homozygous genomic DNA was examined using 22 Simple Sequence Length Polymorphism (SSLP) markers distributed evenly across the genome (Lukowitz *et al.*, 2000); Table 2.4) to determine linkage to a single chromosome arm.

Once linkage had been established to markers 2-AC005917-4269 and 2-AC005623-5887 with the pooled genomic DNA, all 45 individual genomic DNA samples were tested those markers and the recombination frequency at each marker was calculated using the following formula:

$$\text{Recombination frequency} = \frac{\text{Number of recombined chromosomes}}{\text{Number of chromosomes examined}}$$

The recombination frequencies between each marker and the mutation determined the position of the mutation between two markers, and the physical locations of the markers allowed the size of the interval containing the mutation to be calculated. Fine mapping was

then performed once flanking markers were identified at either side of the mutation. 480 homozygous F2 mutants were analysed with new markers to further reduce the size of the interval.

2.3 Molecular biology methods

2.3.1 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a molecular biology technique that exponentially amplifies a target sequence of DNA into millions of copies. For standard PCR conditions that did not require 3'-5' exonuclease proofreading activity, 0.1pmol plasmid-1µg genomic DNA was added to a PCR mix of: 1x *MangoTaq* buffer (Bioline); 2.5mM MgCl₂ (Bioline); 0.1mM dNTPs (Fermentas); 8µM forward primer (Sigma); 8µM reverse primer (Sigma) and 0.3U *MangoTaq* DNA polymerase (Bioline), in a final volume of 50µl in a 0.2ml PCR tube (Starlab). DNA was then amplified in an Applied Biosystems 2720 Thermal Cycler using the following protocol: 1 cycle at 94°C for 3mins, 35 cycles of 94°C for 30s, 55°C-58°C for 30s and 72°C for 15s-30s per kbp extension, followed by 10mins at 72°C.

For the amplification of DNA that required 3'-5' exonuclease activity proof-reading activity for cloning purposes, 0.1pmol plasmid-1µg genomic DNA was added to a PCR mix of: 1x *Pyrobest* buffer II (Takara); 0.2mM dNTPs (Fermentas); 1U *Pyrobest* DNA polymerase (Takara); 0.16µM forward primer (Sigma) and 0.16µM reverse primer in a final volume of 100µl in a 0.2ml PCR tube (Starlab). DNA was then amplified in an Applied Biosystems 2720 Thermal Cycler using the following protocol: 1 cycle at 98°C for 1min and 35 cycles at 98°C for 30s, 55°C-58°C for 30s, 72°C for 1min per kbp extension. 0.3U of *MangoTaq* DNA polymerase (Bioline) was then added and the tube was incubated at 72°C for 10mins for A-tailing.

2.3.2 Sequencing reactions

To sequence plasmid inserts, 150ng-300ng DNA was added to 1x BigDye 3.1 Terminator Ready Reaction Premix (Applied Biosystems), 1x BigDye 3.1 sequencing buffer (Applied Biosystems) and 3.2 pmol primer (Sigma) in a final volume of 20µl in a 0.2ml PCR tube (Starlab). DNA was then amplified in an Applied Biosystems 2720 Thermal Cycler using the following protocol: 35 cycles of 96°C for 10s, 50°C for 5s and 60°C for 4mins. PCR products were precipitated in 0.3M sodium acetate, 70% ethanol at room temperature for 15mins, then tubes were spun at 5000rpm in a centrifuge (Sigma 4-15C) for 5mins. The supernatant was removed and 100µl of 70% ethanol was added to the tubes. Tubes were spun at 5000rpm in a centrifuge (Sigma 4-15C) for 5mins, the supernatant was removed and the DNA pellets were air-dried. DNA was then sent for sequencing on an ABI Prism 377 DNA sequencer (Applied Biosystems, DNA sequencing service, Department of Zoology, University of Oxford).

2.3.3 Genomic DNA extraction for PCR

To extract small amounts of plant genomic DNA for PCR analysis, a single young leaf was macerated in a 1.5ml Eppendorf tube, and 400µl of extraction buffer (250mM Tris-HCl pH 7.5, 250mM NaCl, 25mM EDTA, 0.5% SDS) was added to the tube and vortexed. Tubes were spun at 13000rpm in a benchtop microcentrifuge (Eppendorf centrifuge 5415 D) for 5mins, and 300µl of the supernatant was transferred to a 1.5ml Eppendorf tube containing 300µl propan-2-ol. Tubes were vortexed and incubated at room temperature for 2mins to precipitate DNA. Tubes were spun at 13000rpm in a benchtop microcentrifuge (Eppendorf centrifuge 5415 D) for 5mins, the supernatant was removed and the DNA pellets were air-dried. 100µl ddH₂O was then added to re-dissolve the DNA.

2.3.4 Genomic DNA extraction for map-based cloning

To extract small amounts of plant genomic DNA for PCR analysis in map-based cloning, a single young leaf was added to a single well of a 96 well microtitre plate (Qiagen), and 400µl of extraction buffer (250mM Tris-HCl pH 7.5, 250mM NaCl, 25mM EDTA, 0.5% SDS) was added. Tissue was macerated with beads (3mm tungsten carbide beads; Qiagen) at 13000rpm for 1min in a Retsch MM 300 macerating machine. Plates were spun at 5000rpm in a centrifuge (Sigma 4-15C) for 20mins. 300µl of the supernatant was transferred to a new microtitre plate and 300µl propan-2-ol was added to each well. Plates were vortexed and incubated at room temperature for 2mins to precipitate DNA. Plates were spun at 5000rpm in a centrifuge (Sigma 4-15C) for 20mins, the supernatant was removed and the DNA pellets were air-dried. 100µl ddH₂O was then added to re-dissolve the DNA. DNA was then diluted 1:10 in ddH₂O, and 10µl was used per 50µl PCR sample.

2.3.5 Quantitative RT-PCR analysis

RNA was extracted from 10d old seedling tissue using the Qiagen RNeasy kit. 1µg of RNA was added to a mix of: 1U DNaseI (Invitrogen); 1x DNaseI buffer (Invitrogen) in a final volume of 10µl in a 0.2ml PCR tube (Starlab). Tubes were incubated at room temperature for 10mins. Tubes were then incubated at 65°C in 2.27mM EDTA (Invitrogen) for 10mins, followed by 10mins at 70°C with 0.5µg oligo(dT) (Invitrogen). A mix of: 1x First Strand buffer (Invitrogen); 0.01M dithiothritol (DTT; Invitrogen); 0.5mM dNTPs (Fermentas) was added to the tubes, which were then incubated at 42°C for 2mins. Finally, 1U Superscript II reverse transcriptase (Invitrogen) was added and the tubes were then incubated at 42°C for 60mins followed by 70°C for 15mins.

cDNA was amplified on the ABI PRISM 7300 Sequence Detection System (Applied Biosystems). Amplification reactions were prepared by adding 10µl SYBR-Green PCR

Master Kit (Applied Biosystems) and 0.4 μ M primer to 10 μ l of 1:10 diluted cDNA per reaction. Each reaction was made in triplicate, and each biological replicate was repeated three times. The efficiency of each set of primers and calculation of the level of induction was determined according to Pfaffl (Pfaffl, 2001). Error bars represent the standard error of the mean calculated from the mean of three biological replicates. For *A. thaliana*, expression levels were normalised with values obtained for the *ORNITHINE TRANSCARBAMYLASE* (*OTC*) gene, which was used as an internal reference gene as described by Cnops (Cnops *et al.*, 2004). For *C. hirsuta*, expression level were normalised with values obtained for the *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (*GAPDH*) gene. Primers used are listed in Table 2.3.

2.3.6 Plasmid construction and analysis of transgenics

To generate the AtML1-pMDC32 vector, the *ATML* promoter was amplified from an *ATML1::GFP* plasmid (Gifford *et al.*, 2003) using ATML1-HindIII-F and ATML1-KpnI-R primers and Pyrobest polymerase (Takara), and subcloned into PCRblunt (Invitrogen). The *AtML1* promoter was sequenced to ensure the correct sequence was present. The 35S promoter was removed from pMDC32 (Curtis and Grossniklaus, 2003) by a HindIII/KpnI (New England Biolabs) double digestion and replaced with the subcloned *AtML1* promoter. H. Jenkins performed these steps to generate the AtML1-pMDC32 vector.

For the *AtML1::PIN1:GFP* construct, a *PIN1:GFP* cassette was PCR amplified from a *PIN1::PIN1:GFP* plasmid (Heisler *et al.*, 2005) using PIN1-F and PIN1-R primers using Pyrobest polymerase (Takara), and subcloned into pCR8/GW-TOPO (Invitrogen). The *PIN1:GFP* cassette was sequenced using M13-F and M13-R primers to ensure the correct sequence was present. The *PIN1:GFP* cassette was then recombined into AtML1-pMDC32 using Gateway Clonase II enzyme mix (Invitrogen). The *AtML1::PIN1:GFP* construct was transformed into plants segregating *pin1-7*. Thirteen independent transgenic lines of

AtML1::PIN1:GFP were obtained from a hygromycin selection, of which 3 were confirmed to be mutant for *pin1-7*. One representative line of *AtML1::PIN1:GFP* in *pin1-7* and one independent line of *AtML1::PIN1:GFP* in wild-type were analysed in this study.

For the *AtML1::CUC2:VENUS* construct, a *CUC2:VENUS* cassette was PCR amplified from a *CUC2::CUC2:VENUS* plasmid (Heisler *et al.*, 2005) using CUC2-F and VENUS-R primers and Pyrobest polymerase (Takara), and subcloned into pCR8/GW-TOPO (Invitrogen). The *CUC2:VENUS* cassette was sequenced using M13-F and M13-R primers to ensure the correct sequence was present. The *CUC2:VENUS* cassette was then recombined into *AtML1*-pMDC32 using Gateway Clonase II enzyme mix (Invitrogen). M. Barkoulas performed these steps to generate the *AtML1::CUC2:VENUS* construct. The *AtML1::CUC2:VENUS* construct was transformed into Col-0 plants and three broad classes of transformants were observed from a hygromycin selection: cup-shaped cotyledons and shootless; fused cotyledons and shootless; and leaf-producing plants (not shootless). For the latter category, 41 independent transgenic lines were recovered, of which nine had smooth leaf margins and six had asymmetric leaf lamina.

For the *ATHB-8::GUS* construct, the *ATHB-8* promoter fragment was digested from the pBJ96 plasmid using a *HinDIII/KpnI* (New England Biolabs) double digestion and ligated into a pRITA plasmid (provided by J. Bowman) that had been linearised by a *HinDIII/KpnI* (New England Biolabs) double digestion, using T4 DNA ligase (Promega). The *ATHB-8::GUS* cassette was removed from the pRITA plasmid by a *NotI* digestion and ligated into a pMLBART plasmid (Eshed *et al.*, 1999), linearised by a *NotI* digestion, using T4 DNA ligase (Promega). The *ATHB-8::GUS* construct was transformed into Ox plants. 10 independent transgenic lines were obtained from a BASTA selection, all of which had positive GUS staining in the vasculature. A single representative transgenic line was chosen for analysis.

To test whether the *A. thaliana* *CUC2::CUC2:VENUS* construct (Heisler *et al.*, 2005) complements the *A. thaliana* *cuc2* mutation, *CUC2::CUC2:VENUS* was transformed into *cuc2-3* plants. Forty six out of fifty T2 lines showed a strong reversion from the smooth *cuc2* leaf margin phenotypes back to wild-type leaf geometries with serrated margins. To additionally test whether *A. thaliana* *CUC2* has the same function as *C. hirsuta* *CUC2*, the *A. thaliana* *CUC2::CUC2:VENUS* construct (Heisler *et al.*, 2005) was transformed into *C. hirsuta mir164a;cuc2* double mutants, causing reversion back to the *mir164a* phenotype in three out of seven T2 lines. This suggests that the *A. thaliana* *CUC2::CUC2:VENUS* construct can complement the *C. hirsuta* *cuc2* mutation, and that *A. thaliana* and *C. hirsuta* *CUC2* functions are equivalent. This experiment was performed by H. Jenkins.

The primers used in these plasmid constructions are listed in Table 2.3.

2.4 Microscopy, histology and histochemistry

2.4.1 Confocal microscopy

Seedlings for confocal microscopy were mounted and observed in water without fixation. To image GFP as a single localisation, a Zeiss LSM510 Meta microscope with a 25x (Plan Neofluar) and a 40x (C Apochromat) lens was used, and the parameters are listed in Table 2.6. To image GFP as a single localisation, a Zeiss LSM510 Meta microscope with a 25x (Plan Neofluar) and a 40x (C Apochromat) lens was used, and the parameters are listed in Table 2.7. To image mGFP and VENUS in a colocalisation, a Zeiss LSM510 Meta microscope with a 25x (Plan Neofluar) and a 40x (C Apochromat) lens was used, and the parameters are listed in Table 2.8. To image eGFP and VENUS in a colocalisation, a Leica DM6000 CS microscope with a 20x lens was used, and the parameters are listed in Table 2.9.

Single plane sections or projections from stacks of 5-20 sections were presented in the figures.

Table 2.6 Confocal microscope parameters for GFP localisation

Parameter	Details
Laserline wavelength	458nm argon laser (60% output, 65.3% attenuation)
Filters	GFP: 475-525nm band pass filter; chlorophyll: 646-700nm filter
Pinhole diameters	GFP: 152nm; chlorophyll: 152nm
Beam splitters	Primary dichroic: 458/543; secondary dichroic: 635
Line averaging	16

Table 2.7 Confocal microscope parameters for VENUS localisation

Parameter	Details
Laserline wavelength	514nm argon laser (60% output, 10.9% attenuation)
Filters	VENUS: 535-590nm band pass filter; chlorophyll: 647-747nm filter
Pinhole diameters	VENUS: 88nm; chlorophyll: 88nm
Beam splitters	Primary dichroic: 458/514; secondary dichroic: 635
Line averaging	16

Table 2.8 Confocal microscope parameters for mGFP/VENUS colocalisation

Parameter	Details
Laserline wavelengths	VENUS: 514nm argon laser (60% output, 10.1% attenuation) mGFP and chlorophyll: 458nm argon laser (60% output, 50% attenuation)
Filters	VENUS: 535-590nm band pass filter; mGFP: 475-525nm band pass filter; chlorophyll: 615-700nm filter
Pinhole diameters	VENUS: 152nm; mGFP: 152nm; chlorophyll: 152nm
Beam splitters	Primary dichroic: 458/514; secondary dichroic: 635
Line averaging	16

Table 2.9 Confocal microscope parameters for eGFP/VENUS colocalisation

Parameter	Details
Laserline wavelengths	VENUS: 514nm argon laser (25% output); eGFP and chlorophyll: 488nm argon laser (25% output)
Filters	VENUS: 520-592nm; eGFP: 495-509nm; chlorophyll: 632-731nm
Pinholes	VENUS: 60.6nm; eGFP: 60.6nm; chlorophyll: 60.6nm
Line averaging	4

2.4.2 Scanning electron microscopy (SEM)

Tissue was added to 3% gluteraldehyde, 25mM phosphate buffer pH=7.0, vacuum infiltrated on ice for 30mins, and then fixed overnight at 4°C. This was followed by dehydration through an ethanol series of 15%-100%. Samples were critically point dried using a Tousimis AutoSamDry 814 critical point dryer, dissected as appropriate, sputter coated with palladium using a Polaron SC7640 sputter coater, and viewed on a JSM-5510 (Jeol) microscope.

2.4.3 GUS staining

GUS staining was performed as previously described (Sessions *et al.*, 1999) by fixing tissue in 90% acetone on ice for 10mins. Tissue was washed twice in ice-cold water and then incubated at 37°C overnight in freshly prepared GUS solution (100mM sodium phosphate buffer, 10mM sodium EDTA, 1mM 5-bromo-4-chloro-3-indoly-β-D-glucuronic acid (Invitrogen)) supplied with ferrocyanide and ferricyanide salts (2mM: *ATHB-8::GUS*, *IAA2::GUS* and *CYCBI;1::GUS*; 10mM: *CUC2::GUS* and *MIR164A::GUS*). Reactions were terminated with 95% ethanol, and leaves were dissected, mounted in 50% glycerol, and viewed using either differential interference contrast microscopy (DIC) with an Olympus BX50 microscope, or dark field microscopy.

2.4.4 Leaf clearings

Leaf clearings were performed by incubating leaves in excess 7:1 ethanol:acetic acid solution overnight at room temperature. Leaves were then transferred to chloral hydrate solution (8g chloral hydrate (Fisher):2ml ddH₂O:1ml glycerol (Fisher)) and were mounted on slides with chloral hydrate solution before viewing with dark field photography.

2.4.5 Chemical treatments

For indole-3-acetic acid (IAA) treatments, IAA (Sigma) was dissolved in ethanol to a stock concentration of 10mM and added to MS liquid medium (1% sucrose, 0.22% MS (Duchefa), 1% 2-(N-Morpholino)-ethanesulphonic acid (MES; Melford)) to a final concentration of 1μM. 0.002% ethanol in MS liquid medium was used for controls. *CUC2::GUS* plants were grown on sterile MS-agar plates and incubated in either 1μM IAA MS liquid medium or 0.002% ethanol MS liquid medium at 20°C and shaken at 100rpm for 24h. Samples were then GUS-stained.

For experiments using 2,4-dichlorophenoxyacetic acid (2,4-D) in spray treatments, 2,4-D (Sigma) was dissolved in dimethylsulfoxide (DMSO; Sigma) to stock concentration of 1mM and diluted to 10μM in ddH₂O in 0.02% Silwet. 1% DMSO, 0.02% Silwet was used as a control treatment. Col-0 plants were grown on soil and sprayed with either 10μM 2,4-D or control spray once per day for a week. Leaf phenotypes were then analysed.

For experiments using 2,4-dichlorophenoxyacetic acid (2,4-D) in continuous treatments, 2,4-D (Sigma) was dissolved in dimethylsulfoxide (DMSO; Sigma) to stock concentration of 1mM and added to MS-agar to a final concentration of 10μM. 1% DMSO in MS-agar was used as a control treatment. *CUC2::CUC2:VENUS C. hirsuta* plants were grown on sterile MS-agar plates with either 10μM 2,4-D or 1% DMSO.

For N-1-naphthylphthalamic acid (NPA) treatments, NPA (Sigma) was dissolved in DMSO (Sigma) to a stock concentration of 100mM and added to MS-agar to a final concentration of either 0.1µM, 0.5µM, 1µM or 5µM. Thus, 0.04%, 0.2%, 0.4%, 2% DMSO, respectively, in MS-agar was used for controls.

2.5 Leaf shape analysis

2.5.1 Silhouettes

For leaf silhouettes, leaves were adhered to white paper using clear adhesive (WHSmith), and were then digitally scanned at 800dpi using a Canoscan LiDE 700F scanner.

2.5.2 Quantification of leaf margin dissection index

Leaf area and perimeter were calculated from silhouettes using ImageJ software. Leaf margin dissection index was then determined by using the formula: $(\text{perimeter}^2)/(4\pi \times \text{area})$ (Bai *et al.*, 2010).

2.5.3 Extended eigenshape analysis

To determine whether the leaf shape of various genotypes differs significantly, extended eigenshape analysis was performed. Extended eigenshape analysis is a multivariate technique that reduces the dimensionality of a dataset to give a smaller number of orthogonal, unrelated eigenshape axes that define a morphospace describing the shape variation existing between genotypes. The position of each leaf along the eigenshape axis within the morphospace reflects the contribution of that eigenshape to the morphology of the leaf.

Leaves were measured as closed curves and interpolated to the minimum number of boundary coordinate points on each side of the leaf. Two landmarks corresponding to homologous parts of the leaf: the base of the petiole and the tip of the leaf were manually defined to improve the topological correspondence of the digitised representations of overall

leaf shape. Extended eigenshape analysis was then performed using Mathematica software. Specimens were aligned by phi functions, and differences of each phi-aligned object from the overall mean shape were used to generate a covariance matrix. A singular value decomposition of this matrix returned a series of orthogonal eigenshape axes that captured the principal components of variance among the outlines. Shape models were then generated to visualise the nature of variation along significant eigenshape axes. Analysis of variance (ANOVA) in conjunction with Tukey's test was used to assess the significant differences among the eigenshape axes between genotypes.

Chapter 3:

**The role of *CUC2* and auxin in
patterning serration development**

Chapter 3: The role of *CUC2* and auxin in patterning serration development

3.1 Introduction

A key question in developmental biology is how morphology is generated. Wide diversity in leaf margin morphology has been documented since antiquity (Theophrastus, 1916), but the genetic basis of leaf margin morphology was first studied over a century ago in nettle (Correns, 1909). The model species *A. thaliana* produces leaves with marginal protrusions termed serrations, thus studying serration development has become an attractive system to investigate the genetic mechanisms of morphogenesis. Moreover, recent work in *A. thaliana* has shown that serration development is regulated by hormones, transcription factors and miRNAs (Hay *et al.*, 2006; Nikovics *et al.*, 2006).

PIN1 is an auxin efflux carrier that regulates the direction of auxin flow by the polarity of its subcellular localisation (Petrasek *et al.*, 2006; Vieten *et al.*, 2007; Wisniewska *et al.*, 2006; Yang and Murphy, 2009). PIN1 forms convergence points at the leaf margin which generate auxin activity maxima; however, PIN1 has a basal subcellular localisation in the pre-procambial cells beneath the convergence point to internalise auxin flow (Hay *et al.*, 2006; Scarpella *et al.*, 2006). PIN1-mediated auxin transport is required for both serration and vasculature development as the chemical inhibition of auxin transport and loss-of-function *pin1* mutants produce leaves with smooth margins and aberrant vasculature (Hay *et al.*, 2006; Scarpella *et al.*, 2006). This led to the hypothesis that the processes of serration and vasculature development are coupled (Scarpella *et al.*, 2006). However, the extent to which these two processes are linked has not been tested.

An additional regulator of serration development is the transcription factor *CUC2* (Aida *et al.*, 1997; Nikovics *et al.*, 2006). *CUC2* is expressed in strips along the margin of the leaf and becomes downregulated at sites of serration development (Nikovics *et al.*, 2006). Loss-of-function *cuc2* mutants produce leaves with smooth margins that phenocopy *pin1* leaves, suggesting that *CUC2* is essential for serration development (Nikovics *et al.*, 2006). *CUC2* transcripts are post-transcriptionally regulated by the miRNA *mir164* (Nikovics *et al.*, 2006). Since loss-of-function *mir164a* mutants produce leaves with deeply serrated leaf margins due to increased levels of *CUC2*, this suggests that *CUC2* represses growth in the sinuses between serrations (Nikovics *et al.*, 2006). Furthermore, overexpression of *MIR164A* represses serration development, suggesting that the ratio of *CUC2:MIR164A* determines serration depth (Nikovics *et al.*, 2006). Interestingly, mutants with reduced auxin signalling, such as *axr1* and *bdl/BDL*, produce leaves with deeply serrated margins (Hamann *et al.*, 2002; Hay *et al.*, 2006; Leyser *et al.*, 1993), which phenocopy *mir164a* mutants. This suggests that correct auxin signalling may be required to regulate serration development in a manner similar to *MIR164A*.

In this chapter, I investigate the regulatory interactions between *CUC2*, PIN1 and auxin. I show that the formation of *CUC2* maxima cannot be uncoupled from PIN1 convergence points and auxin activity maxima. I further demonstrate that *CUC2* and auxin activity maxima form in complementary domains along the margin. I then show that these dynamic expression patterns of *CUC2*, PIN1 and auxin occur reiteratively along the proximo-distal axis of the margin. This creates interspersed peaks of *CUC2* and auxin activity maxima along the leaf margin. By taking a molecular genetics approach, I show that interspersed peaks of *CUC2* expression are required for PIN1 convergence points and auxin activity maxima to form. I then demonstrate that high auxin activity feeds back to repress *CUC2* expression. Thus, I show that a negative feedback loop between auxin and *CUC2* is re-

iteratively employed along the proximo-distal axis of the leaf to pattern serration development.

3.2 Results

3.2.1 *CUC2* and *PIN1* activities regulate serration initiation

Wild-type *A. thaliana* plants produce leaves with serrated margins (Figure 3.1 A), whereas loss-of-function *cuc2* and *pin1* mutants produce leaves with smooth margins (Figure 3.1 B-C; Hay *et al.*, 2006; Nikovics *et al.*, 2006). To understand whether *CUC2* and *PIN1* are required for serration initiation, I monitored leaf development in *cuc2* and *pin1* loss-of-function mutants using SEM. Wild-type leaves initiate as rounded structures with smooth margins (Figure 3.1 D), and initiate their first pair of serrations when the leaf is approximately 250µm in length (Figure 3.1 E; Nikovics *et al.*, 2006). Since serration development is an iterative process that occurs basipetally along the leaf margin, the second pair of serrations initiate when the leaf is approximately 500µm in length (Figure 3.1 F). *cuc2* and *pin1* mutant leaves initiate as morphologically similar structures to wild type (Figure 3.1 G, J). However, serration initiation fails to occur when *cuc2* and *pin1* leaves are between 250µm-500µm in length (Figure 3.1 H-I, K-L). These data, therefore, indicate that *CUC2* and *PIN1* functions are required for serration initiation.

3.2.2 Interspersed peaks of auxin and *CUC2* activity pattern serration development

Since *CUC2* and auxin transport both regulate the process of serration initiation, I hypothesised that they may interact in the same regulatory network. To investigate this, I analysed the spatio-temporal dynamics between *CUC2* and auxin activity using colocalised *CUC2::CUC2:VENUS* and *DR5::GFP*, which is an auxin sensitivity reporter. During leaf initiation, *DR5::GFP* is expressed at the tip and *CUC::CUC2:VENUS* is expressed at the

Figure 3.1 *CUC2* and *PIN1* function are required for serration initiation at the margins of *A. thaliana* leaves. **(A-C)** Fifth rosette leaf and half silhouette of fifth rosette leaf of Col-0 (A, A'), *cuc2-3* (B, B') and *pin1-7* (C, C'). **(D-F)** Scanning electron micrographs of fifth rosette leaf of Col-0 approximately 75µm in length (D), 250µm in length (E) and 500µm in length (F). **(G-I)** Scanning electron micrographs of fifth rosette leaf of *cuc2-3* approximately 75µm in length (G), 250µm in length (H) and 500µm in length (I). **(J-L)** Scanning electron micrographs of fifth rosette leaf of *pin1-7* approximately 75µm in length (J), 250µm in length (K) and 500µm in length (L). Scale bars: 1cm A-C; 0.5cm A'-C'; 100µm D-L.

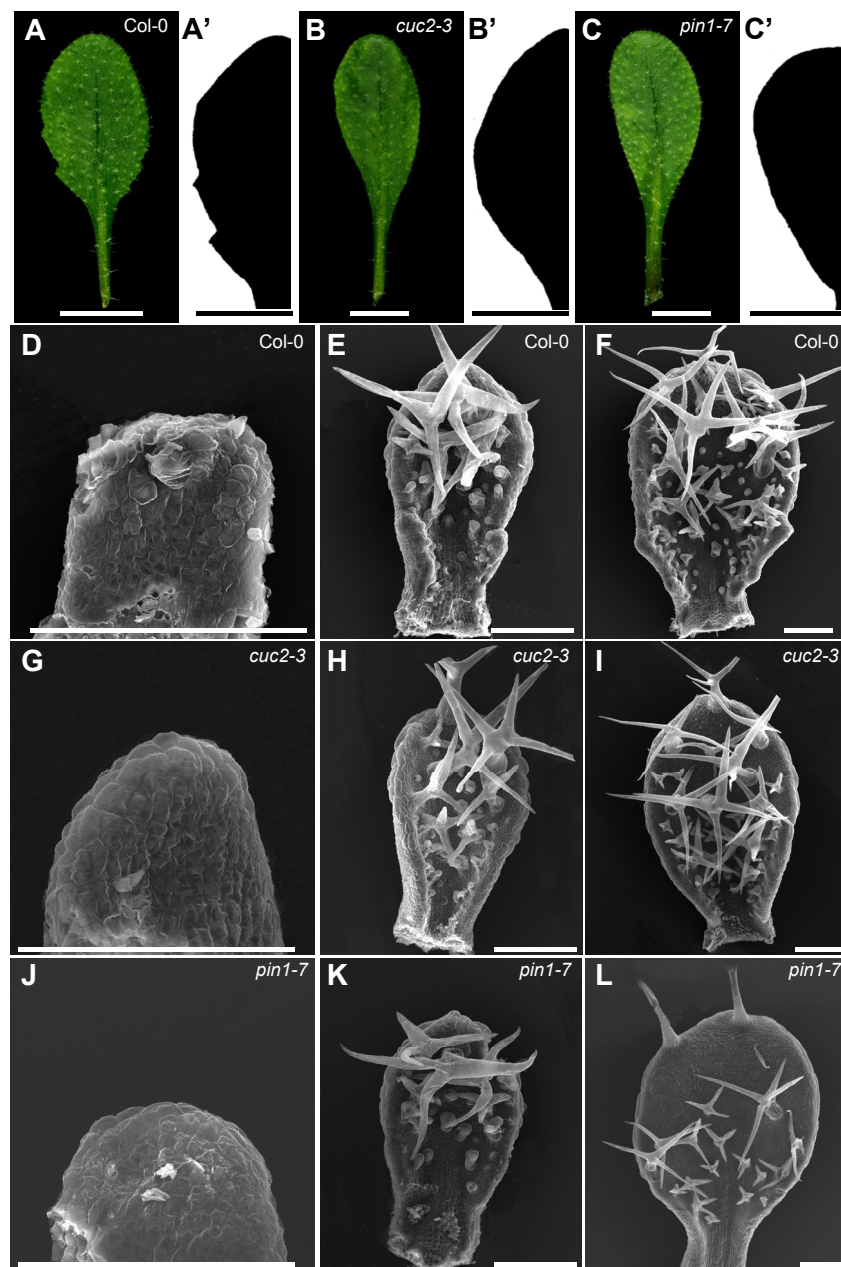
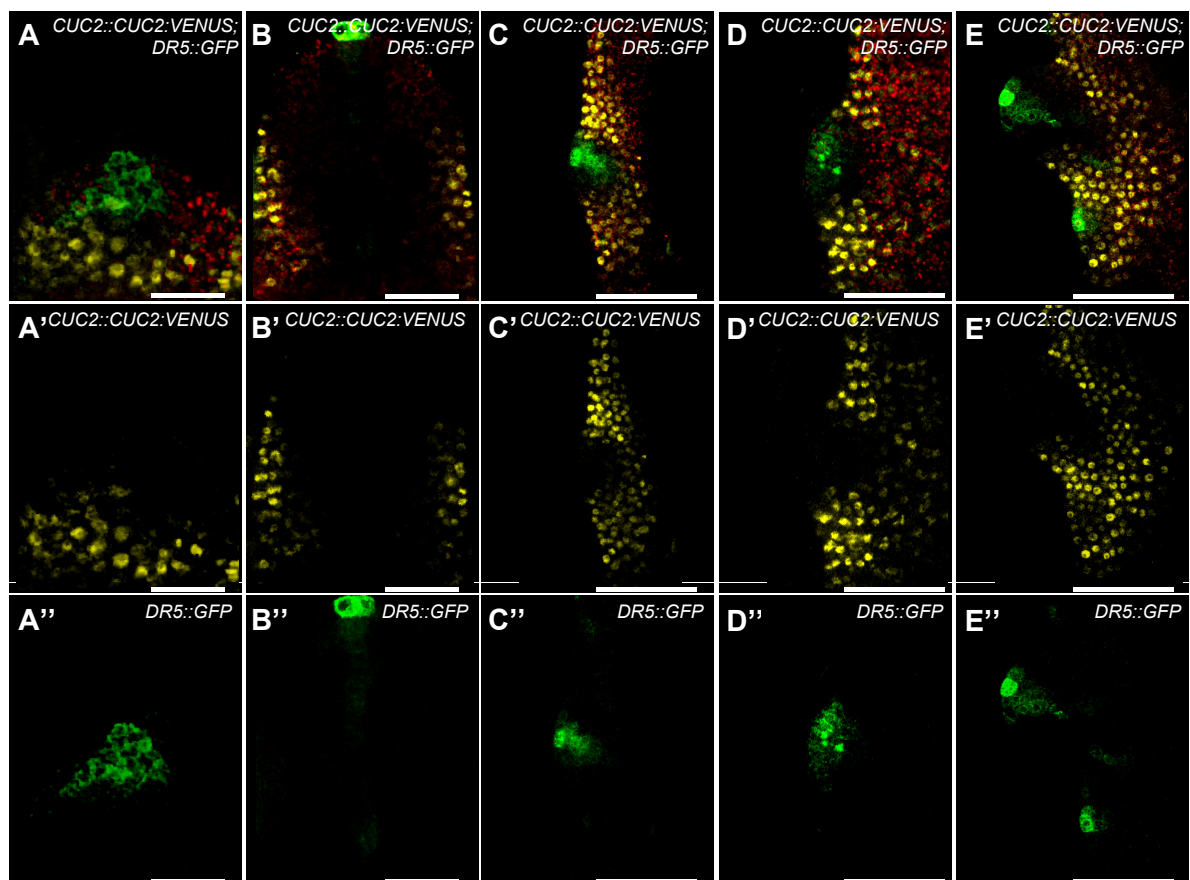


Figure 3.2 Formation of an auxin activity maximum coincides with the downregulation of *CUC2* expression at the *A. thaliana* leaf margin. (A-E) Confocal micrograph of colocalised *DR5::GFP* (green) and *CUC2::CUC2:VENUS* (yellow) expression in fifth rosette leaf approximately 75µm in length (A), 130µm in length (B), 200µm in length (C), 300µm in length (D) and 500µm in length (E). **(A'-E')** Split channel confocal micrograph of *CUC2::CUC2:VENUS* (yellow) expression in fifth rosette leaf approximately 75µm in length (A'), 130µm in length (B'), 200µm in length (C'), 300µm in length (D') and 500µm in length (E'). **(A''-E'')** Split channel confocal micrograph of *DR5::GFP* expression in fifth rosette leaf approximately 75µm in length (A''), 130µm in length (B''), 200µm in length (C''), 300µm in length (D'') and 500µm in length (E''). Scale bars: 50µm A-E.



base of the leaf (Figure 3.2 A). As the leaf grows to approximately 130µm in length, *CUC2::CUC2:VENUS* is expressed in a continuous strip along the edge of the margin (Figure 3.2 B). Once the leaf is approximately 200µm in length, a *DR5::GFP* maximum is expressed at the margin of the leaf within the strip of *CUC2* expression. However, there is a concomitant reduction in *CUC2::CUC2:VENUS* expression. This causes *DR5::GFP* and *CUC2::CUC2:VENUS* to have complementary domains of expression and interspersed peaks of activity (Figure 3.2 C). The complementary pattern of *DR5::GFP* and *CUC2::CUC2:VENUS* expression is maintained as the serration grows (Figure 3.2 D). Once the leaf is approximately 500µm in length, a second *DR5::GFP* maximum forms along the margin in a basipetal sequence. This correlates with downregulation of *CUC2::CUC2:VENUS* expression (Figure 3.2 E), creating interspersed peaks of *CUC2* and auxin activity. These data indicate that a regulatory mechanism generates *CUC2* and auxin activity maxima iteratively along the leaf margin to pattern serration development, and that the formation of an auxin activity maximum coincides with the downregulation of *CUC2* expression.

3.2.3 Downregulation of *CUC2* expression coincides with PIN1 convergence point and auxin activity maxima formation

Since a patterning mechanism appears to exist between *CUC2* and auxin activities, I next investigated the dynamics between *CUC2* and PIN1 expression by visualising *CUC2::CUC2:VENUS* and *PIN1::PIN1:GFP* throughout leaf development. During leaf initiation, *PIN1::PIN1:GFP* is expressed at the margins and in the midvein, whereas *CUC2::CUC2:VENUS* is expressed at the base of the leaf (Figure 3.3 A). As the leaf grows to approximately 130µm in length, *CUC2::CUC2:VENUS* and *PIN1::PIN1:GFP* are expressed in overlapping domains at the leaf margin (Figure 3.3 B). When the leaf is approximately 200µm in length, *PIN1::PIN1:GFP* convergence points form concomitantly

Figure 3.3 Formation of a PIN1 convergence point coincides with the downregulation of CUC2 expression at the *A. thaliana* leaf margin. (A-C) Confocal micrograph of colocalised *PIN1::PIN1:GFP* (green) and *CUC2::CUC2:VENUS* (yellow) expression in fifth rosette leaf approximately 75µm in length (A), 130µm in length (B), 200µm in length (C), 300µm in length (D) and 500µm in length (E). **(A'-C')** Split channel confocal micrograph of *CUC2::CUC2:VENUS* (yellow) expression in fifth rosette leaf approximately 75µm in length (A'), 130µm in length (B'), 200µm in length (C'), 300µm in length (D') and 500µm in length (E'). **(A''-C'')** Split channel confocal micrograph of *PIN1::PIN1:GFP* expression in fifth rosette leaf approximately 75µm in length (A''), 130µm in length (B''), 200µm in length (C''), 300µm in length (D'') and 500µm in length (E''). Scale bars: 50µm A-C.

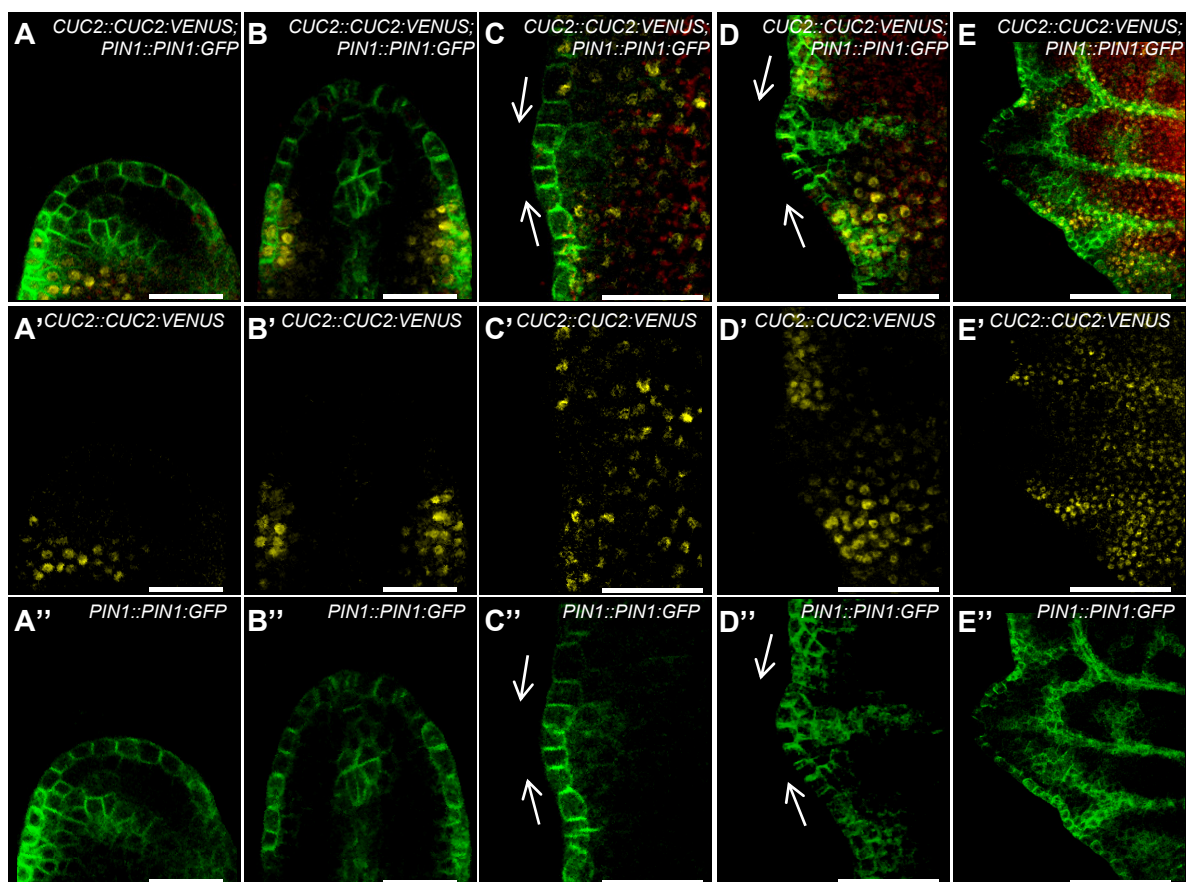
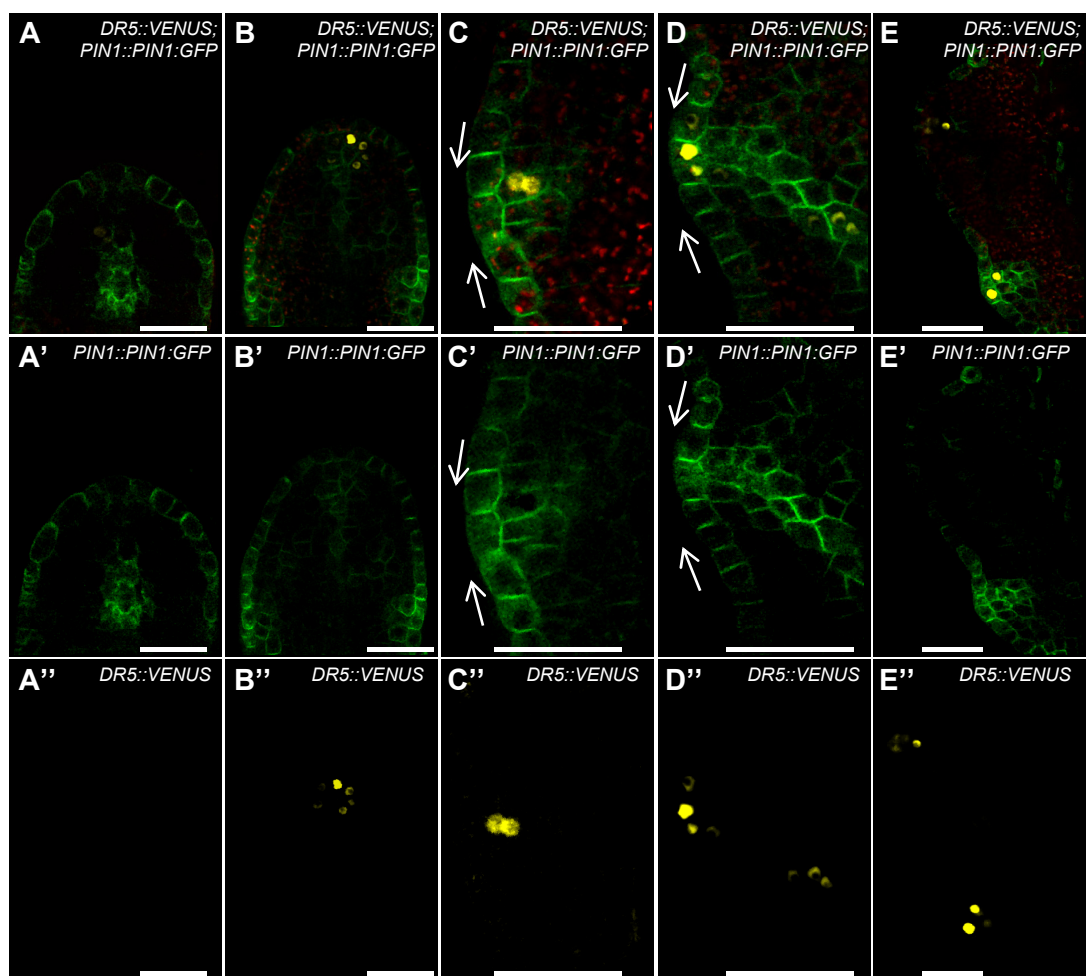


Figure 3.4 PIN1 convergence point formation coincides with auxin activity maxima formation at the *A. thaliana* leaf margin. (A-E) Confocal micrograph images of colocalised *PIN1::PIN1:GFP* (green) and *DR5::VENUS* (yellow) expression in fifth rosette leaf approximately 75µm in length (A), 125µm in length (B), 200µm in length (C), 250µm in length (D), and 500µm in length (E). **(A'-E')** Confocal micrograph images of *DR5::GFP* expression (green) in fifth rosette leaf approximately 75µm in length (A'), 125µm in length (B'), 200µm in length (C'), 250µm in length (D'), and 500µm in length (E'). **(A''-E'')** Confocal micrograph images of *DR5::VENUS* expression (yellow) in fifth rosette leaf approximately 75µm in length (A''), 125µm in length (B''), 200µm in length (C''), 250µm in length (D''), and 500µm in length (E''). Scale bars: 50µm A-E.



with the repression of *CUC2::CUC2:VENUS* expression (Figure 3.3 C). These expression patterns then persist during serration outgrowth (Figure 3.3 D). Subsequent *PIN1::PIN1:GFP* convergence points form in a basipetal sequence, correlating with the downregulation of *CUC2::CUC2:VENUS* expression (Figure 3.3 E), and thereby creating interspersed PIN1 convergence points and *CUC2* activity maxima along the leaf margin.

Subsequently, I investigated whether PIN1 convergence points can be temporally uncoupled from auxin activity maxima by visualising the dynamics between *PIN1::PIN1:GFP* and *DR5::VENUS* expression. During leaf initiation, *PIN1::PIN1:GFP* is expressed throughout the margin, whilst a *DR5::VENUS* maximum forms at the tip of the leaf (Figure 3.4 A-B). PIN1 convergence points form at the margin once the leaf is approximately 200µm in length, and this is coupled with the subepidermal expression of *DR5::VENUS* maxima (Figure 3.4 C). These expression patterns persist as the serration develops (Figure 3.4 D). Subsequent PIN1 convergence points then form basipetally, correlating with the formation of *DR5::VENUS* maxima and serration outgrowth (Figure 3.4 E). These data, therefore, suggest that PIN1 convergence point formation coincides with auxin activity maximum formation.

3.2.4 *CUC2* promotes PIN1 convergence points to control auxin activity maxima

Since formation PIN1 convergence points is coupled with *CUC2* maxima, I hypothesised that *CUC2* may be required for PIN1 convergence point formation. To determine the regulatory relationship between *CUC2* and auxin during serration development, I analysed *DR5::GFP* expression during *cuc2* leaf development. In wild type, *DR5::GFP* is expressed at the tips of leaves during early leaf development (Figure 3.5 A). *DR5::GFP* maxima then form at sites of serration development when the leaf is approximately 200µm in length (Figure 3.5 B), and this expression persists during serration outgrowth (Figure 3.5 C). Subsequent *DR5::GFP* maxima form in a basipetal sequence along

Figure 3.5 *CUC2* function is required for auxin activity maxima to form at the *A. thaliana* leaf margin. (A-E) Confocal micrograph of *DR5::GFP* (green) expression in fifth rosette leaf of Col-0 approximately 120µm in length (A), 200µm in length (B), 300µm in length (C), 500µm in length (D) and close-up of margin of fifth rosette leaf 500µm in length (E). **(F-J)** Confocal micrograph of *DR5::GFP* (green) expression in fifth rosette leaf of *cuc2-3* approximately 120µm in length (F), 200µm in length (G), 300µm in length (H), 500µm in length (I) and close-up of margin of fifth rosette leaf 500µm in length (J). **(K)** Quantification of auxin concentration in initiating Col-0 and *cuc2-3* leaves; average derived from the mean of three biological replicates; error bars represent standard error of the mean. Scale bars: 50µm A-J.

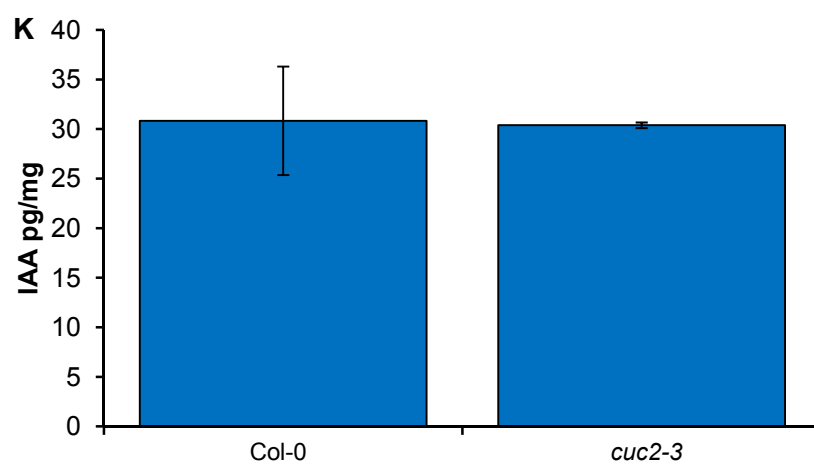
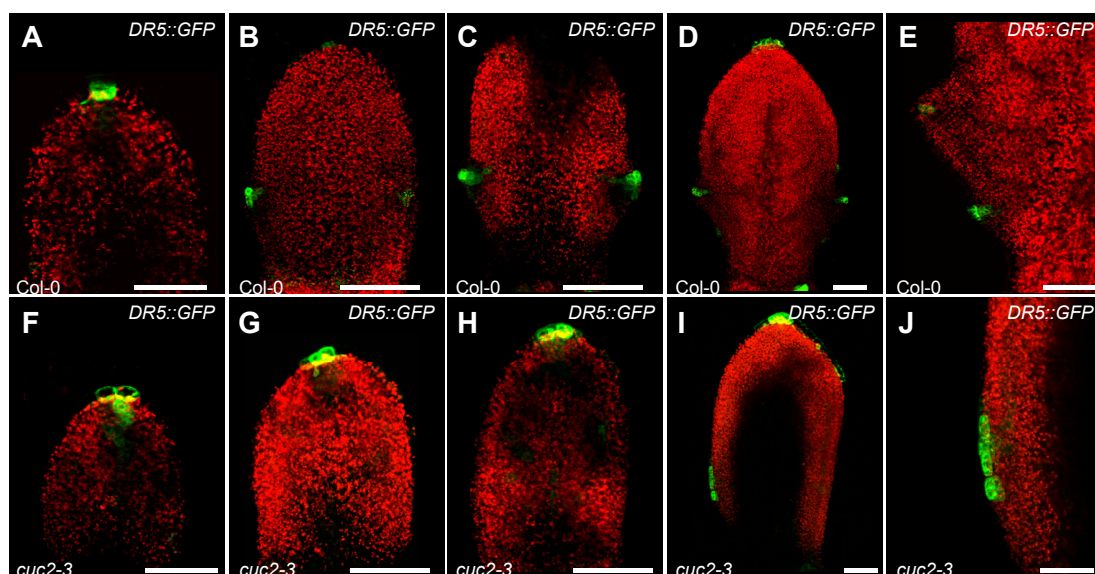
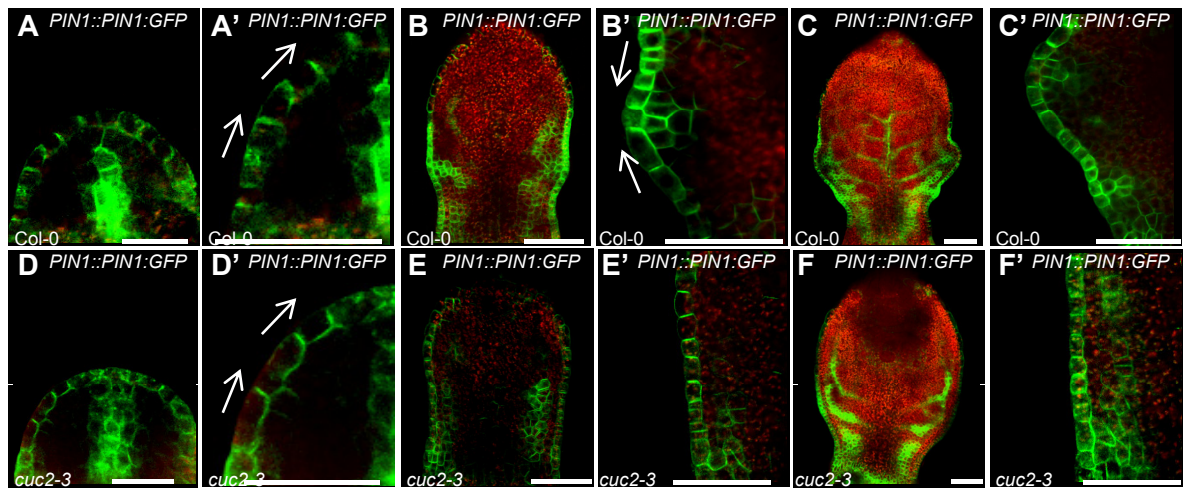


Figure 3.6 *CUC2* function is required for *PIN1* convergence point formation at the margins of *A. thaliana* leaves. **(A-C)** Confocal micrograph of *PIN1::PIN1:GFP* expression in fifth rosette leaf and close-up of margin of Col-0 approximately 120µm in length (A, A'), 200µm in length (B, B') and 500µm in length (C, C'). **(D-F)** Confocal micrograph of *PIN1::PIN1:GFP* expression in fifth rosette leaf and close-up of margin of *cuc2-3* approximately 120µm in length (D, D'), 200µm in length (E, E') and 500µm in length (F, F'). Scale bars: 50µm A-F.



the leaf margin once the leaf is approximately 500µm in length (Figure 3.5 D-E). Whilst *DR5::GFP* is expressed at the tips of *cuc2* leaves during early leaf development (Figure 3.5 F), *DR5::GFP* expression is absent from the margins of *cuc2* leaves that are approximately 200-300µm in length (Figure 3.5 G-H). However, diffuse *DR5::GFP* expression then forms around the margins of *cuc2* leaves approximately 500µm in length (Figure 3.5 I-J). These data suggest that *CUC2* promotes the formation of localised auxin activity maxima.

One possible explanation for aberrant auxin activity in *cuc2* leaves is that auxin biosynthesis is perturbed in *cuc2* mutants. To test this, we measured levels of auxin in *cuc2* leaves in comparison to wild type (Figure 3.5 K; IAA quantifications were determined by K. Ljung). However, there is no significant difference in the level of auxin between *cuc2* and wild-type leaves. These data suggest that aberrant auxin activity in *cuc2* leaves is not likely to be a result of perturbations in auxin biosynthesis.

Since PIN1 convergence points generate auxin activity maxima (Hay *et al.*, 2006; Scarpella *et al.*, 2006), an alternative hypothesis for aberrant auxin activity in *cuc2* leaves is that *CUC2* regulates PIN1 convergence point formation. I tested this hypothesis by analysing *PIN1::PIN1::GFP* expression during *cuc2* leaf development. During wild-type leaf initiation, *PIN1::PIN1::GFP* has an apical localisation pointing towards the leaf tip (Figure 3.6 A). Once the leaf is approximately 200µm in length, *PIN1::PIN1::GFP* forms convergence points at the margin (Figure 3.6 B). Subsequent *PIN1::PIN1::GFP* convergence points form in a basipetal sequence once the leaf is approximately 500µm in length (Figure 3.6 C). In *cuc2* mutant leaves, *PIN1::PIN1::GFP* is apically localised towards the leaf tip during early leaf development (Figure 3.6 D). However, *PIN1::PIN1::GFP* convergence points fail to form in *cuc2* leaves between 200µm-500µm in length (Figure 3.6 E-F). Despite this, PIN1 subcellular localisation remains polar (Figure 3.6 E-F). This, therefore, suggests that *CUC2* promotes the formation of PIN1 convergence points at the margins of leaves to promote serration initiation.

3.2.5 Interspersed *CUC2* activity regulates serration development

To investigate whether interspersed peaks of *CUC2* activity underpin serration development, I expressed *CUC2:VENUS* throughout the epidermis of wild-type leaves using the *AtML1* promoter. Whilst endogenous *CUC2* is expressed in interspersed peaks of activity along the margin (Figure 3.7 A), *CUC2* is expressed in a continuous strip around the leaf margin in *AtML1::CUC2:VENUS* plants (Figure 3.7 B). This expression causes 22% of *AtML1::CUC2:VENUS* plants to produce smooth leaf margins compared to the serrated margins of control plants (Figure 3.7 C-D), indicating that interspersed peaks of *CUC2* maxima are required for serration development.

To distinguish whether the smooth leaf margin phenotypes of *AtML1::CUC2:VENUS* plants are caused by failure to initiate serrations or from secondary growth defects, I monitored leaf morphology through development using SEM. Wild-type leaves initiate as rounded structures (Figure 3.7 E), however, the first pair of serrations initiate when leaves are approximately 250µm in length (Figure 3.7 F). The second pair of serrations then initiate basipetally when the leaf is approximately 500µm in length (Figure 3.7 G). In contrast, *AtML1::CUC2:VENUS* leaves initiate from the SAM as rounded structures (Figure 3.7 H), but serrations fail to initiate when the leaf is between 250µm-500µm in length (Figure 3.7 I-J). These data suggest that interspersed peaks of *CUC2* maxima are required for serration initiation.

3.2.6 Interspersed *CUC2* activity regulates auxin distribution

To investigate whether interspersed peaks of *CUC2* maxima are required to generate auxin activity maxima, I observed *DR5::GFP* expression in the leaves of *AtML1::CUC2:VENUS* plants in comparison to *CUC2::CUC2:VENUS* plants. During wild-type leaf initiation, *CUC2::CUC2:VENUS* is expressed at the margin and *DR5::GFP* is expressed at the tip (Figure 3.8 A). *DR5::GFP* maxima form at the margin of leaves

Figure 3.7 Interspersed *CUC2* expression is required for serration initiation. (A) Confocal micrograph of *CUC2::CUC2:VENUS* expression (yellow, arrowhead) in fifth rosette leaf of Col-0. (B) Confocal micrograph of *AtML1::CUC2:VENUS* expression (yellow, arrowhead) in fifth rosette leaf of Col-0. (C-D) Fifth rosette leaf and half silhouette of fifth rosette leaf of Col-0 (C, C') and *AtML1::CUC2:VENUS*. (E-G) Scanning electron micrographs of Col-0 fifth rosette leaf approximately 100µm in length (E), 250µm in length (F) and 500µm in length (G). (H-J) Scanning electron micrographs of *AtML1::CUC2:VENUS* fifth rosette leaf approximately 100µm in length (H), 250µm in length (I) and 500µm in length (J). Scale bars: 50µm A-B, H-J; 1cm C-D.

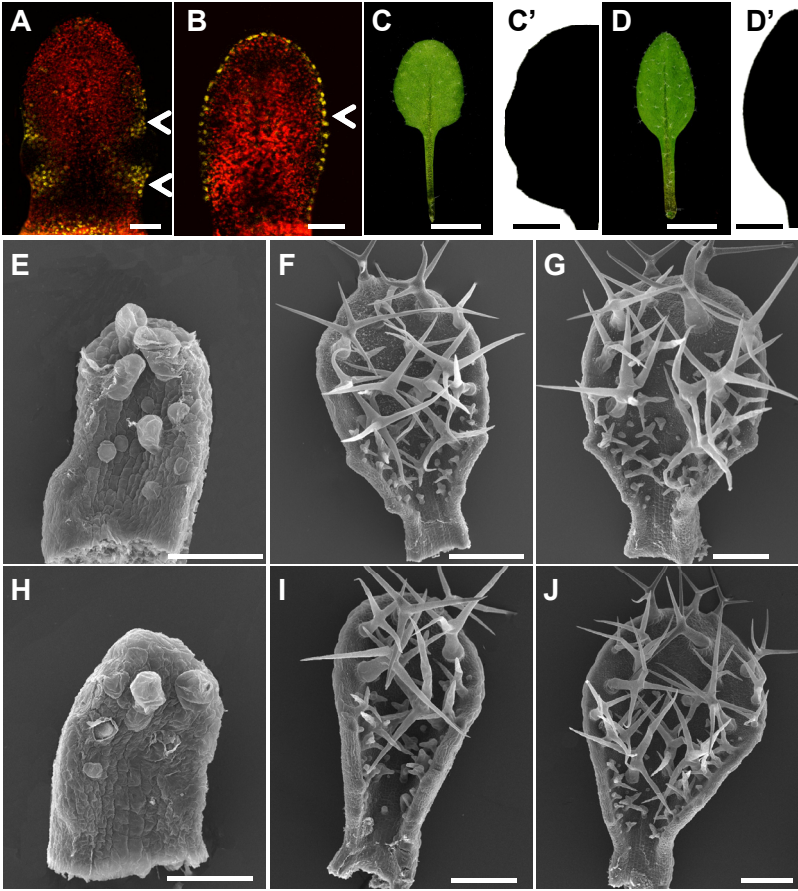
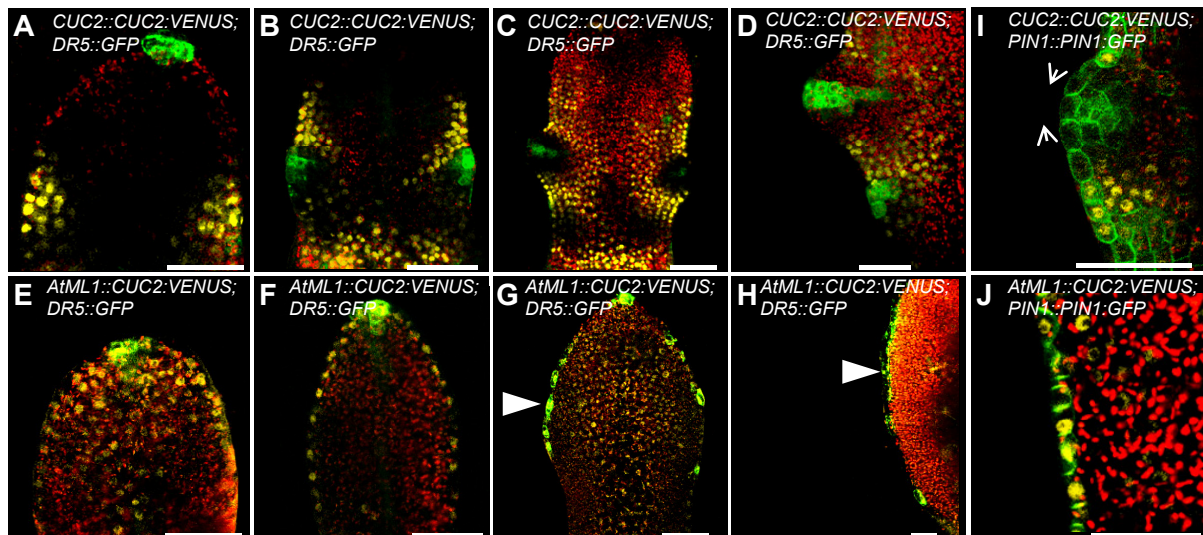


Figure 3.8 Interspersed *CUC2* expression is required for the formation of auxin activity maxima and PIN1 convergence points. (A-D) Confocal micrograph of colocalised *DR5::GFP* (green) and *CUC2::CUC2:VENUS* (yellow) expression in fifth rosette leaf approximately 125µm in length (A), 200µm in length (B), 300µm in length (C) and close-up of fifth rosette leaf 500µm in length (D). **(E-H)** Confocal micrograph of colocalised *DR5::GFP* (green) and *AtML1::CUC2:VENUS* (yellow) expression in fifth rosette leaf approximately 125µm in length (E), 200µm in length (F), 300µm in length (G) and close-up of fifth rosette leaf 500µm in length (H); arrowheads indicate broad auxin activity. **(I)** Confocal micrograph of colocalised *PIN1::PIN1:GFP* (green) and *CUC2::CUC2:VENUS* (yellow) expression in close-up of fifth rosette leaf approximately 300µm in length; arrows indicate the direction of auxin transport. **(J)** Confocal micrograph of colocalised *PIN1::PIN1:GFP* (green) and *AtML1::CUC2:VENUS* (yellow) expression in close-up of fifth rosette leaf approximately 300µm in length. Scale bars: 50µm A-J.



approximately 200µm in length, correlating with the downregulation of *CUC2::CUC2:VENUS* expression (Figure 3.8 B); this creates interspersed peaks of high auxin and *CUC2* activity maxima which are maintained as the serration grows (Figure 3.8 C). Subsequent *DR5::GFP* and *CUC2::CUC2:VENUS* maxima are generated in a basipetal sequence (Figure 3.8 D). During *AtML1::CUC2:VENUS* leaf initiation, *DR5::GFP* is expressed at the leaf tip (Figure 3.8 E). *DR5::GFP* maxima fail to form at the margins of leaves approximately 200µm in length (Figure 3.8 F). However, when leaves are 300-500µm in length, *DR5::GFP* has a broad expression pattern around the margins (Figure 3.8 G-H). This is similar to the expression pattern of *DR5::GFP* in *cuc2* loss-of-function mutants (Figure 3.5 A-J), and suggests that interspersed peaks of *CUC2* expression are required for auxin activity maxima to form.

PIN1 convergence points establish auxin activity maxima in wild-type leaves (Hay *et al.*, 2006; Scarpella *et al.*, 2006), and I showed that PIN1 convergence points fail to form in *cuc2* leaves, correlating with aberrant auxin activity. Thus, I hypothesised that PIN1 expression is aberrant at *AtML1::CUC2:VENUS* margins. To test this, I examined *PIN1::PIN1:GFP* expression in *AtML1::CUC2:VENUS* leaves. In wild-type leaves, *PIN1::PIN1:GFP* forms convergence points at sites of *CUC2::CUC2:VENUS* downregulation (Figure 3.8 I). In contrast, *PIN1::PIN1:GFP* does not form convergence points in *AtML1::CUC2:VENUS* leaves (Figure 3.8 J). This suggests that interspersed peaks of *CUC2* activity are required for PIN1 convergence points to form along the margin, and that aberrant auxin distribution in *AtML1::CUC2:VENUS* leaves arises from a lack of directional auxin transport.

3.2.7 Auxin represses *CUC2* expression to control serration development

As *CUC2* is specifically downregulated at sites of high auxin activity, I hypothesised that a repressive interaction between auxin and *CUC2* could exist. To investigate this, I

Figure 3.9 Broadened auxin activity correlates with reduced levels of *CUC2* expression in *A. thaliana* leaves. (A-D) Confocal micrograph of *DR5::VENUS* expression (yellow) in sixth (A), fifth (B), fourth (C) and close-up of fourth rosette leaf (D), respectively, of Col-0. **(E-H)** Confocal micrograph of *DR5::VENUS* expression (yellow) in sixth (E), fifth (F), fourth (G) and close up of fourth rosette leaf (H), respectively, of *pin1-7*. **(I-L)** Eighth (I), seventh (J), sixth (K) and fifth (L) rosette leaf, respectively, stained for *CUC2::GUS* in Col-0. **(M-P)** Eighth (M), seventh (N), sixth (O) and fifth (P) rosette leaf, respectively, stained for *CUC2::GUS* in *pin1-7*. Scale bars: 50µm A-H; 100µm I-P.

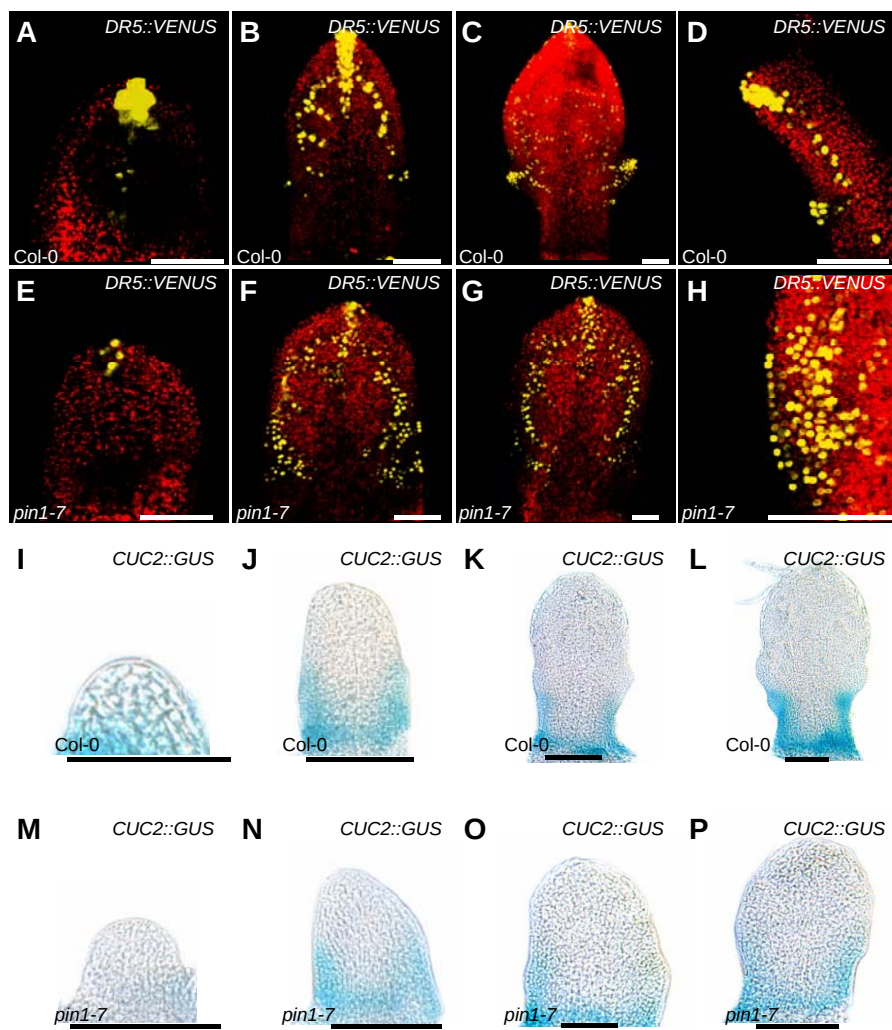
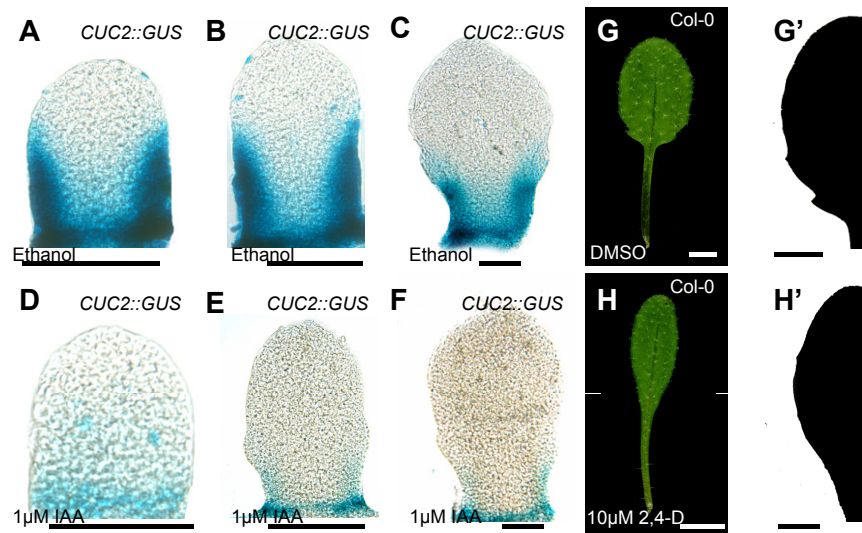


Figure 3.10 Exogenous auxin application represses serration development and *CUC2* expression. (A-C) Eighth (A), seventh (B) and sixth (C) rosette leaf of control ethanol-treated Col-0 stained for *CUC2::GUS* expression. (D-F) Eighth (D), seventh (E) and sixth (F) rosette leaf of 1 μ M IAA-treated Col-0 stained for *CUC2::GUS* expression. (G-H) Fifth rosette leaf and half silhouette of fifth rosette leaf of control DMSO-treated Col-0 (G, G') and 10 μ M 2,4-D-treated Col-0 (H, H'). Scale bars: 100 μ m A-F; 1cm G-H.



examined *CUC2* expression in backgrounds with elevated levels of auxin activity. In wild-type leaves, *DR5::VENUS* maxima are expressed at the leaf tip and along the leaf margin in a basipetal sequence (Figure 3.9 A-D), however, in *pin1* mutant leaves, *DR5::VENUS* expression is broad along the leaf margin (Figure 3.9 E-H). This suggests that auxin activity accumulates at the margins of *pin1* leaves. Examination of *CUC2::GUS* expression in *pin1* leaves suggests that *CUC2* transcription is reduced during early and late stages of leaf development in *pin1* leaves compared to wild type (Figure 3.9 I-P). This indicates that high auxin activity may repress *CUC2* transcription.

To directly test whether auxin represses *CUC2* transcription, I applied exogenous auxin to plants expressing a transcriptional *CUC2::GUS* reporter. In control-treated plants, *CUC2::GUS* is expressed along the margin during early leaf development (Figure 3.10 A-B), and is downregulated at sites of serration development (Figure 3.10 C). However, *CUC2::GUS* expression is reduced during early and late stages of leaf development in plants treated with auxin (Figure 3.10 D-F). This suggests that auxin can repress *CUC2* at the transcriptional level, and that leaves are competent to respond to high level of auxin at early and late stages of leaf development. Interestingly, exogenous auxin application prevents serration development (Figure 3.10 G-H), thereby phenocopying *pin1* mutant leaves. This suggests that peaks of auxin activity are required to pattern serration development by repressing *CUC2* expression.

3.2.8 Auxin signalling represses *CUC2* expression by promoting *MIR164A* transcription

Two mutants with reduced auxin signalling, *axr1* and *bdl/BDL*, produce leaves with more deeply serrated margins compared to wild type (Figure 3.11 A-C), thereby phenocopying *mir164a* leaves. Since the deeply serrated margins of *mir164a* mutants are caused by increased levels of *CUC2* (Hay *et al.*, 2006; Nikovics *et al.*, 2006), I hypothesised

Figure 3.11 *CUC2* is misexpressed in auxin signalling mutants and is responsible for the more serrated leaf margin phenotypes of auxin signalling mutants. (A-F) Fifth rosette leaf and half silhouette of fifth rosette leaf of Col-0 (A, A'), *axr1-3* (B, B'), *bdl/BDL* (C, C'), *cuc2-3* (D, D'), *axr1-3;cuc2-3* (E, E') and *bdl/BDL;cuc2-3* (F, F'). **(G)** Quantification of relative *CUC2* transcript level in Col-0, *axr1-3* and *bdl/BDL*; average derived from the mean of three biological replicates; error bars represent standard error of the mean. **(H-I)** Confocal micrograph of *CUC2::CUC2:VENUS* expression in fifth rosette leaf approximately 120µm in length in Col-0 (H) and *axr1-3* (I). Scale bars: 0.5cm A-F; 0.25cm A'-F'; 50µm H-I.

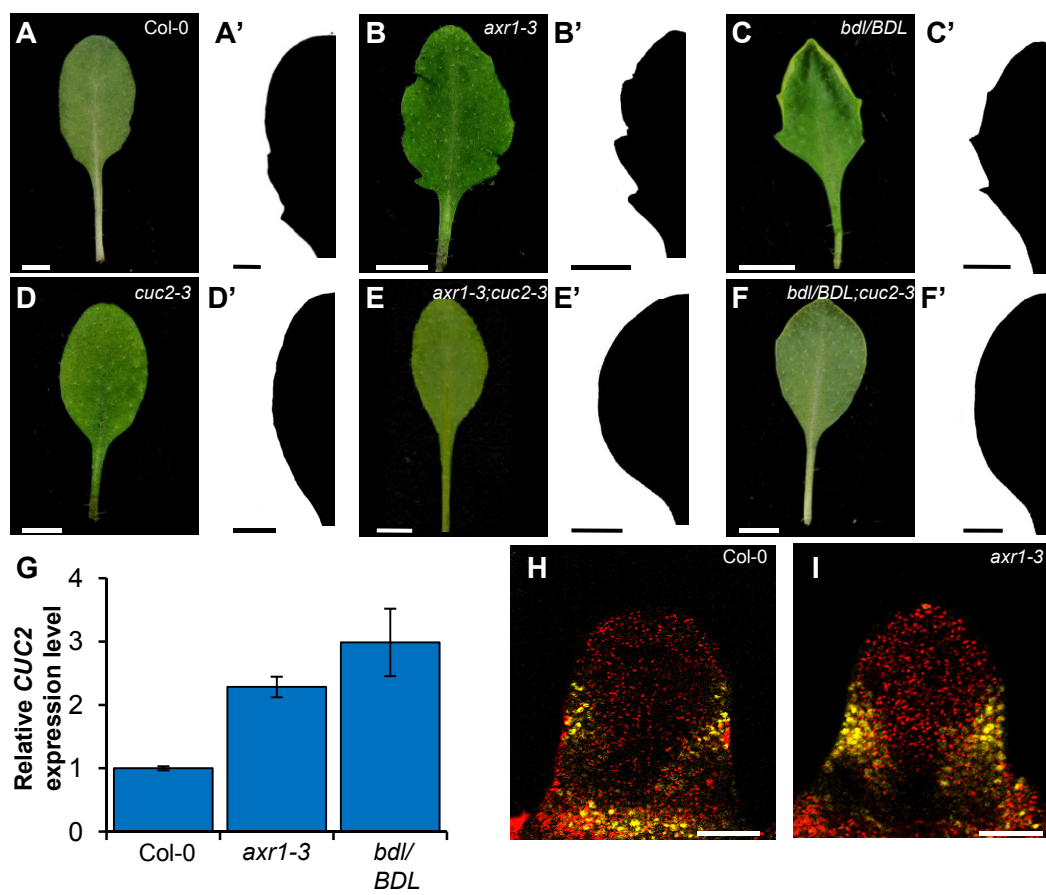
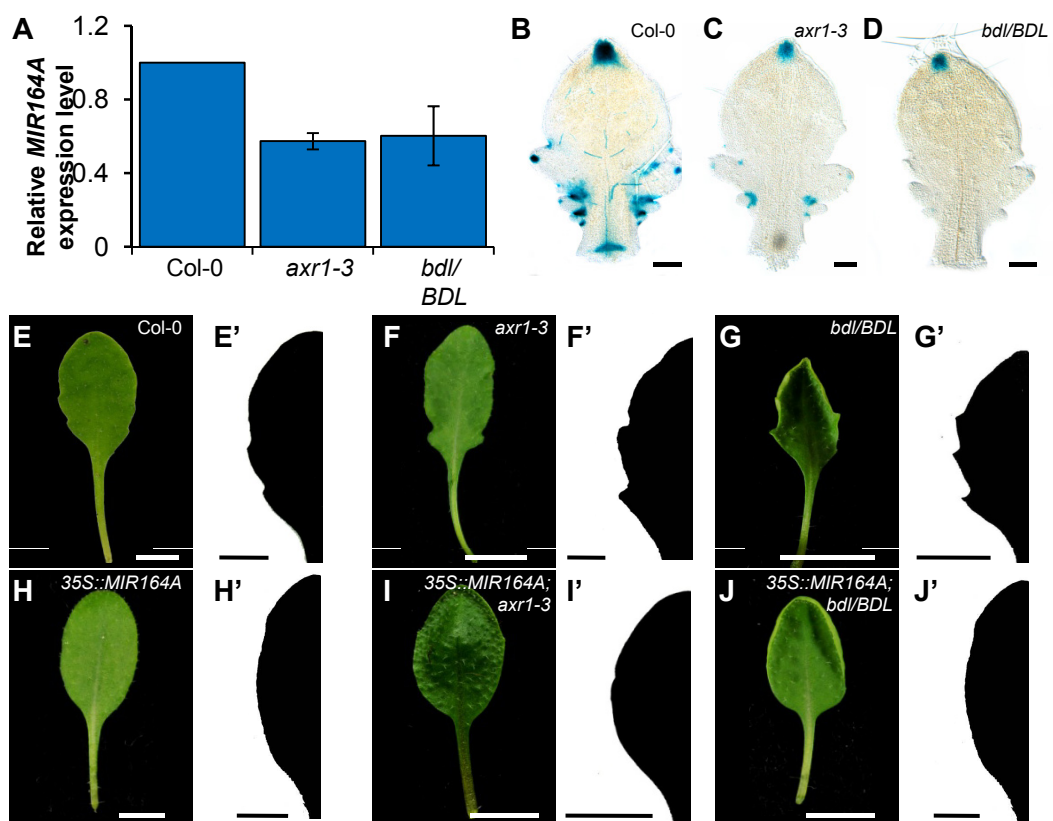


Figure 3.12 Auxin signalling promotes *MIR164A* transcription to regulate serration development at the margins of *A. thaliana* leaves. (A) Quantification of relative *MIR164A* transcript level in Col-0, *axr1-3* and *bdl/BDL*; average derived from the mean of three biological replicates; error bars represent standard error of the mean. (B-D) Fifth rosette leaf stained for *MIR164A::GUS* in Col-0 (B), *axr1-3* (C) and *bdl/BDL* (D). (E-J) Fifth rosette leaf and half silhouette of fifth rosette leaf of Col-0 (E, E'), *axr1-3* (F, F'), *bdl/BDL* (G, G'), *35S::MIR164A* (H, H'), *axr1-3;35S::MIR164A* (I, I') and *bdl/BDL;35S::MIR164A* (J, J'). Scale bars: 100µm B-D; 1cm E-J; 0.5cm E'-J'



that *CUC2* levels may be elevated in *axr1* and *bdl/BDL*. To investigate whether *CUC2* is required for the more deeply serrated phenotypes of *axr1* and *bdl/BDL*, I conducted genetic analyses. Both *axr1;cuc2* and *bdl/BDL;cuc2* produce leaves with smooth margins, similar to *cuc2* single mutants (Figure 3.11 D-F). This suggests that *CUC2* is required for serration development in *axr1* and *bdl/BDL* mutants. To determine whether auxin signalling regulates *CUC2* expression, levels of *CUC2* transcript were assayed in *axr1* and *bdl/BDL*. Quantitative RT-PCR showed that *CUC2* transcript levels are significantly increased in both *axr1* and *bdl/BDL* compared to wild type (Figure 3.11 G; *t*-test $p < 0.05$). Furthermore, *CUC2::CUC2:VENUS* expression is increased in *axr1* leaves compared with wild-type leaves (Figure 3.11 H-I). These data, therefore, suggest that auxin signalling represses *CUC2* expression, and that increased levels of *CUC2* are responsible for the more deeply serrated phenotypes of *axr1* and *bdl/BDL*.

Since *miR164* represses *CUC2* expression and *axr1* and *bdl/BDL* phenocopy *mir164a* leaves (Hay *et al.*, 2006; Nikovics *et al.*, 2006), I hypothesised that auxin signalling may regulate *MIR164A* expression. To investigate this, levels of *MIR164A* transcript were assayed in *axr1* and *bdl/BDL*. Quantitative RT-PCR showed that levels of *MIR164A* transcript are significantly reduced in *axr1* and *bdl/BDL* compared to wild type (Figure 3.12 A; *t*-test $p < 0.05$), suggesting that auxin signalling promotes *MIR164A* expression. To test whether auxin signalling promotes *MIR164A* transcription, the expression pattern of the *MIR164A::GUS* transcriptional reporter was examined in *axr1* and *bdl/BDL* leaves. *MIR164A::GUS* expression is reduced in the sinuses of both *axr1* and *bdl/BDL* leaves compared to wild type (Figure 3.12 B-D), suggesting that auxin signalling promotes *MIR164A* transcription.

To further examine whether reduced levels of *MIR164A* contribute to the more deeply serrated phenotypes of *axr1* and *bdl/BDL*, *MIR164A* was overexpressed in these

backgrounds. *35S::MIR164A* single mutants produce leaves with smooth margins, and both *axr1;35S::MIR164A* and *bdl/BDL;35S::MIR164A* produce leaves with smooth margins (Figure 3.12 E-J). This suggests that reduced *MIR164A* transcript levels in the *axr1* and *bdl/BDL* mutants may be responsible for the deeply serrated phenotypes of *axr1* and *bdl/BDL* leaf margins.

3.2.9 *MP* does not regulate *CUC2* expression to control serration development

As auxin signalling regulates *CUC2* and *MIR164A* expression to control serration development, it is likely that at least one ARF represses *CUC2* expression. *MP* encodes *ARF5*, and weak loss-of-function *mp* mutants produce leaves that are smoother compared to wild type and produce naked inflorescences (Hay *et al.*, 2006), thus phenocopying *pin1* mutants. I, therefore, hypothesised that *MP* may have a role in repressing *CUC2* expression. To test this, the expression patterns of the *CUC2::GUS* transcriptional reporter and the *CUC2::CUC2:VENUS* post-transcriptional reporter were observed in *mp* leaves. Both *CUC2::GUS* and *CUC2::CUC2:VENUS* expression in *mp* is similar to wild type throughout leaf development (Figure 3.13 A-L). This therefore suggests that *MP* does not regulate *CUC2* expression.

To investigate whether *CUC2* is required for the production of shallow serrations in *mp* leaves, I performed genetic analyses. *cuc2;mp* double mutants produce smooth margin leaves similar to *cuc2* single mutants (Figure 3.14 A-D), suggesting that *CUC2* is active in the *mp* leaf. To determine whether *MP* is required for the deeply serrated leaf margins of *mir164a* mutant leaves, the margin phenotypes of *mir164a;mp* leaves were assessed. *mir164a;mp* double mutant leaves produce deeply serrated margins similar to those of the *mir164a* single mutant (Figure 3.14 E-F). Taken together, these data suggest that *MP* does not regulate the expression of *MIR164A* or *CUC2*. However, it cannot be excluded that *MP* acts redundantly with additional ARFs to control *CUC2* expression.

Figure 3.13 *MP* does not regulate *CUC2* expression. (A-C) Sixth (A), fifth (B) and fourth (C) rosette leaves, respectively, stained for *CUC2::GUS* expression in Col-0. (D-F) Sixth (D), fifth (E) and fourth (F) rosette leaves, respectively, stained for *CUC2::GUS* expression in *mp-319*. (G-I) Confocal micrographs of *CUC2::CUC2:VENUS* expression (yellow) in Col-0 sixth (G), fifth (H) and close-up of fourth rosette leaf (I). (J-L) Confocal micrographs of *CUC2::CUC2:VENUS* expression (yellow) in *mp-319* sixth (J), fifth (K) and close-up of fourth rosette leaf (L). Scale bars: 100µm A-F; 50µm G-L.

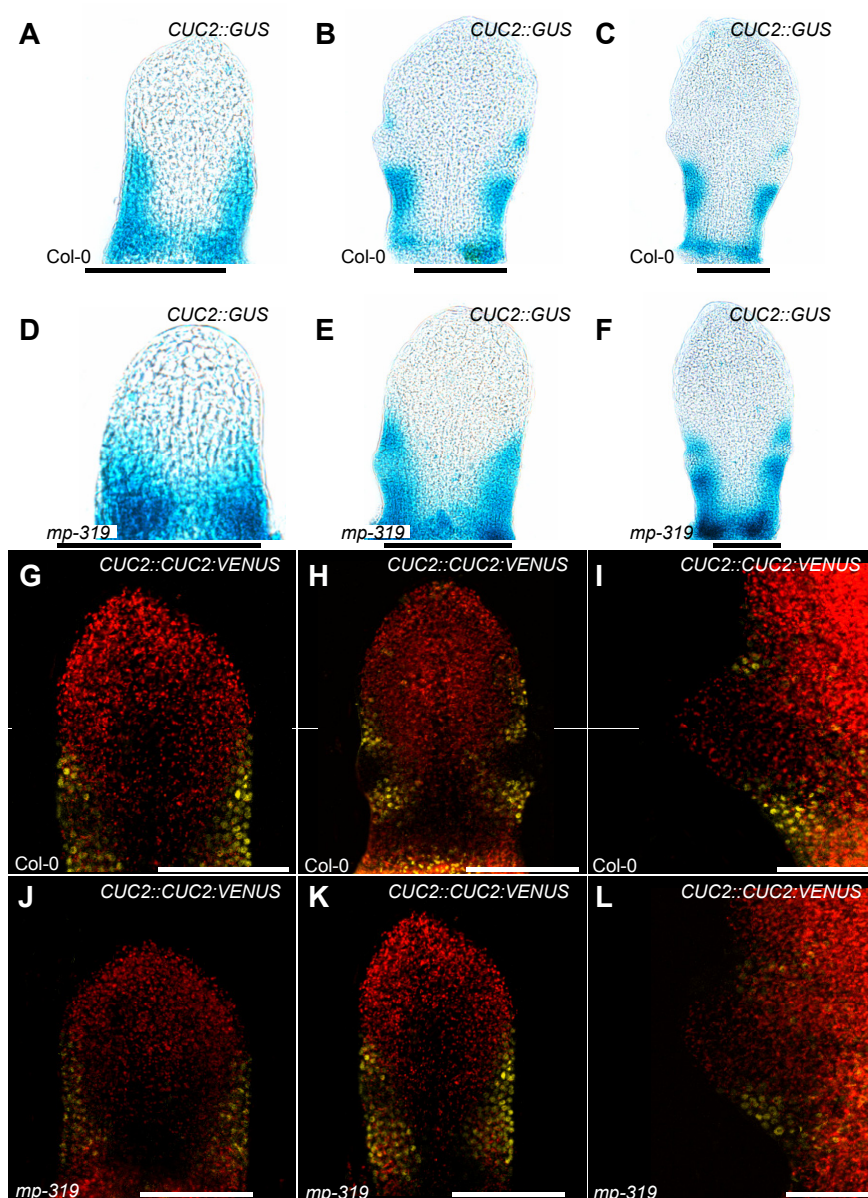
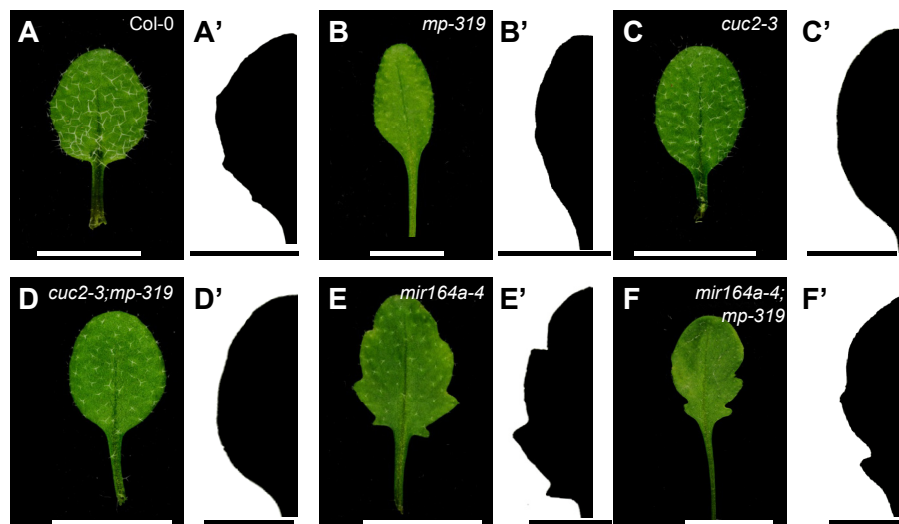


Figure 3.14 *CUC2* and *MIR164A* are required for serration development in *mp* leaves.

(A-D) Fifth rosette leaf and half silhouette of fifth rosette leaf of Col-0 (A, A'), *mp-319* (B, B'), *cuc2-3* (C, C') and *cuc2-3;mp-319* (D, D'), *mir164a-4* (E, E') and *mir164a-4;mp-319* (F, F'). Scale bars: 1cm A-H; 0.5cm A'-D'.



3.2.10 Epidermal PIN1 expression is sufficient to control serration and vasculature development

PIN1 is expressed in the epidermis and pre-precambial cells, and is required for both serration and vasculature development (Hay *et al.*, 2006; Scarpella *et al.*, 2006). However, it is unclear whether epidermal expression alone is sufficient to pattern both processes. To investigate this, I expressed PIN1:GFP in the epidermis under the *AtML1* promoter (Figure 3.15 A-D) where it is able to form convergence points in wild-type leaves (Figure 3.15 E). *pin1;AtML1::PIN1:GFP* leaves produce significantly more serrations compared with *pin1* leaves (Figure 3.15 F-J; *t*-test $p < 0.01$), suggesting that epidermal PIN1 expression is sufficient to pattern serration development.

I next sought to determine whether epidermal PIN1 expression rescues serration formation in *pin1* mutants by generating auxin activity maxima. In wild-type leaves, *DR5::VENUS* is expressed in discrete maxima along the margin (Figure 3.16 A-C), whereas *DR5::VENUS* is expressed broadly along the margins of *pin1* leaves (Figure 3.16 D-F). In both *pin1;AtML1::PIN1:GFP* and *AtML1::PIN1:GFP* plants, *DR5::VENUS* is expressed in discrete maxima along the margin, coinciding with serration initiation and PIN1:GFP convergence points (Figure 3.16 G-L). This suggests that epidermal PIN1 expression is sufficient for PIN1 convergence points to form at leaf margins, which drives the formation of auxin activity maxima and serration outgrowth at the margin.

Since secondary vein development is thought to be coupled with formation of marginal PIN1 convergence points (Hay *et al.*, 2006; Scarpella *et al.*, 2006), I investigated whether epidermal expression of PIN1 is sufficient to pattern vasculature development. *pin1* leaves produce significantly more secondary veins than wild type (Figure 3.17 A-B, E; *t*-test $p < 0.001$), whereas *pin1;AtML1::PIN1:GFP* leaves produce significantly fewer secondary veins than *pin1* (Figure 3.17 A-E; *t*-test $p < 0.05$). This suggests that epidermal PIN1

Figure 3.15 Epidermal PIN1 expression is sufficient to direct the formation of serrations at the *A. thaliana* leaf margin. (A-D) Serial single optical section confocal micrographs of *AtML1::PIN1:GFP* expression (green) in fifth rosette leaf. **(E)** Confocal micrograph of *AtML1::PIN1:GFP* expression (green) in close-up of fifth rosette leaf. **(F-I)** Fifth rosette leaf and half silhouette of fifth rosette leaf of Col-0 (F, F'), *pin1-7* (G, G'), *AtML1::PIN1:GFP* (H, H') and *pin1-7;AtML1::PIN1:GFP* (I, I'). **(J)** Quantification of average number of serrations in Col-0, *pin1-7*, *AtML1::PIN1:GFP* and *pin1-7;AtML1::PIN1:GFP*; error bars represent standard error of the mean, where n=15-20. Scale bars: 50µm A-E; 1cm F-I; 0.5cm F'-I'.

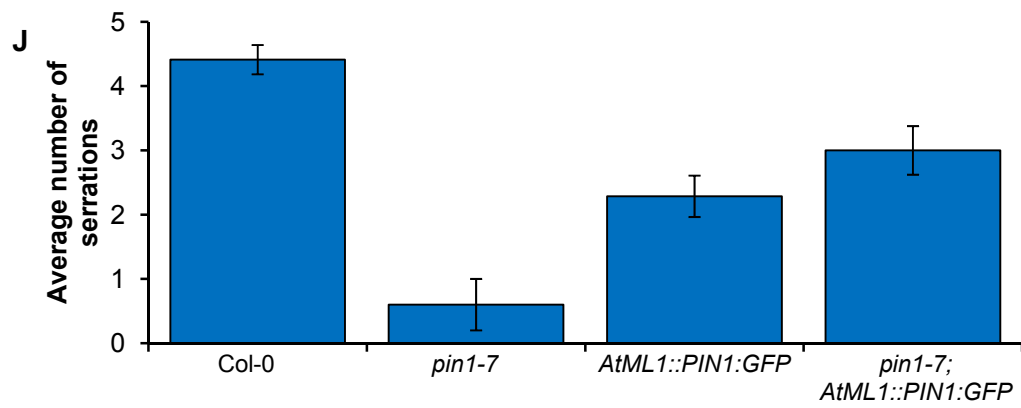
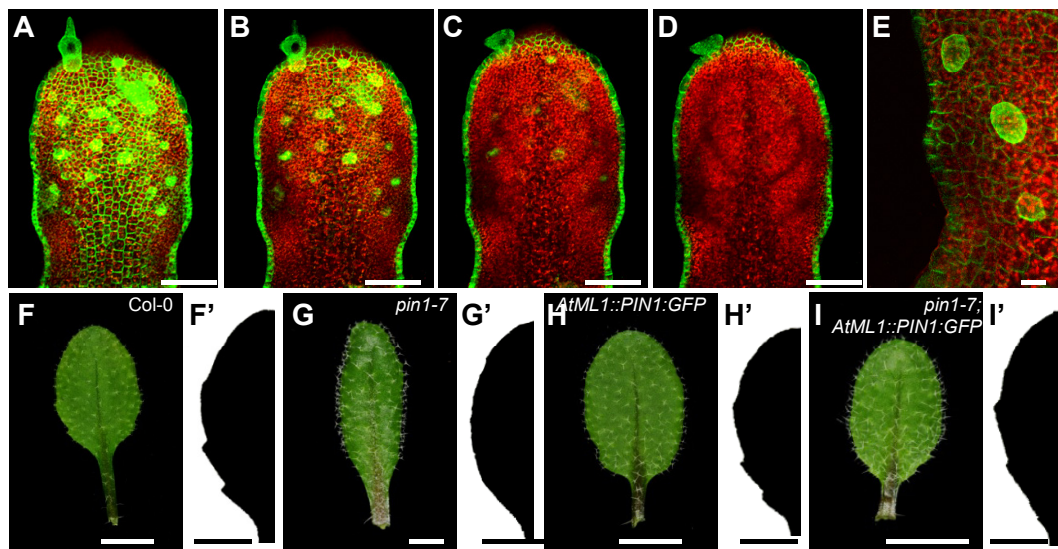
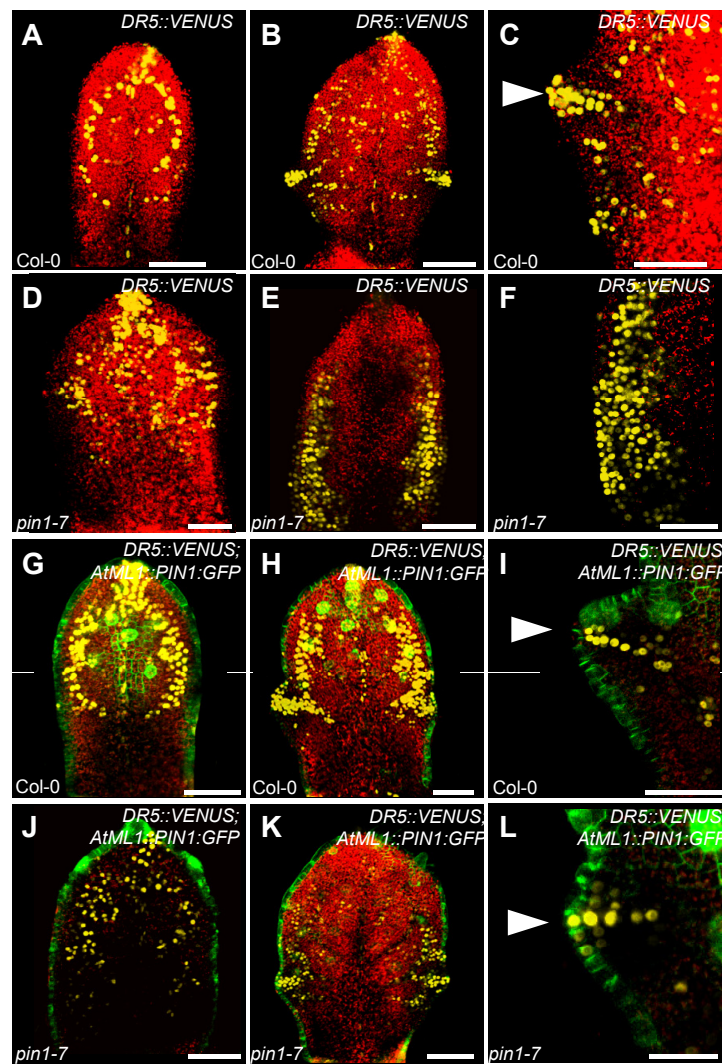


Figure 3.16 Epidermal PIN1 expression rescues serration development in *pin1* mutant leaves by establishing auxin activity maxima at the leaf margin. (A-C) Confocal micrographs of *DR5::VENUS* (yellow) expression in Col-0 sixth (A), fifth (B) and close-up of fourth (C) rosette leaf; white triangle indicates auxin activity focus. **(D-F)** Confocal micrographs of *DR5::VENUS* (yellow) expression in *pin1-7* sixth (D), fifth (E) and close-up of fourth (F) rosette leaf. **(G-I)** Confocal micrographs of colocalised *DR5::VENUS* (yellow) and *AtML1::PIN1::GFP* (green) expression in Col-0 sixth (G), fifth (H) and close-up of fourth (I) rosette leaf; white triangle indicates auxin activity focus. **(J-L)** Confocal micrographs of colocalised *DR5::VENUS* (yellow) and *AtML1::PIN1::GFP* (green) expression in *pin1-7* sixth (J), fifth (K) and close-up of fourth (L) rosette leaf; white triangle indicates auxin activity focus. Scale bars: 50µm A-L.



expression is sufficient to pattern secondary vein formation. Interestingly, *pin1* leaves produce more midveins compared to wild type, which stereotypically produce a single midvein per leaf (Figure 3.17 A-B, F; *t*-test $p < 0.001$). However, *pin1;AtML1::PIN1:GFP* leaves produce significantly fewer midveins compared with *pin1* (Figure 3.17 B, D, F; *t*-test $p < 0.01$).

3.2.11 CUC2 regulates serration development without affecting secondary vein number

To further investigate whether secondary veins can be uncoupled from PIN1 convergence points, I analysed vasculature development, using an *ATHB-8::GUS* vasculature reporter, in *cuc2* leaves lacking marginal PIN1 convergence points. In wild-type leaves, *ATHB-8::GUS* is initially expressed in the presumptive midvein, and is then expressed in the secondary veins which connect to the midvein (Figure 3.18 A). In *cuc2* leaves, *ATHB-8::GUS* is expressed in the presumptive midvein secondary veins at similar stages to wild type (Figure 3.18 B). To validate this, I observed a second vasculature marker, *IAA2::GUS*, in *cuc2* leaves. *IAA2::GUS* is expressed at similar stages in both *cuc2* and wild-type leaves (Figure 3.18 C-D). This suggests that secondary vein development is not altered in *cuc2* leaves. Furthermore, *cuc2* leaves produce the same number of secondary veins as wild-type leaves (Figure 3.18 E), suggesting that secondary vein formation can be uncoupled from PIN1 convergence points.

Interestingly, secondary veins are not freely ending in *cuc2* leaves and do not terminate at the leaf margin as in wild type (Figure 3.18 A-B). Hydathodes are structures that are often associated with serration tips and connect to secondary veins (Aloni *et al.*, 2003; Scarpella *et al.*, 2006), and significantly fewer are formed in *cuc2* compared to wild-type leaves (Figure 3.18 F; *t*-test $p < 0.001$). Thus, although the mechanism for determining the

Figure 3.17 Epidermal expression of *PIN1* is sufficient for secondary veins development.

(A-D) Leaf clearings of fifth rosette leaf of Col-0 (A), *pin1-7* (B), *AtML1::PIN1:GFP* (C) and *pin1-7;AtML1::PIN1:GFP* (D). **(E)** Quantification of average number of secondary veins in Col-0, *pin1-7*, *AtML1::PIN1:GFP* (*L1::PIN1*) and *pin1-7;AtML1::PIN1:GFP* (*pin1;L1::PIN1*); error bars represent standard error of the mean, where n=15-20. **(F)** Quantification of average number of midveins in Col-0, *pin1-7*, *AtML1::PIN1:GFP* (*L1::PIN1*) and *pin1-7;AtML1::PIN1:GFP* (*pin1;L1::PIN1*); error bars represent standard error of the mean, where n=15-20. Scale bars: 1cm A-D.

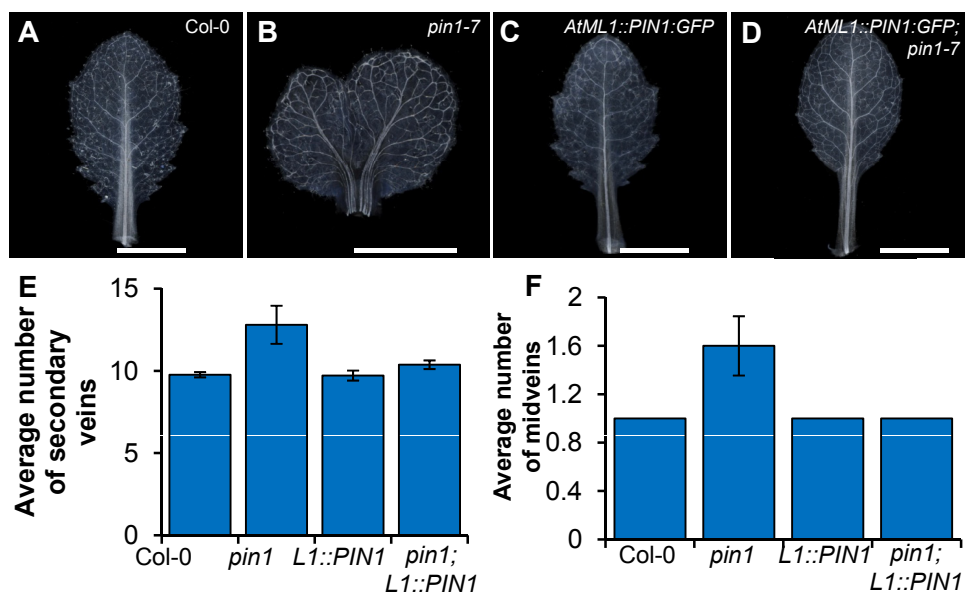
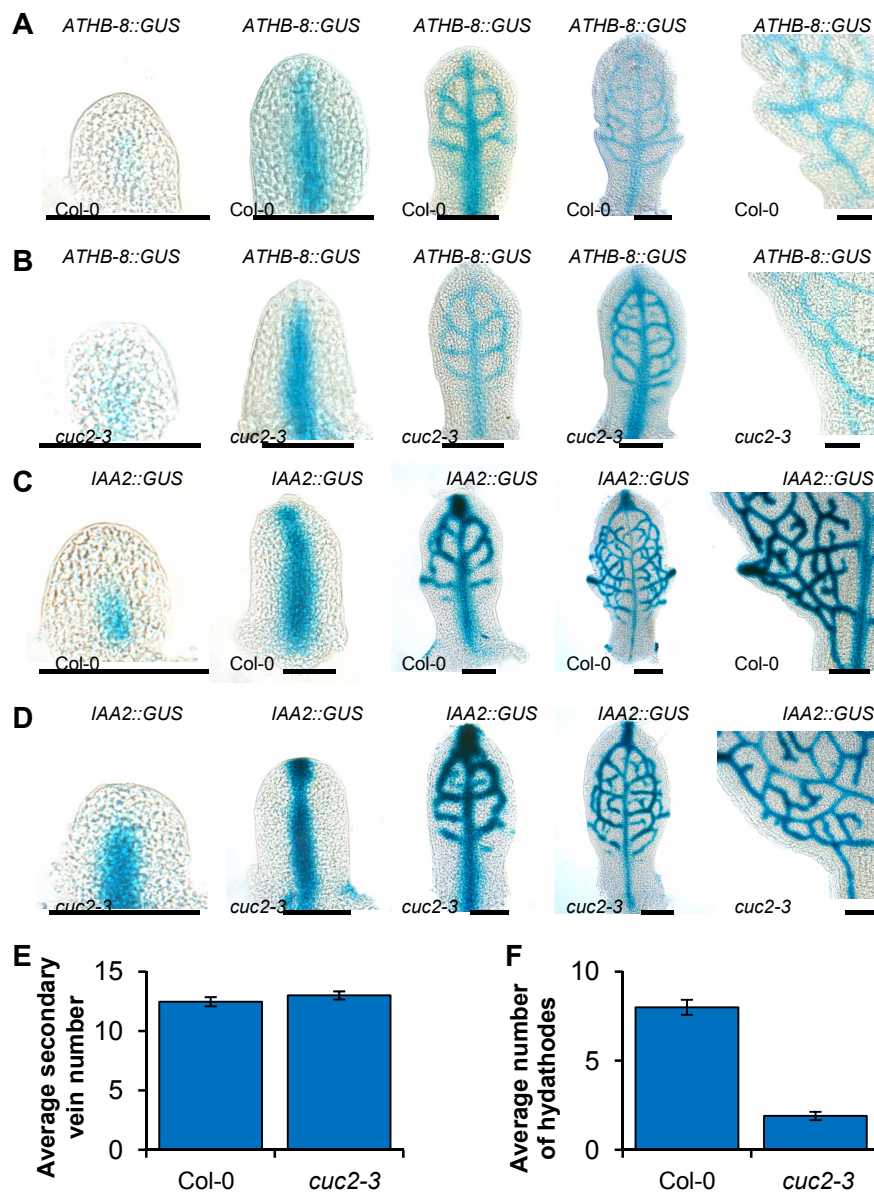


Figure 3.18 *CUC2* function is not required for vasculature development. (A-B) Eighth, seventh, sixth, fifth and close-up of fourth rosette leaf, respectively, stained for *ATHB-8::GUS* in Col-0 (A) and *cuc2-3* (B). (C-D) Eighth, seventh, sixth, fifth and close-up of fourth rosette leaf, respectively, stained for *IAA2::GUS* in Col-0 (C) and *cuc2-3* (D). (E) Quantification of average number of secondary veins in Col-0 and *cuc2-3* fifth rosette leaves; error bars represent standard error of the mean, where n=15. (F) Quantification of average number of hydathodes in Col-0 and *cuc2-3* fifth rosette leaves; error bars represent standard error of the mean, where n=15. Scale bars: 100µm A-D.



number of secondary veins is not disrupted in *cuc2* leaves, the patterning of secondary vein development is.

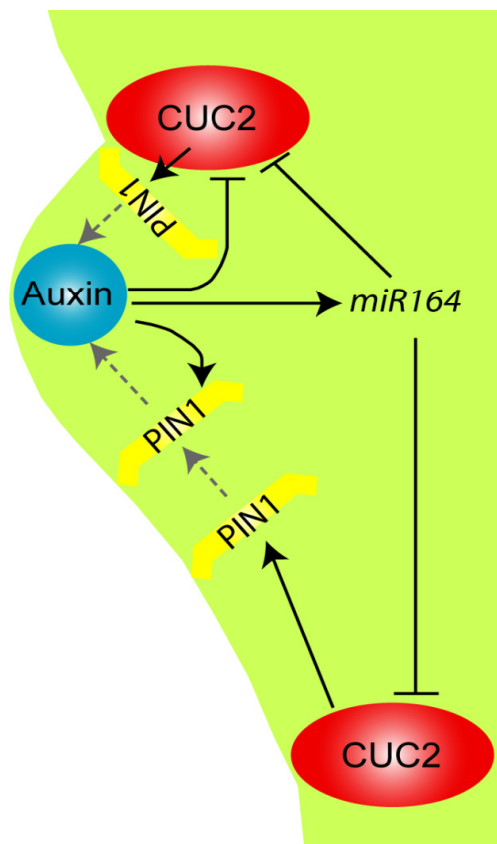
3.3 Discussion

3.3.1 Interlinking feedback loops pattern serration development

The data presented in this chapter suggest that *A. thaliana* serration development is patterned by interspersed peaks of *CUC2* and auxin activity maxima along the leaf margin. At a mechanistic level, *CUC2*, PIN1 and auxin appear to interact in two interlinking feedback loops (Figure 3.19). In the first loop, PIN1 forms epidermal convergence points at the margin causing auxin transport to occur against the gradient (Hay *et al.*, 2006), whereby PIN1 proteins localise to the face of the cell adjacent to the neighbouring cell with the highest concentration of auxin. This leads to the accumulation of high auxin activity, which feeds back to promote PIN1 expression (Vieten *et al.*, 2005), thereby creating a positive feedback loop between auxin and PIN1. In this study, I showed that a second loop operates where *CUC2* promotes the formation of PIN1 convergence points and foci of high auxin activity (Figures 3.5-3.6). Auxin activity maxima then feed back to repress *CUC2* expression in two main ways: firstly, auxin represses *CUC2* transcription in a process which is thought to regulate serration initiation (Figure 3.10 A-F); secondly, auxin promotes *MIR164A* transcription, leading to post-transcriptional repression of *CUC2* transcript level, which regulates serration depth (Figures 3.11-3.12).

Excitingly, a computational model was implemented to test whether the molecular level interactions described in this chapter could plausibly generate the observed patterns of gene expression and auxin distribution at the leaf margin, as well as generate the observed leaf morphologies (modelling performed by A. Runions in Bilsborough *et al.*, 2011). The model shows that these molecular level interactions can generate peaks of auxin and *CUC2*

Figure 3.19 Interlinking feedback loops regulate serration development. PIN1 (yellow trapezia) forms convergence points at sites of serration initiation, and drive the transport of auxin to create auxin activity foci (blue circle). Auxin feeds back to promote PIN1 transcription and localisation, creating a positive feedback loop. *CUC2* (red oval) also promotes the formation of PIN1 convergence points and auxin activity foci, however, auxin feeds back to repress *CUC2* expression by promoting the transcription of *MIR164A* and repressing *CUC2* transcription.



activity iteratively in a basipetal sequence along the leaf margin. Crucially, the model also demonstrates that the negative feedback loop between *CUC2* and auxin stabilises the patterns of serration development. This suggests that the interlinking of the positive feedback loop between PIN1 and auxin with the negative feedback loop between *CUC2*, PIN1 and auxin may have evolved to create a robust patterning mechanism to specify iterative outgrowth formation.

Serration development is an iterative process where sequential serrations are produced basipetally along the proximo-distal axis of the margin, and the data in this study show that the same mechanism is deployed for the production of each serration (Figures 3.2-3.4, 3.7-3.8). However, it is not clear which factors determine the periodicity and duration of the patterning mechanism. In *A. thaliana* leaves, the cell cycle arrest front occurs basipetally along the proximo-distal axis of the leaf (Donnelly *et al.*, 1999). The rate of progression of the cell cycle arrest front is controlled by type II *TCP* genes, and mutants with reduced *TCP* expression produce deeply serrated leaf margins (Nath *et al.*, 2003). Furthermore, the type II *TCP* gene *LA* has been shown to repress the activity of *CUC* genes in tomato leaves (Blein *et al.*, 2008). Thus, it is possible that progression of the cell cycle arrest front is linked to the gene regulatory networks described in this chapter via TCP-dependent regulation of *CUC* gene expression. This could regulate the periodicity between successive serration development and the duration of leaf margin competence to generate serrations.

Interestingly, computational modelling has shown that *CUC2* and auxin activity maxima are created in a basipetal order as space is created for them at the base of the leaf where there are higher growth rates (modelling performed by A. Runions in Bilsborough *et al.*, 2011). This, therefore, suggests that growth could feed back to regulate the periodicity of serration development by interacting with the interlinking feedback loops described in this chapter.

3.3.2 Epidermal auxin flow patterns serration and secondary vein development

PIN1 forms convergence points along the *A. thaliana* leaf margin, directing auxin flow through the epidermis to create auxin activity maxima that define sites of serration development (Hay *et al.*, 2006; Scarpella *et al.*, 2006). PIN1 also has a basal localisation in the pre-procambial cells beneath the convergence point, which is predicted to internalise auxin flow (Hay *et al.*, 2006; Scarpella *et al.*, 2006). However, it was not known whether epidermal PIN1 expression alone can pattern serration and secondary vein development, or whether internal auxin flow is also required. Crucially, in this chapter, I demonstrated that epidermal PIN1 expression is sufficient for PIN1 convergence points to form and to regulate serration and secondary vein development (Figures 3.15-3.17).

One possible explanation why PIN-mediated auxin flow in the epidermis is sufficient for serration and secondary vein development is that a redundantly acting auxin efflux carrier protein may compensate for PIN1-mediated auxin flow in pre-procambial cells. Reducing the level of a single PIN protein can cause the upregulation of other PIN proteins (Vieten *et al.*, 2005). PIN3, PIN4 and PIN7 are expressed in leaf primordia, although they are not expressed in the pre-procambial cells in wild type (Scarpella *et al.*, 2006). However, it is possible that, when PIN1 is expressed in the epidermis but is absent in the pre-procambium, one of these PIN proteins is activated in the pre-procambial cells to internalise auxin flow. It will, therefore, be interesting to determine the expression patterns of additional PIN proteins in *pin1;AtML1::PIN1:GFP* leaves.

3.3.3 Serration development can be uncoupled from secondary vein formation

The formation of PIN1 convergence points at the leaf margin is associated with serration and secondary vein development (Hay *et al.*, 2006; Scarpella *et al.*, 2006). Additionally, freely ending secondary veins terminate at the tips of serrations and are associated with hydathodes (Aloni *et al.*, 2003; Scarpella *et al.*, 2006). On the basis of these

data, it was assumed that PIN1 convergence point, serration and secondary vein formation are intimately linked processes. However, in this chapter, I suggested that PIN1 convergence point formation and serration development can be uncoupled from secondary vein formation. This is because PIN1 convergence points do not form at the margins of *cuc2* leaves, and significantly fewer serrations form in *cuc2* leaves compared to wild type (Figure 3.6). However, there is no difference in secondary vein numbers between *cuc2* and wild-type leaves. This suggests that PIN1 convergence points are not required for secondary veins to form, and that secondary vein formation is not required to drive serration development in leaves.

Interestingly, secondary veins form as closed loop structures in *cuc2* leaves, in contrast to freely ending secondary veins associated with serrations in wild-type leaves (Figure 3.18 A-B). Furthermore, significantly fewer hydathodes are produced in *cuc2* leaf margins compared with wild type (Figure 3.18 F). PIN1 convergence points do not form at the margins of *cuc2* leaves, suggesting that PIN1 convergence points are required to direct secondary veins to terminate at the leaf margin, in association with hydathodes. This may have important implications on leaf metabolism. Serrations have been shown to be more highly metabolically active compared to other parts of the leaf (Baker-Brosh and Peet, 1997; Parkhurst and Louks, 1972; Roth-Nebelsick, 2001; Royer and Wilf, 2006). Since hydathodes aid water exchange, it is possible that specification of secondary veins terminating at serration tips may have evolved to improve photosynthetic rates and overall individual fitness levels.

3.4 Conclusions

- Interspersed peaks of *CUC2* and auxin activity pattern serration development.
- A negative feedback loop between auxin and *CUC2* regulates serration development.
- Epidermal auxin flow is sufficient to pattern serration and vasculature development.
- PIN1 convergence points and serration development can be uncoupled from vasculature development.

Chapter 4: The role of *CUC* and auxin in patterning leaflet development in *C. hirsuta*

Chapter 4: The role of *CUC* and auxin in patterning leaflet development in *C. hirsuta*

4.1 Introduction

Leaf shape is extremely variable between extant plant species, raising the key question of whether conserved gene regulatory networks regulate diverse leaf forms. One way to address this is by studying the genetic mechanisms that regulate leaf form in model species in comparison to closely-related species with divergent leaf forms. *C. hirsuta* is a compound leafed species that is closely-related to the simple leafed model species *A. thaliana*, with an estimated divergence time of fourteen million years between these species (Koch *et al.*, 2001). *C. hirsuta* is a diploid species with a relatively small genome, is self compatible and genetically tractable and, therefore, represents an attractive system to investigate the genetic basis of leaf dissection (Hay and Tsiantis, 2006). In Chapter 3, I demonstrated that *PIN1* and *CUC2* interact in a negative feedback loop to control serration development in the *A. thaliana* leaf (Figure 3.19). Since *A. thaliana* is thought to have evolved from a more complex leafed ancestor (Piazza *et al.*, 2010), this raises the question of how *PIN1* and *CUC* genes interact to control leaflet development in the compound leafed species *C. hirsuta*.

Recently, studies in *C. hirsuta* have highlighted the importance of PIN1-mediated auxin transport in controlling leaflet development (Barkoulas *et al.*, 2008). Loss-of-function *chpin1* mutants produce simplified leaves, and chemical inhibition of polar auxin transport also causes simplified leaves to develop, suggesting that PIN1-mediated polar auxin transport is required for leaflet development (Barkoulas *et al.*, 2008). PIN1 convergence point form at the margin of the rachis which are associated with auxin activity maxima (Barkoulas *et al.*,

2008). Furthermore, auxin activity maxima are thought to promote leaflet outgrowth by repressing *KNOX* expression and stimulating cell division (Barkoulas *et al.*, 2008). Despite the importance of PIN1-mediated auxin activity maxima in leaflet development, it is not known how PIN1 convergence points are established along the rachis.

Additionally, there are three *ChCUC* genes in the *C. hirsuta* genome, and reducing levels of these transcripts causes leaf simplification (Blein *et al.*, 2008), suggesting that *ChCUC* genes are essential for leaflet development. *ChCUC* gene maxima form at the distal sides of leaflets where they are thought to repress growth (Blein *et al.*, 2008). Interestingly, *ChCUC* genes promote *STM* expression in the *C. hirsuta* leaf and are required for leaflet development in *KNOX*-misexpressing *C. hirsuta* backgrounds (Blein *et al.*, 2008). This, therefore, suggests that *ChCUC* and *KNOX* genes form a positive feedback loop to regulate leaflet development. In Chapter 3, I showed that *CUC2* forms a negative feedback loop with PIN1 to control serration development in *A. thaliana*. However, it is not known whether a negative feedback loop operates between *ChCUC* and PIN1 to regulate leaflet development in *C. hirsuta*.

In this chapter, I investigate the interactions between *CUC*, PIN1 and auxin in the *C. hirsuta* leaf. I show that *CUC2* maxima form before PIN1 convergence points, and that PIN1 convergence points can be temporally uncoupled from auxin activity maxima formation. I then demonstrate that *ChCUC* genes are required to promote PIN1 convergence point and auxin activity maxima formation. Additionally, I show that *ChCUC* genes promote auxin biosynthesis in *C. hirsuta* leaves. Through genetic and pharmacological experiments I demonstrate that auxin represses *CUC2* expression in the *C. hirsuta* leaf, thereby demonstrating that a negative feedback loop exists between *CUC*, *PIN1* and auxin. Finally, by analysing vasculature development, I show that *ChCUC* genes and PIN1 regulate both

secondary vein and leaflet development, and suggest that *PIN1* convergence points cannot be uncoupled from secondary vein development in *C. hirsuta* leaves.

4.2 Results

4.2.1 *ChCUC* and *PIN1* are required for leaflet initiation

Wild-type *C. hirsuta* plants produce compound leaves with leaflets along a central rachis (Figure 4.1 A; Hay and Tsiantis, 2006). Reducing *ChCUC* and *PIN1* activity causes the simplification of leaves (Figure 4.1 B-E), suggesting that *ChCUC* and *PIN1* are required for leaflet development in *C. hirsuta* (Blein *et al.*, 2008). To quantitatively determine the effect of reducing *ChCUC* and *PIN1* expression on leaf shape, I performed extended eigenshape analysis (Figure 4.1 F). Two eigenshape axes have significant differences in shape between genotypes (Figure 4.1 F; ANOVA, $p < 0.005$). ES1 accounts for 30.53% of total observed shape variance, and shapes along ES1 show changes in the length-width ratio of the terminal leaflet. *chpin1-1* is significantly different in shape compared to wild type and *35S::MIR164B;CUC3RNAi* (ANOVA, Tukey post-hoc: $p < 0.001$). ES6 accounts for 3.35% of total shape variance, and shapes along ES6 show changes in the width of the terminal leaflet base. *35S::MIR164B;CUC3RNAi* is significantly different in shape compared to wild type (ANOVA, Tukey post-hoc: $p < 0.001$) and clusters with *chpin1-1* in morphospace (Figure 4.1 F). Thus, morphometric analysis suggests that both *ChCUC* and *PIN1* regulate similar pathways to control terminal leaflet width. However, *PIN1* may have additional and distinct roles with *ChCUC* in controlling the length-width ratio in *C. hirsuta* terminal leaflet development.

To investigate whether *PIN1* and *ChCUC* genes regulate leaflet initiation or leaflet separation, I analysed *pin1* and *35S::MIR164B;CUC3RNAi* leaf development. Wild-type leaves initiate as rounded structures with smooth margins (Figure 4.2 A-B), however leaflets

Figure 4.1 *PIN1* and *CUC* genes are required for leaflet development. (A-F) Fifth rosette leaf of Ox (A), *35S::MIR164B* (B), *CUC3RNAi* (C), *35S::MIR164B;CUC3RNAi* (D) and *chpin1* (E). (F) Eigenshape analysis of terminal leaflet shape changes in response to reduced *CUC* and *PIN1* expression. Silhouettes represent modelled shape variation at equal steps through the morphospace range of ES1 and ES6. Each point in the morphospace represents an individual leaf, with blue diamonds, yellow squares and green triangles representing Ox, *35S::MIR164B;CUC3RNAi* and *chpin1*, respectively. Connected lines represent the morphospace occupied by each genotype, with blue, yellow and green lines representing Ox, *35S::MIR164B;CUC3RNAi* and *chpin1*, respectively. n=20-22. Scale bars: 1cm A-E.

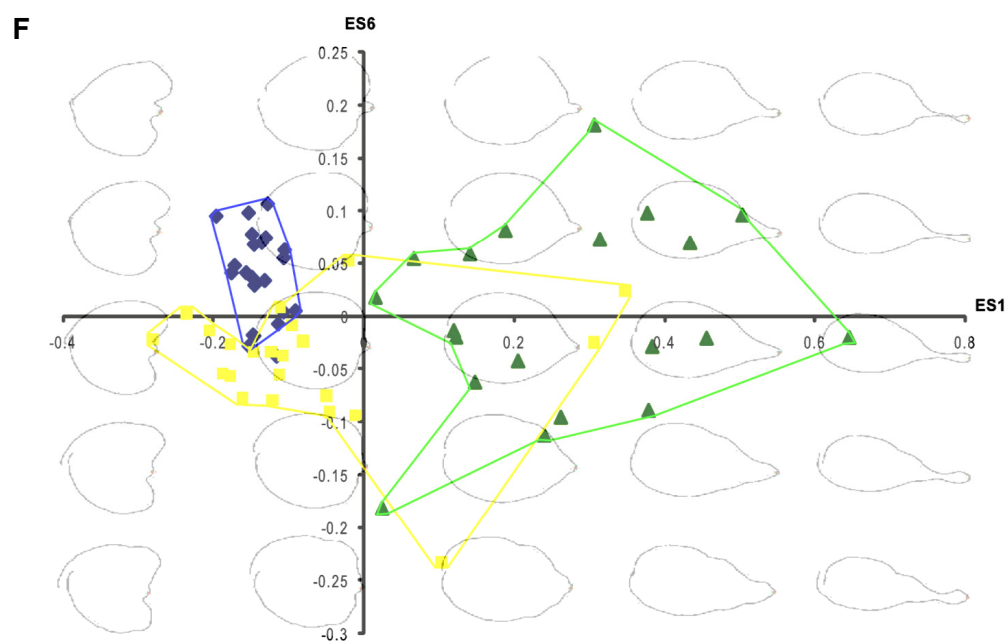
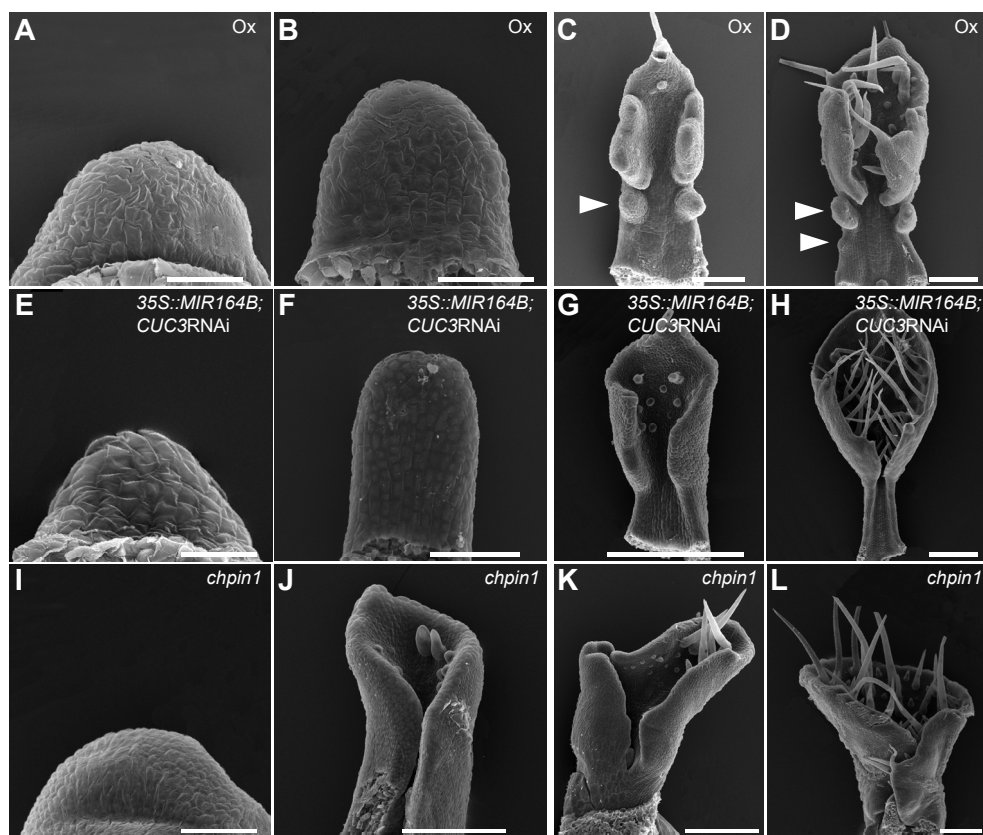


Figure 4.2 *CUC* and *PIN1* functions are required for leaflet initiation in *C. hirsuta* leaves. **(A-D)** Scanning electron micrographs of fifth rosette leaf of Ox approximately 50µm in length (A), 100µm in length (B), 200µm in length (C) and 400µm in length (D); white triangles indicate initiating leaflets. **(E-H)** Scanning electron micrographs of fifth rosette leaf of *35S::MIR164B;CUC3RNAi* approximately 50µm in length (E), 100µm in length (F), 200µm in length (G) and 400µm in length (H). **(I-L)** Scanning electron micrographs of fifth rosette leaf of *chpin1* approximately 50µm in length (I), 100µm in length (J), 200µm in length (K) and 400µm in length (L). Scale bars: 25µm A, E, I; 50µm B-C, F-G, J-K; 100µm D, H, L.



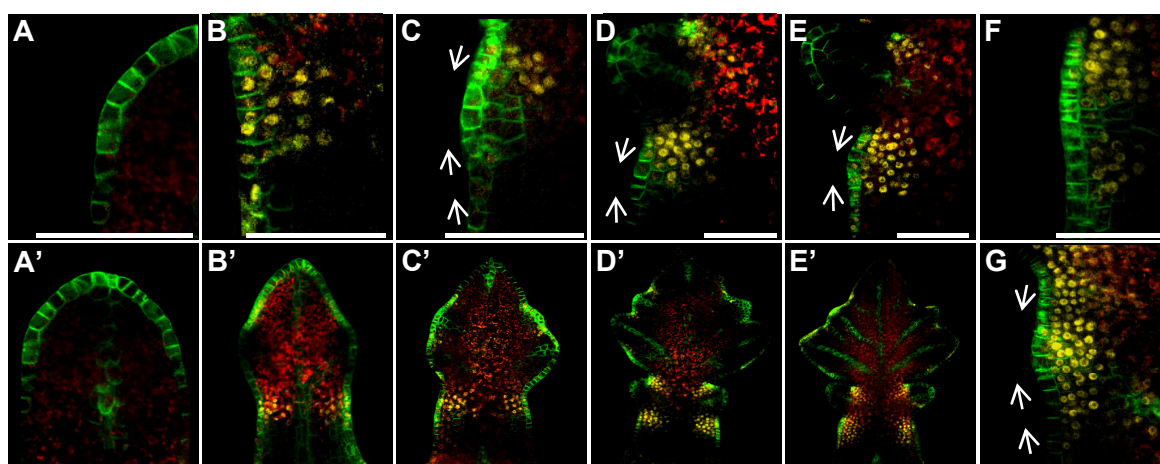
initiate when leaves are approximately 200µm in length (Figure 4.2 C). Subsequent leaflets form in a basipetal sequence along the proximo-distal axis of the margin when the leaf is approximately 400µm in length (Figure 4.2 D). In contrast, *35S::MIR164B;CUC3RNAi* and *chpin1* fail to initiate leaflets when approximately 200µm in length or at later stages of leaf development (Figure 4.2 E-L). These data suggest that both *ChCUC* and *PIN1* promote leaflet initiation when leaves are 200µm-400µm in length.

4.2.2 *CUC2* maxima precede the formation of *PIN1* convergence points

Since *PIN1* and *ChCUC* genes appear to regulate leaflet initiation in overlapping genetic pathways, I next investigated the spatio-temporal dynamics between *CUC2::CUC2:VENUS* and *PIN1::PIN1:GFP* expression. In early leaf development, *PIN1::PIN1:GFP* is expressed at the margins and in the midvein (Figure 4.3 A). Once the leaf is approximately 150µm in length, *CUC2::CUC2:VENUS* is expressed at the junction between the terminal leaflet and the rachis (Figure 4.3 B). *PIN1::PIN1:GFP* then forms convergence points at the rachis margin when the leaf is approximately 200µm in length, and *CUC2::CUC2:VENUS* expression is restricted to the distal side of the convergence point (Figure 4.3 C). Subsequent *PIN1::PIN1:GFP* convergence points form in a basipetal sequence, and *CUC2::CUC2:VENUS* expression is again restricted to the distal side of the convergence points (Figure 4.3 D-E).

However, *PIN1::PIN1:GFP* forms convergence points within continuous strips of *CUC2::CUC2:VENUS* expression once the leaf is 600-650µm in length (Figure 4.3 F-G). Thus, the distribution of *PIN1* and *CUC2* during the patterning of the final pair of leaflets is similar to the patterning mechanism employed during serration development in *A. thaliana* where *PIN1::PIN1:GFP* convergence points form within continuous strips of *CUC2::CUC2:VENUS* (Figure 3.3).

Figure 4.3 *CUC2* expression precedes the formation of PIN1 convergence points at the *C. hirsuta* leaf margin. (A-F) Confocal micrograph images of colocalised *PIN1::PIN1:GFP* (green) and *CUC2::CUC2:VENUS* (yellow) expression in close-ups of fifth rosette leaf of Ox approximately 100µm in length (A, A'), 150µm in length (B, B'), 200µm in length (C, C'), 350µm in length (D, D'), 500µm in length (E, E'), 600µm in length (F) and 650µm in length (G). Arrows indicate the direction of auxin transport. Scale bars: 50µm A-G.



4.2.3 PIN1 convergence can be temporally uncoupled from auxin activity maxima

Auxin activity maxima and PIN1 convergence points form at sites of leaflet development (Barkoulas *et al.*, 2008), however, it is unclear what the sequence of emergence is. To investigate the spatio-temporal dynamics between PIN1 and auxin activity, I examined *PIN1::PIN1:GFP* and *DR5::VENUS* colocalisations through leaf development. During leaf initiation, *DR5::VENUS* maxima form at the leaf tip and *PIN1::PIN1:GFP* is expressed around the margin (Figure 4.4 A). *PIN1::PIN1:GFP* forms convergence points at the margins of leaves approximately 175µm in length, but *DR5::VENUS* expression is absent (Figure 4.4 B). *DR5::VENUS* is expressed in a single subepidermal cell beneath the *PIN1::PIN1:GFP* convergence point once the leaf is approximately 250µm in length (Figure 4.4 C). These data suggest that the formation of *PIN1::PIN1:GFP* convergence points can be temporally uncoupled from auxin activity maxima. Subsequent *PIN1::PIN1:GFP* convergence points and *DR5::VENUS* maxima form in a basipetal sequence (Figure 4.4 D-E). Interestingly, when the leaf is 600-650µm in length, *PIN1::PIN1:GFP* convergence points are associated with several subepidermal cells that express *DR5::VENUS* (Figure 4.4 F-G).

4.2.4 CUC genes are required for PIN1 convergence points and auxin activity maxima to form

In Chapter 3, I demonstrated that *CUC2* promotes the formation of auxin activity maxima at the *A. thaliana* leaf margin (Figure 3.5). I, therefore, hypothesised that *CUC* genes may also regulate auxin activity maxima formation at the *C. hirsuta* leaf margin. To investigate this, I observed *DR5::VENUS* expression in *35S::MIR164B;CUC3RNAi* leaves. During wild-type leaf initiation, *DR5::VENUS* is expressed at the leaf tip (Figure 4.5 A), and then at the margin of the terminal leaflet when the leaf is approximately 150µm in length (Figure 4.5 B). *DR5::VENUS* maxima form at the rachis margin once the leaf is approximately 200µm in length (Figure 4.5 C). Subsequent *DR5::VENUS* maxima form in a

Figure 4.4 PIN1 convergence points precede the formation of auxin activity maxima at the margin of the *C. hirsuta* leaf. (A-F) Confocal micrograph images of *PIN1::PIN1:GFP* (green) and *DR5::VENUS* (yellow) expression in close-ups of fifth rosette leaf of Ox approximately 100µm in length (A, A'), 175µm in length (B, B'), 225µm in length (C, C'), 350µm in length (D, D'), 500µm in length (E, E'), 600µm in length (F) and 650µm in length (G). Arrows indicate the direction of auxin transport. Scale bars: 50µm A-G.

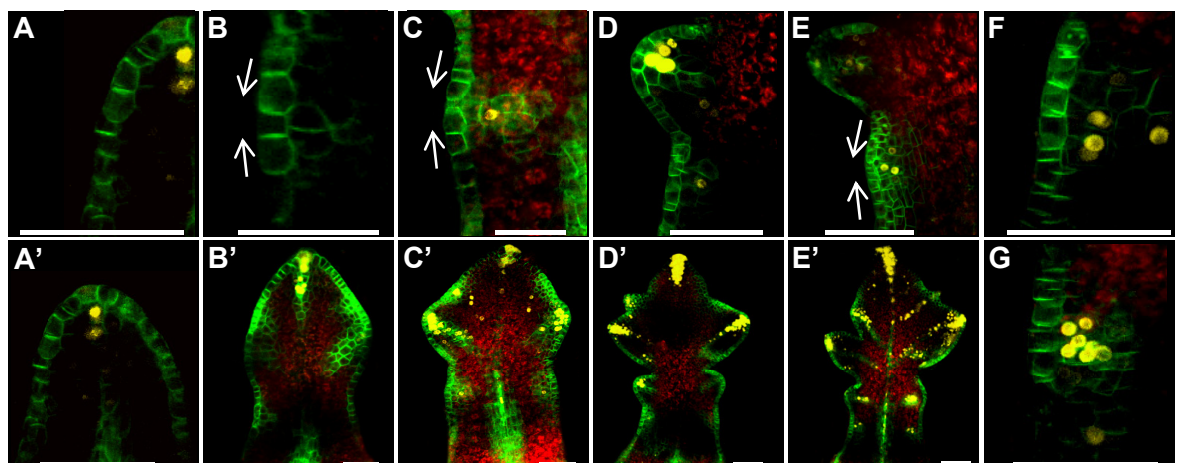
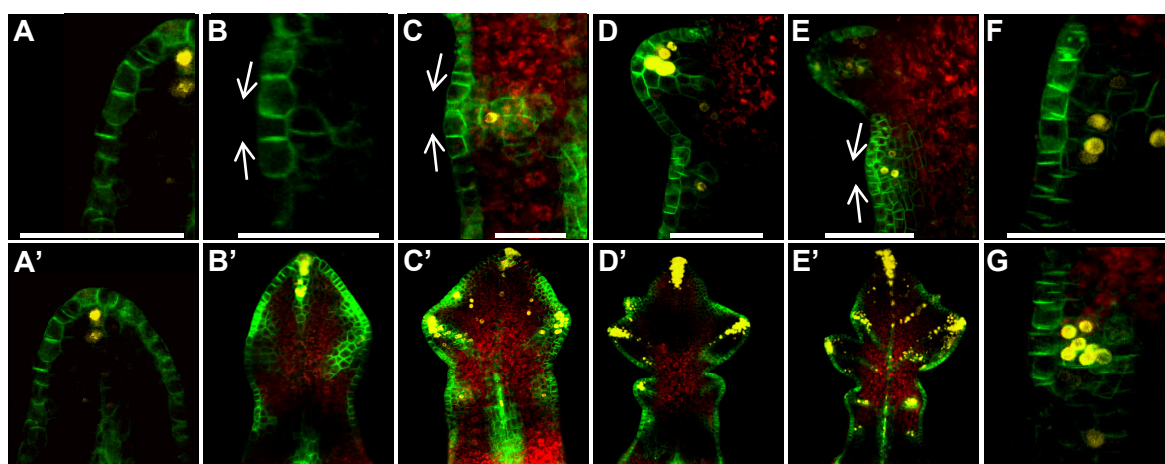


Figure 4.5 *CUC* genes are required for auxin activity maxima to form along the rachis of the *C. hirsuta* leaf. **(A-D)** Confocal micrograph images of *CUC2::CUC2:VENUS* (yellow) expression in Ox fifth rosette leaf approximately 100µm in length (A), 150µm in length (B), 200µm in length (C) and 400µm in length (D); white triangles indicate auxin activity foci. **(E-H)** Confocal micrograph images of *CUC2::CUC2:VENUS* (yellow) expression in *35S::MIR164B;CUC3RNAi* fifth rosette leaf approximately 100µm in length (E), 150µm in length (F), 200µm in length (G) and 400µm in length (H). White triangles indicate foci of auxin activity on the rachis of the leaf. **(I)** Quantification of auxin concentration in initiating Ox and *35S::MIR164B;CUC3RNAi* leaves; average derived from the mean of three biological replicates; error bars represent standard error of the mean. Scale bars: 50µm A-H.

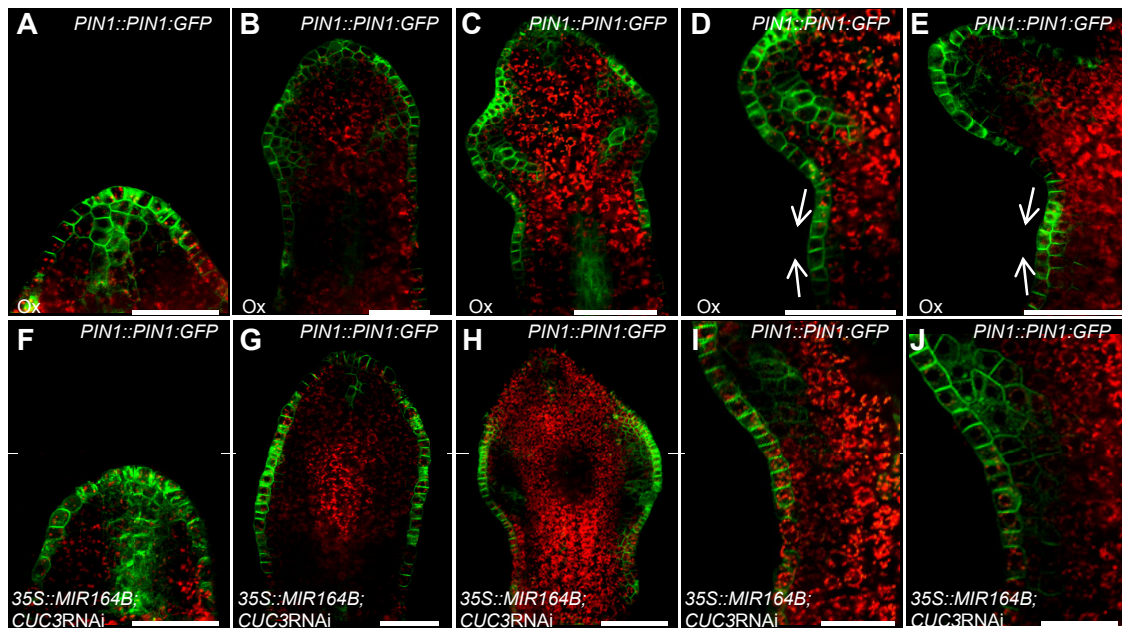


basipetal sequence along the rachis margin once the leaf is approximately 400µm in length (Figure 4.5 D). In contrast, *DR5::VENUS* expression is reduced during *35S::MIR164B;CUC3RNAi* leaf initiation compared to wild type (Figure 4.5 E-F). Moreover, when *35S::MIR164B;CUC3RNAi* leaves are 200µm-400µm in length, *DR5::VENUS* expression is absent from the rachis but remains expressed at the margins of the terminal leaflet (Figure 4.5 G-H). This suggests that *CUC* genes are required for the formation of auxin activity maxima in the rachis.

To investigate whether reduced auxin activity results from decreased auxin levels in the *35S::MIR164B;CUC3RNAi* leaf, we quantified auxin levels. *35S::MIR164B;CUC3RNAi* leaves contain significantly lower levels of auxin compared to wild type (Figure 4.5 I; *t*-test $p < 0.05$; K. Ljung performed auxin quantifications). This suggests that *CUC* genes may promote auxin biosynthesis in the *C. hirsuta* leaf, and that reduced levels in *35S::MIR164B;CUC3RNAi* leaves may contribute to reduced auxin activity and inhibition of leaflet initiation. This is in contrast to *cuc2* mutants in *A. thaliana* where auxin activity maxima fail to form at the leaf margin, but auxin levels are not altered (Figure 3.5).

Since I demonstrated in Chapter 3 that *CUC2* promotes PIN1 convergence points in *A. thaliana* leaves, I hypothesised that *CUC* genes may also promote PIN1 convergence points in the *C. hirsuta* rachis. To investigate this, I analysed *PIN1::PIN1:GFP* expression in *35S::MIR164B;CUC3RNAi* leaves. During wild-type leaf initiation, *PIN1::PIN1:GFP* is expressed around the margin (Figure 4.6 A), and this expression pattern persists as the leaf grows to approximately 150µm in length (Figure 4.6 B). Once the leaf is approximately 200µm in length, *PIN1::PIN1:GFP* convergence points form at the rachis margin (Figure 4.6 C-D). Subsequent convergence points form basipetally when the leaf is approximately 400µm in length (Figure 4.6 E). During *35S::MIR164B;CUC3RNAi* leaf initiation, *PIN1::PIN1:GFP* is expressed around the margins (Figure 4.6 F-G). However, when

Figure 4.6 *CUC* function is required for PIN1 convergence points to form along the rachis of the *C. hirsuta* leaf. (A-E) Confocal micrograph images of *PIN1::PIN1:GFP* expression (green) in Ox fifth rosette leaf approximately 100µm in length (A), 150µm in length (B), 200µm in length (C), close-up of leaf 200µm in length (D) and close-up of leaf 400µm in length (E). (F-J) Confocal micrograph images of *PIN1::PIN1:GFP* expression (green) in *35S::MIR164B;CUC3RNAi* fifth rosette leaf approximately 100µm in length (F), 150µm in length (G), 200µm in length (H), close-up of leaf 200µm in length (I) and close-up of leaf 400µm in length (J). Arrows indicate the direction of polar auxin transport. Scale bars: 50µm A-J.



35S::MIR164B;CUC3RNAi leaves are 200µm-400µm in length, PIN1::PIN1:GFP convergence points fail to form along the rachis (Figure 4.6 H-J). This suggests that *ChCUC* genes are required to promote PIN1 convergence point formation at the margin of the rachis.

4.2.5 Auxin represses *CUC2* expression in the rachis of the *C. hirsuta* leaf

In Chapter 3, I showed that auxin represses *CUC2* expression to regulate serration development in *A. thaliana* leaves (Figure 3.10 A-F). I, therefore, hypothesised that auxin may also repress *CUC2* expression to control leaflet development in *C. hirsuta* leaves. To investigate this, I examined *CUC2::CUC2:VENUS* expression in *chpin1* leaves. *CUC2::CUC2:VENUS* expression is absent during wild-type leaf initiation (Figure 4.7 A), but is expressed at the junction between the terminal leaflet and the rachis when the leaf is approximately 150µm in length (Figure 4.7 B). As the first pair of leaflets grow out, *CUC2::CUC2:VENUS* expression is restricted to the distal side of the leaflets (Figure 4.7 C). Further *CUC2::CUC2:VENUS* maxima form in a basipetal sequence along the proximo-distal axis of the leaf, defining the distal side of subsequent leaflets (Figure 4.7 D-E). In contrast, *CUC2::CUC2:VENUS* expression is absent from *chpin1* leaves 100µm-400µm in length (Figure 4.7 F-I). However, faint *CUC2::CUC2:VENUS* expression is activated at the junction between the terminal leaflet and the rachis when *chpin1* leaves are approximately 500µm in length (Figure 4.7 J). This suggests that reduced auxin transport represses *CUC2* expression in the *C. hirsuta* rachis.

To directly test whether auxin represses *CUC2* expression in the *C. hirsuta* leaf, I treated *CUC2::CUC2:VENUS* plants with auxin. In control DMSO-treated plants, *CUC2::CUC2:VENUS* is absent during leaf initiation (Figure 4.8 A), but is expressed at the junction between the terminal leaflet and the rachis when leaves are approximately 200µm in length (Figure 4.8 B). Subsequent *CUC2::CUC2:VENUS* maxima are expressed at the distal sides of leaflets later in leaf development (Figure 4.8 C). However, *CUC2::CUC2:VENUS*

Figure 4.7 *PIN1* function is required for *CUC2* expression in *C. hirsuta* leaf development. (A-E) Confocal micrograph images of *CUC2::CUC2:VENUS* expression (yellow) in Ox fifth rosette leaf approximately 100µm in length (A), 150µm in length (B), 350µm in length (C), 400µm in length (D) and close-up of leaf 500µm in length (E). (F-J) Confocal micrograph images of *CUC2::CUC2:VENUS* expression (yellow) in fifth rosette leaf in *chpin1* approximately 100µm in length (F), 150µm in length (G), 350µm in length (H), 400µm in length (I) and close-up of leaf 500µm in length (J). Scale bars: 50µm A-J.

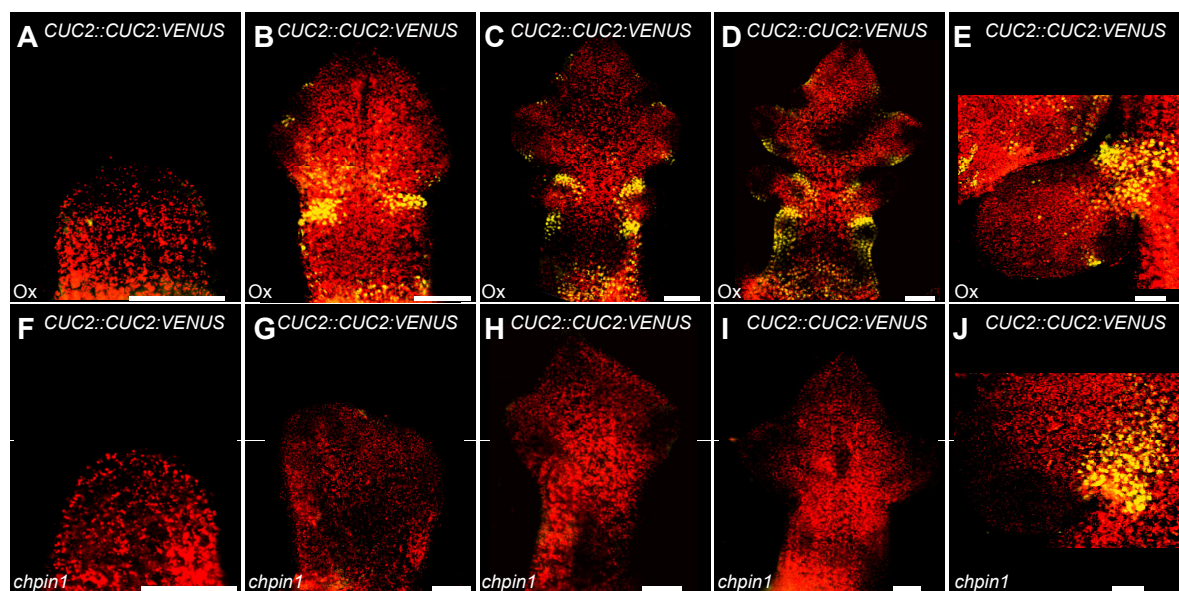
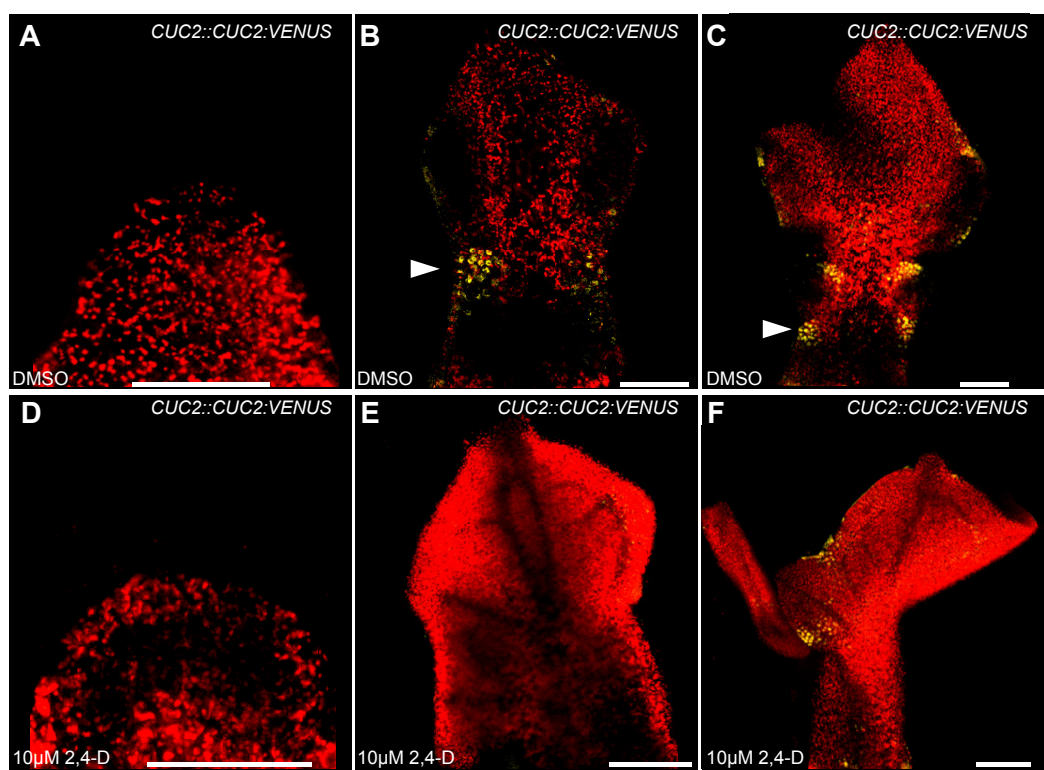


Figure 4.8 Auxin likely represses *CUC2* expression during early stages of *C. hirsuta* leaf development. (A-C) Confocal micrograph images of *CUC2::CUC2:VENUS* expression (yellow) in control-treated fifth rosette leaves approximately 75µm in length (A), 200µm in length (B) and 400µm in length (C); white triangles indicate foci of *CUC2* expression along the rachis. (D-F) *CUC2::CUC2:VENUS* expression (yellow) in 10µM 2,4-D-treated fifth rosette leaves approximately 75µm in length (D), 200µm in length (E) and 400µm in length (F). Scale bars: 50µm A-F.



expression is absent from leaves 75µm-200µm in length of auxin-treated plants (Figure 4.8 D-E). Later in development, faint *CUC2::CUC2:VENUS* expression occurs at the margin of the terminal leaflet of auxin-treated leaves (Figure 4.8 F). These data suggest that auxin has the ability to repress *CUC2* expression in the rachis of the *C. hirsuta* leaf, and that a negative feedback loop exists between *ChCUC*, PIN1 and auxin.

4.2.6 *CUC* and *PIN1* regulate secondary vein number in *C. hirsuta* leaves

Although PIN1 convergence points correlate with the formation of secondary veins in the *A. thaliana* leaf (Scarpella *et al.*, 2006), in Chapter 3 I showed that secondary vein development takes place in the absence of PIN1 convergence points in *cuc2* leaves (Figure 3.18). To investigate whether secondary vein development can be uncoupled from PIN1 convergence points in *C. hirsuta* leaves, I quantified secondary vein number in *pin1* and *35S::MIR164B;CUC3RNAi* leaves which lack PIN1 convergence points (Figure 4.9 A-F). Both *35S::MIR164B;CUC3RNAi* and *pin1* leaves produce significantly more secondary veins compared with wild type (Figure 4.9 A-F; *t*-test *p*<0.005). Correlating with this, *35S::MIR164B;CUC3RNAi* and *pin1* leaves produce significantly higher numbers of hydathodes compared with wild type (Figure 4.9 G). These data suggest that both *PIN1* and *ChCUC* are required for secondary vein development in *C. hirsuta* leaves, and that secondary vein formation cannot be uncoupled from PIN1 convergence points in *C. hirsuta* leaves.

4.2.7 Characterisation of wild-type vasculature development in *C. hirsuta* leaves

To characterise vasculature development during wild-type *C. hirsuta* leaf development, I analysed an *ATHB-8::GUS* vasculature reporter. As leaves initiate from the SAM, *ATHB-8::GUS* is expressed in the centre of the leaf primordium and extends through the SAM into the stem (Figure 4.10 A). As the leaf grows from 50µm to 125µm in length, *ATHB-8::GUS* expression remains in the centre of the leaf, marking the presumptive midvein (Figure 4.10 B-D). When the leaf is 150µm-200µm in length, marginal outgrowths become

Figure 4.9 *CUC* and *PIN1* are required for secondary vein development in *C. hirsuta* leaves. (A-E) Leaf clearings of fifth rosette leaf of Ox (A), *35S::MIR164B* (B), *CUC3RNAi* (C), *35S::MIR164B;CUC3RNAi* (D) and *chpin1* (E). **(F)** Quantification of average number of secondary veins in terminal leaflet of fifth rosette leaf of Ox, *35S::MIR164B* (*35S::M*), *CUC3RNAi* (*cuc3*), *35S::MIR164B;CUC3RNAi* (*35S::M;cuc3*) and *chpin1* (*pin1*); error bars represent standard error of the mean, where n=15. **(G)** Quantification of average number of hydathodes in fifth rosette leaf of Ox, *35S::MIR164B* (*35S::M*), *CUC3RNAi* (*cuc3*), *35S::MIR164B;CUC3RNAi* (*35S::M;cuc3*) and *chpin1-1* (*pin1*); error bars represent standard error of the mean, where n=15. Scale bars:1cm A-E.

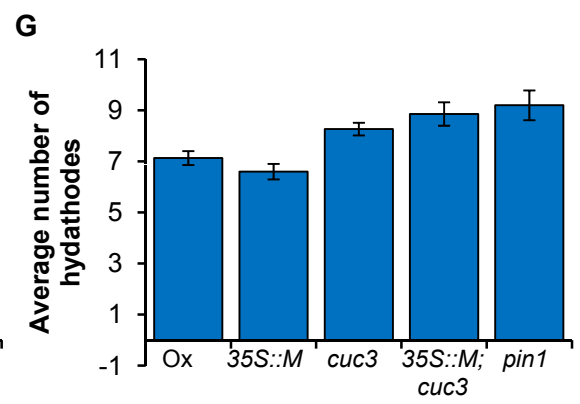
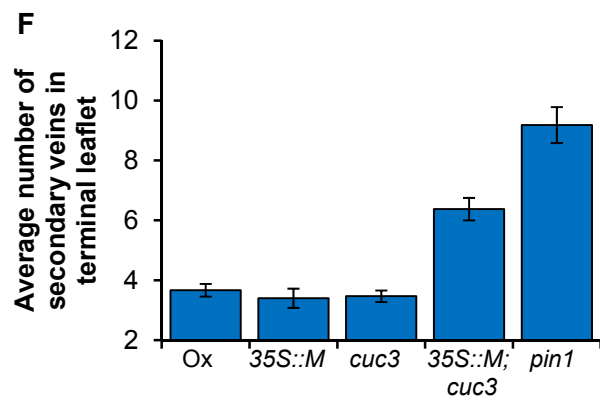
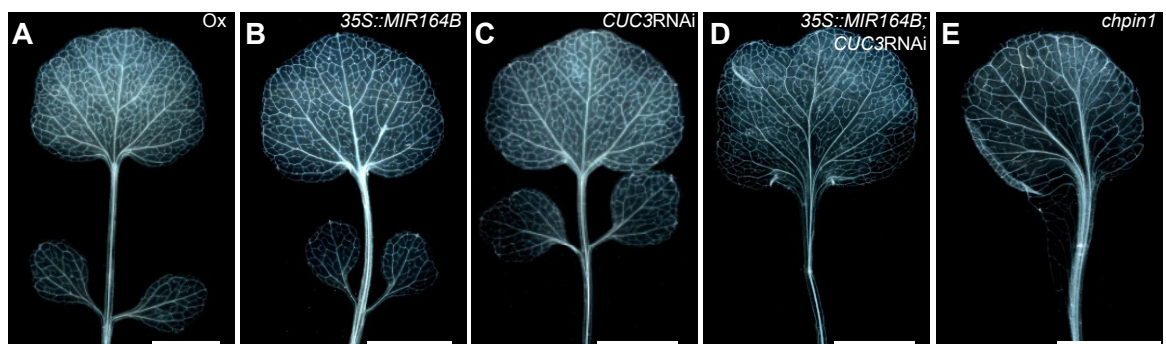
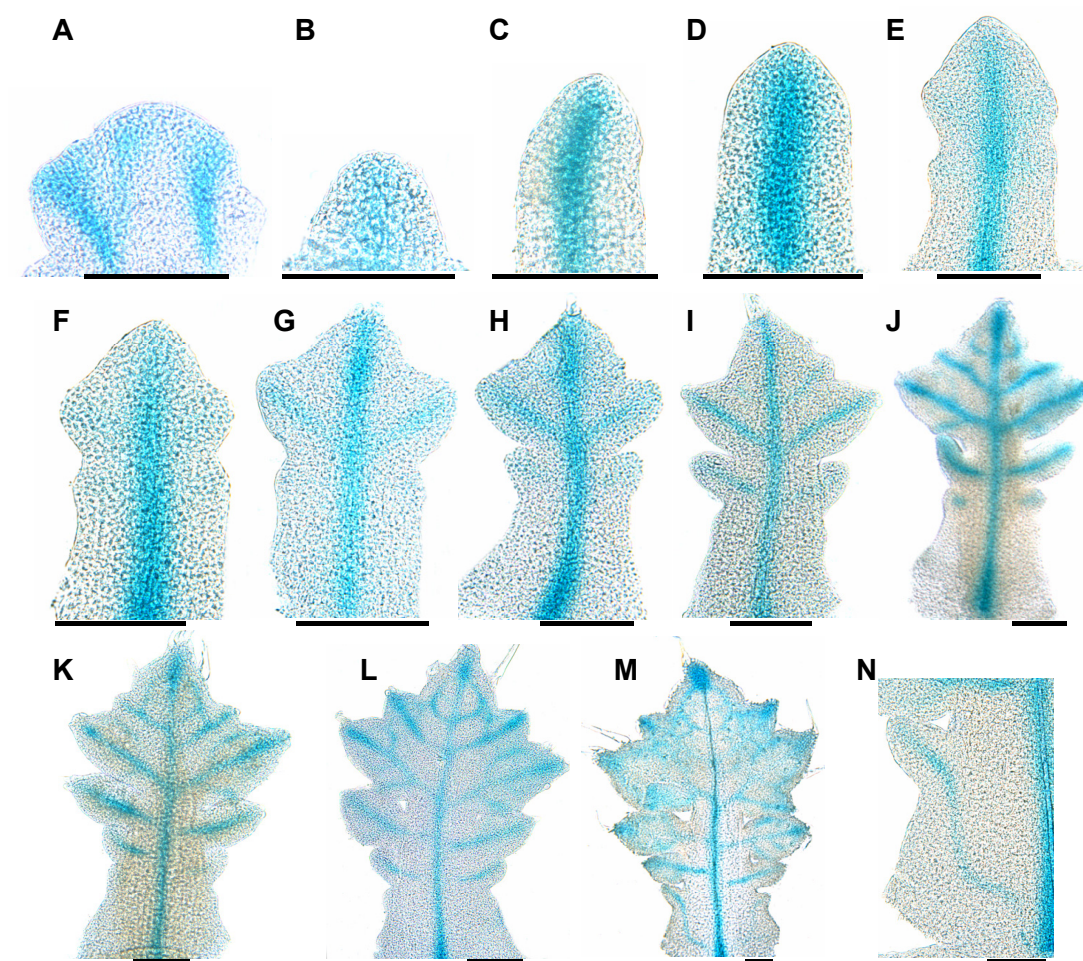


Figure 4.10 Progression of vasculature development during *C. hirsuta* leaf development.

(A-M) Meristem with initiating fifth leaf primordium (A), and fifth rosette leaf approximately 50µm in length (B), 100µm in length (C), 125µm in length (D), 150µm in length (E), 200µm in length (F), 225µm in length (G), 250µm in length (H), 300µm in length (I), 350µm in length (J), 400µm in length (K), 450µm in length (L) and 500µm in length (M) and close-up of fifth rosette leaf 500µm in length (N) stained for *ATHB-8::GUS* expression.

Scale bars: 100µm A-N.



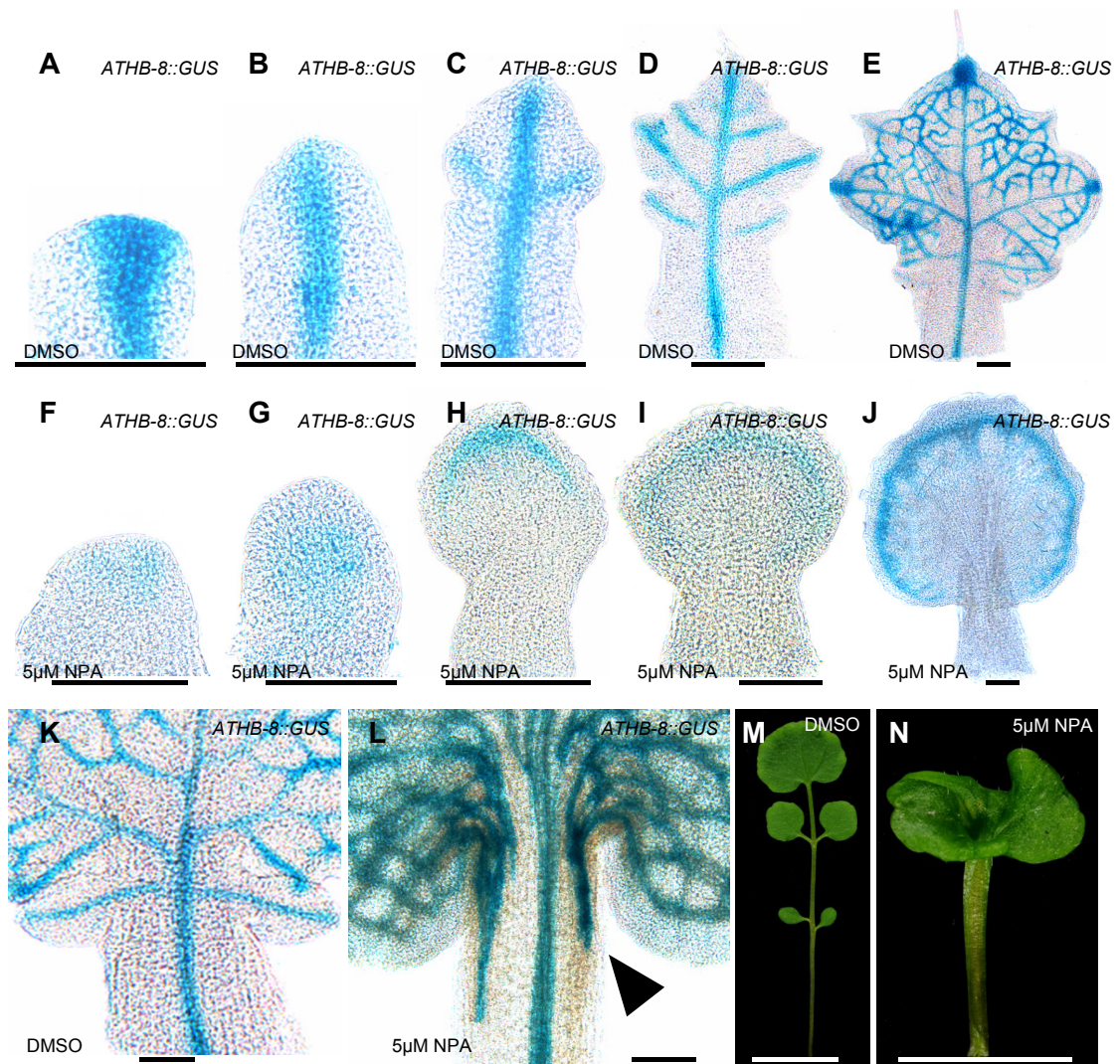
visible at the margin of the rachis and the terminal leaflet, but *ATHB-8::GUS* expression remains in the presumptive midvein (Figure 4.10 E-F).

Once the leaf is 225µm in length, *ATHB-8::GUS* is expressed in secondary veins that connect the outgrowths of the terminal leaflet to the midvein (Figure 4.10 G). Shortly after this, when the leaf is 250µm-300µm in length, *ATHB-8::GUS* is expressed in secondary veins connecting the first pair of lateral leaflets to the midvein (Figure 4.10 H-I). *ATHB-8::GUS* then expresses in the secondary veins connecting the second pair of leaflets to the midvein, when the leaf is 350µm-450µm in length (Figure 4.10 J-L). Finally, once the leaf is 500µm in length, *ATHB-8::GUS* is expressed in the secondary veins connecting the final pair of leaflets to the midvein (Figure 4,10 M-N). However, *ATHB-8::GUS* expression does not run parallel to the midvein, but instead loops to the base of the leaf to connect with the midvein (Figure 4.10 M-N).

4.2.8 Polar auxin transport regulates secondary vein development in *C. hirsuta* leaves

Since I showed that PIN1-mediated auxin transport is required for secondary vein development in *C. hirsuta* leaves, I next sought to determine how auxin transport regulates secondary vein development. To investigate this, I treated *ATHB-8::GUS* expressing plants with the chemical NPA to inhibit polar auxin transport. In contrast to wild type, during initiation of NPA-treated leaves, diffuse *ATHB-8::GUS* expression occurs in the centre of the leaf (Figure 4.11 A-B, F-G). When the leaf is 225µm-300µm in length, *ATHB-8::GUS* is expressed in an open-ended loop around the edge of the terminal leaflet, but is not expressed in the rachis (Figure 4.11 C-D, H-I). *ATHB-8::GUS* is then expressed in open-ended strands that connect to the loop and extend basipetally into the terminal leaflet (Figure 4.11 E, J). Whereas *ATHB-8::GUS* is expressed in a single vascular strand in the rachis of control DMSO-treated leaves 500µm in length (Figure 4.11 K), *ATHB-8::GUS* expression in the

Figure 4.11 Polar auxin transport is required for vasculature development in *C. hirsuta* leaf development. (A-E) Fifth rosette leaf stained for *ATHB-8::GUS* expression in control DMSO-treated leaves approximately 100µm in length (A), 150µm in length (B), 225µm in length (C), 300µm in length (D) and 400µm in length (E). (F-J) Fifth rosette leaf stained for *ATHB-8::GUS* expression in 5µM NPA-treated leaves approximately 100µm in length (F), 150µm in length (G), 225µm in length (H), 300µm in length (I) and 400µm in length (J). (K) Control DMSO-treated fifth rosette leaf approximately 500µm in length stained for *ATHB-8::GUS* expression. (L) 5µM NPA-treated fifth rosette leaf approximately 500µm in length stained for *ATHB-8::GUS* expression. (M-N) Fifth rosette leaf from control DMSO-treated plant (M) and 5µM NPA-treated plant (N); black triangle indicates open-ended veins. Scale bars: 100µm A-L; 1cm M-N.



open-ended strands of the terminal leaflet converge to form several open-ended strands in the rachis of NPA-treated leaves (Figure 4.11 L). These vascular defects of NPA-treated leaves are also associated with aberrant leaf morphologies as NPA-treated leaves are simplified with wider rachises compared to wild type (Figure 4.11 M-N). These data therefore suggest that polar auxin transport is required for both leaflet and vasculature development in *C. hirsuta*.

4.2.9 *CUC* genes and *PIN1* are required for serration development in *A. thaliana* leaves

In Chapter 3, I demonstrated that *PIN1* convergence points can be uncoupled from secondary vein development in *cuc2* leaves (Figures 3.6, 3.18). However, I showed that *PIN1* convergence points cannot be uncoupled from secondary vein development when all three *ChCUC* genes are downregulated in *C. hirsuta*. I, therefore, investigated the effect of reducing all three *A. thaliana CUC* genes on secondary vein development in *A. thaliana* leaves. *35S::MIR164A;cuc3* produces leaves with smooth margins that phenocopy *pin1* leaves (Figure 4.12 A-E).

To quantitatively determine the effect of reducing *CUC* and *PIN1* expression on leaf shape, I performed extended eigenshape analysis. Two eigenshape axes have significant differences in shape between genotypes (Figure 4.12 F; ANOVA, $p < 0.005$). ES1 accounts for 23.05% of total observed shape variance, and shapes along ES1 show changes in the width of the blade and petiole. Both *35S::MIR164A;cuc3* (ANOVA, Tukey post-hoc: $p < 0.01$) and *pin1* (ANOVA, Tukey post-hoc: $p < 0.01$) are significantly different in shape compared to Col-0. Furthermore, *35S::MIR164A;cuc3* clusters with *pin1* in morphospace (Figure 4.12 F). ES3 accounts for 5.12% of total observed shape variance, and shapes along ES3 show changes in the width of the base of the petiole. *pin1* is significantly different in shape compared to Col-0 (ANOVA, Tukey post-hoc: $p < 0.01$), and clusters with *35S::MIR164A;cuc3* in morphospace (Figure 4.12 F). Thus, morphometric analysis suggests

Figure 4.12 Phenotypic similarities and differences between reduced *A. thaliana* leaves with reduced *CUC* and *PIN1* activities. (A-E) Fifth rosette leaf and half silhouette of fifth rosette leaf of Col-0 (A, A'), *35S::MIR164A* (B, B'), *cuc3-105* (C, C'), *35S::MIR164A;cuc3-105* (D, D') and *pin1-7* (E, E'). **(F)** Eigenshape analysis of leaf shape changes in response to reduced *CUC* and *PIN1* expression. Silhouettes represent modelled shape variation at equal steps through the morphospace range of ES1 and ES3. Each point in the morphospace represents an individual leaf, with blue diamonds, yellow squares and green triangles representing Col-0, *35S::MIR164A;cuc3-105* and *pin1-7*, respectively. Connected lines represent the morphospace occupied by each genotype, with blue, yellow and green lines representing Col-0, *35S::MIR164A;cuc3-105* and *pin1-7*, respectively. n=10-20. Scale bars: 1cm A-E; 0.5cm A'-E'.

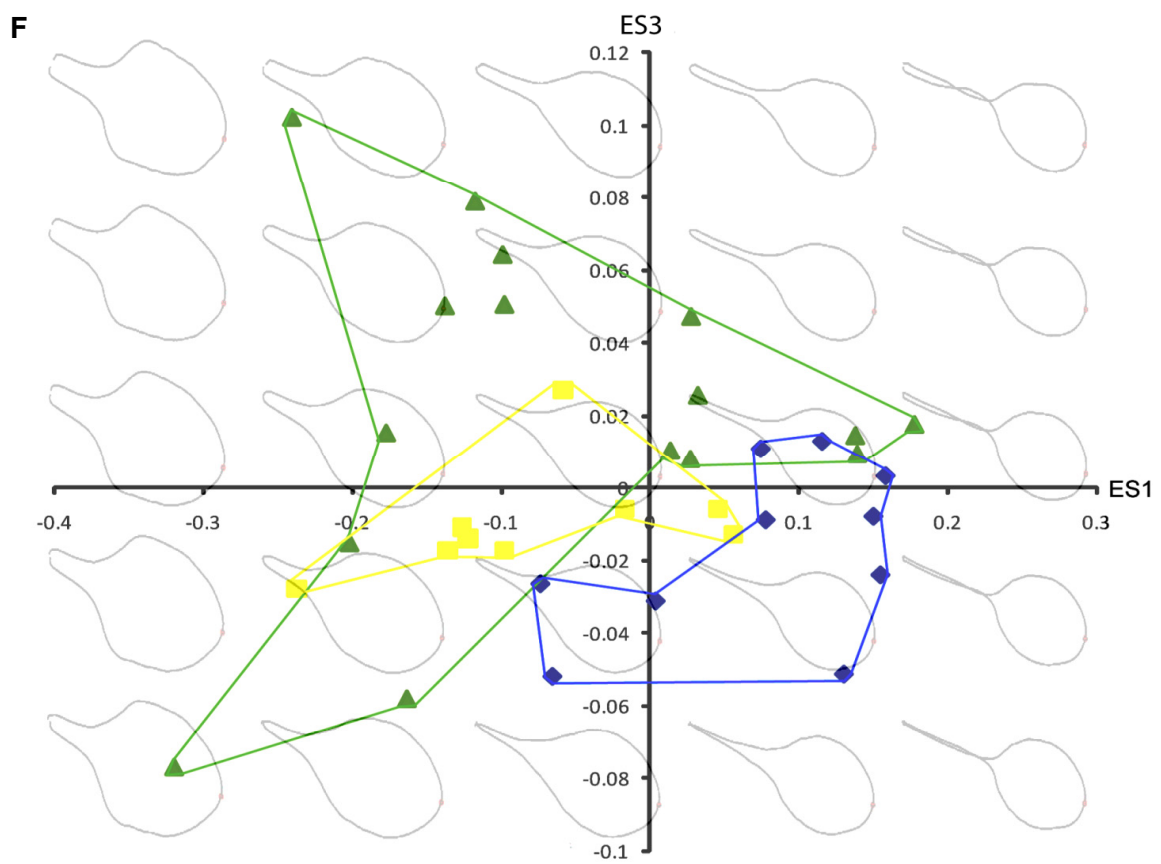
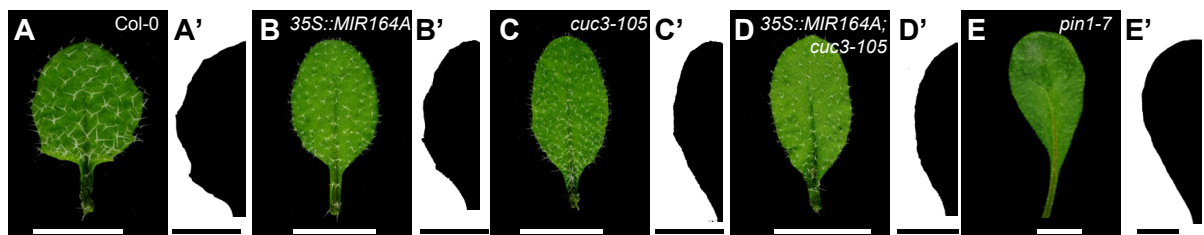
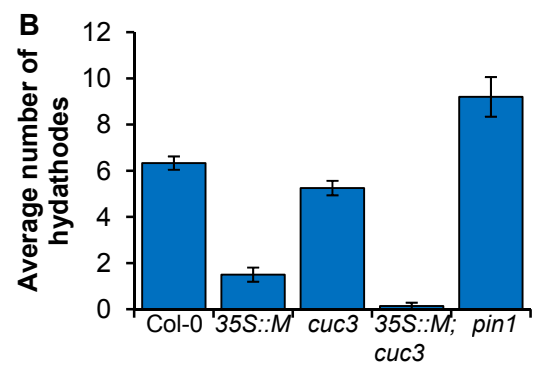
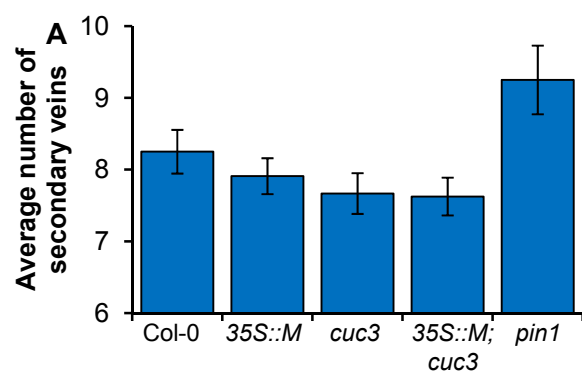


Figure 4.13 *CUC* and *PIN1* functions are required for vasculature development in *A. thaliana* leaves. **(A)** Quantification of average number of secondary veins in fifth rosette leaf of Col-0, *35S::MIR164A* (*35S::M*), *cuc3-105* (*cuc3*), *35S::MIR164A;cuc3-105* (*35S::M*) and *pin1-7* (*pin1*); error bars represent standard error of the mean, where n=15. **(B)** Quantification of average number of hydathodes in fifth rosette leaf of Col-0, *35S::MIR164A* (*35S::M*), *cuc3-105* (*cuc3*), *35S::MIR164A;cuc3-105* (*35S::M*) and *pin1-7* (*pin1*); error bars represent standard error of the mean, where n=15.



that *CUC* genes and *PIN1* have common functions in the development of blade and petiole width, however, *PIN1* alone restricts the width of the petiole base.

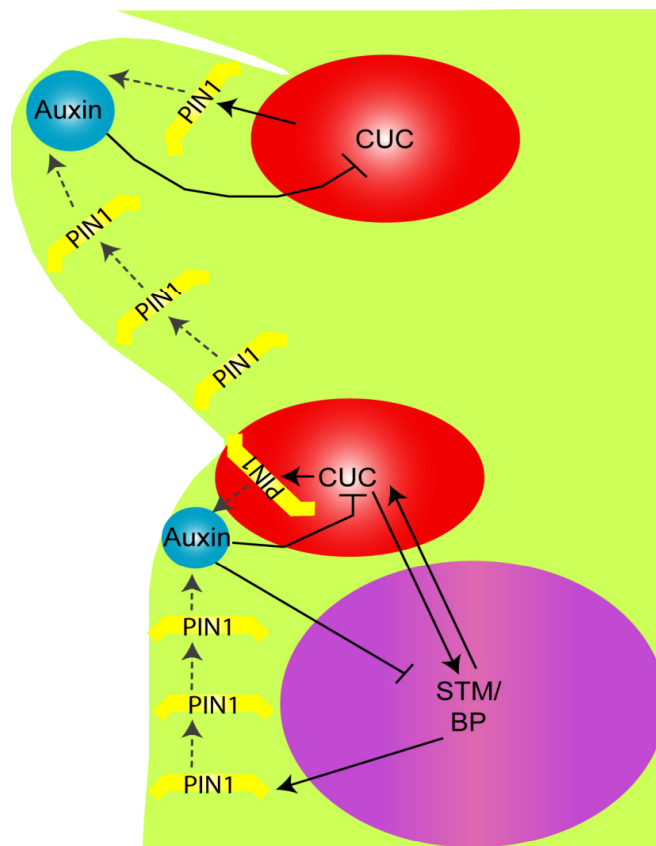
To investigate the effect of reducing *CUC* gene and *PIN1* on vasculature development, I quantified secondary vein number in *35S::MIR164A;cuc3* and *pin1* leaves from leaf clearings. *pin1* produces leaves with significantly higher numbers of secondary veins compared to wild type (Figure 4.13 A; *t*-test $p < 0.05$), whereas secondary vein number in *35S::MIR164A;cuc3* is not significantly different to wild type. *pin1* produces leaves with significantly higher numbers of hydathodes compared with wild type (Figure 4.13 B; *t*-test $p < 0.001$), whereas *35S::MIR164A;cuc3* produces leaves with significantly fewer hydathodes than wild type (Figure 4.13 B; *t*-test $p < 0.005$). These data suggest that *PIN1* regulates both secondary vein and hydathode development, whereas *CUC* genes promote hydathode development but not secondary vein formation. This is in contrast to *C. hirsuta* where *ChCUC* and *PIN1* regulate leaflet and hydathode development in similar manners.

4.3 Discussion

4.3.1 Interlinking feedback loops pattern leaflet development in *C. hirsuta*

The data in this chapter, combined with previous work, suggest that three feedback loops interact to pattern leaflet development in *C. hirsuta* leaves (Figure 4.14; Barkoulas *et al.*, 2008; Blein *et al.*, 2008). It is thought that a negative feedback loop between auxin and *KNOX* exists to pattern leaflet development. This is because auxin represses *KNOX* gene expression in the rachis to allow leaflet outgrowth, whilst *KNOX* activity feeds back to promote the establishment of new auxin activity maxima (Barkoulas *et al.*, 2008). Additionally, a positive feedback loop between *ChCUC* and *KNOX* is proposed to promote leaflet development as *ChCUC* gene activity is required for *KNOX* expression, whilst reducing *ChCUC* activity prevents leaflet development in *KNOX*-overexpressing

Figure 4.14 Interlinking feedback loops regulate leaflet development. (A) PIN1 (yellow trapezia) forms convergence points at sites of leaflet development, leading auxin transport (broken arrows) to create auxin activity foci (blue circle) at the tips of leaflets. *ChCUC* (red oval) and *KNOX* (purple oval) genes act to promote the formation of PIN1 convergence points, however, auxin feeds back to repress *ChCUC* and *KNOX* expression. A positive feedback loop also exists between *ChCUC* and *KNOX* genes.



backgrounds (Blein *et al.*, 2008). In this chapter, I suggest that an additional negative feedback loop exists between auxin and *CUC* to regulate leaflet initiation. I showed that *ChCUC* genes promote PIN1 convergence point and auxin activity maxima formation at the margin of the rachis (Figures 4.5-4.6), whilst auxin activity maxima feed back to restrict *CUC2* expression to the distal side of the initiating leaflet (Figure 4.8). Furthermore, this negative feedback loop appears to generate interspersed peaks of *CUC2* and auxin activity along the *C. hirsuta* rachis to pattern leaflet development. This is similar to the patterning of serration development described in Chapter 3 (Figure 3.19). It would, therefore, be interesting to test whether the molecular interactions between *ChCUC*, *KNOX*, auxin and PIN1 described above can plausibly generate the patterns of gene expression and leaf geometries observed in *C. hirsuta* by performing computational modelling.

Interestingly, I also showed that the negative feedback loop between *ChCUC*, PIN1 and auxin likely occurs iteratively along the *C. hirsuta* rachis in a basipetal sequence as interspersed peaks of auxin and *CUC* activity are generated basipetally along the proximo-distal axis of the *C. hirsuta* leaf (Figures 4.3-4.4). However, the mechanism that regulates the periodicity of peaks of *ChCUC* and auxin activity in *C. hirsuta* leaves remains unclear. Intriguingly, the type II TCP transcription factor *LA* regulates the transition from cell division to cell differentiation in the compound leaves of tomato (Ori *et al.*, 2007; Shleizer-Burko *et al.*, 2011). Gain-of-function *La* mutants produce simplified leaves due to precocious cell differentiation and, correlating with this, *La* leaves have significantly lower levels of *SLCUC* genes compared to wild type (Blein *et al.*, 2008). Thus, it would be interesting to test whether TCP transcription factors repress the iterative expression of *ChCUC* along the *C. hirsuta* rachis. The onset of *TCP* expression could prevent *ChCUC* expression, meaning that PIN1 convergence points would not be able to organise, and hence new axes of lateral growth could not occur.

4.3.2 A conserved feedback loop between *CUC*, PIN1 and auxin regulates leaflet and serration development

In Chapters 3 and 4, I showed that a negative feedback loop operates between *CUC*, PIN1 and auxin to promote serration initiation in *A. thaliana* and leaflet initiation in *C. hirsuta* (Figure 3.19, Figure 4.14). Despite the conservation of this feedback loop, there are striking differences between the margin morphologies of *C. hirsuta* and *A. thaliana* leaves. This suggests that the negative feedback loop may be differentially integrated with growth in the two species. Interestingly, it has been shown that a small number of cells in the rachis divide to generate each leaflet in *C. hirsuta* (Barkoulas *et al.*, 2008), whereas cell division is not associated with serration development in *A. thaliana* (Donnelly *et al.*, 1999). Correlating with these differences in growth, *KNOX* genes are expressed in *C. hirsuta* leaves where they prevent the transition from cell division to differentiation, whereas *KNOX* expression is excluded from *A. thaliana* leaves (Hay and Tsiantis, 2006). Significantly, *ChCUC* genes have been shown to interact with *KNOX* in a positive feedback loop in *C. hirsuta* leaves, suggesting that the differences in morphology between leaflets and serrations may result from an additional feedback loop between *ChCUC* and *KNOX* genes that prevents the transition from cell division to differentiation in *C. hirsuta*.

Furthermore, *CUC* genes have been shown to regulate leaflet development in a wide variety of species, including tomato and pea, leading to the suggestion that *CUC* genes are a universal regulator of compound leaf form (Blein *et al.*, 2008). Since auxin transport is also required for leaf dissection in tomato and pea (DeMason and Chawla, 2004; Koenig *et al.*, 2009), it is possible that *CUC* genes may also regulate the formation of PIN1 convergence points in most compound leafed species.

4.3.3 A change in gene expression may signal the initiation of the final leaflet pair

In this chapter, I observed that patterns of *CUC2::CUC2:VENUS*, *PIN1::PIN1:GFP*, *DR5::VENUS* and *ATHB-8::GUS* expression differ between the first and final leaflets produced. *CUC2* maxima precede PIN1 convergence point formation in the first pair of leaflets, and *CUC2* maxima are restricted to the distal side of these leaflets (Figure 4.3 B-C). However, PIN1 convergence points form within a strip of *CUC2* expression that extends to the base of the leaf in the final leaflet pair (Figure 4.3 F-G). One hypothesis is that the expansion of *CUC2* to the base of the leaf acts as a signal to stop leaflet production. This is because *CUC2* has been shown to act as a growth repressor in *A. thaliana* leaves (Nikovics *et al.*, 2006). If this repressive function of *CUC2* has been conserved in *C. hirsuta* leaves, the expansion of *CUC2* to the base of the leaf may reduce growth and prevent subsequent leaflets developing. It would, therefore, be interesting to determine the effect of differential *CUC2* and PIN1 expression on leaf morphology.

In addition, *DR5::VENUS* is expressed in a single subepidermal cell during the initiation of the first pair of leaflets (Figure 4.4 B-C). However, *DR5::VENUS* is expressed in several cells during the initiation of the final pair of leaflets (Figure 4.4 F-G). Since I showed that *CUC* genes may promote auxin biosynthesis, it is possible that the expanded domain of *CUC2* expression in the final leaflet may be responsible for the increased auxin activity due to an upregulation of auxin biosynthesis. However, it is unclear what causes *CUC2* expression to expand to the base of the leaf as the final pair of leaflets initiate. Furthermore, it is not known whether the expansion of auxin activity seen in final leaflets acts as a signal to stop subsequent leaflet production, or whether it is simply an output from expanded *CUC2* expression.

Finally, by observing *ATHB-8::GUS* expression patterns, I showed that secondary veins connect the tip of the first pair of leaflets to the midvein in the shortest possible

distance, by forming perpendicular to the midvein (Figure 4.10 I). However, secondary veins connect the tip of the final pair of leaflets to the midvein by looping towards the base of the leaf (Figure 4.10 N). This may act as a mechanism to prevent subsequent leaflet forming. It is likely that there must be an auxin sink for leaflet outgrowth since reducing polar auxin transport prevents leaflet initiation (Barkoulas *et al.*, 2008). If the auxin sink for the final pair of leaflet occurs at the base of the leaf, there would not be sufficient space for subsequent leaflets to connect to the midvein of the leaf. This is similar to the auxin transport canalisation-based model for shoot branching where auxin export from the bud is required for bud outgrowth (Bennett *et al.*, 2006; Li and Bangerth, 1999; Prusinkiewicz *et al.*, 2009)

4.4 Conclusions

- A conserved negative feedback loop between *CUC*, PIN1 and auxin regulates leaflet development in *C. hirsuta* and serration development in *A. thaliana*.
- PIN1 convergence point formation cannot be uncoupled from secondary vein development, suggesting that the two processes are linked in *C. hirsuta* leaves.
- Changes in *CUC2*, PIN1, auxin and vasculature reporter expression may signal the cessation of leaflet production in *C. hirsuta* leaves.

**Chapter 5: A possible role for
auxin, *CUC* and *KNOX* genes in
elaborating lobed leaf
morphologies**

Chapter 5: A possible role for auxin, *CUC* and *KNOX* genes in elaborating lobed leaf morphologies

5.1 Introduction

Understanding the genetic mechanisms that underlie morphological diversity is an area of intense investigation in developmental biology. Recent studies highlighted that changes in the *cis*-regulatory regions of genes can generate morphological diversity in both plants and animals (Frankel *et al.*, 2011; Hay and Tsiantis, 2006; Hersh and Carroll, 2005). Comparative studies seeking to determine the gene regulatory networks controlling compound leaf morphology have compared the model species *A. thaliana*, which produces simple leaves, with the closely-related crucifer *C. hirsuta*, which produces compound leaves (Barkoulas *et al.*, 2008; Hay and Tsiantis, 2006). These studies showed that *KNOX* expression is excluded from *A. thaliana* leaves, whereas *KNOX* expression in the leaves of *C. hirsuta* is necessary and sufficient for leaflet development (Hay and Tsiantis, 2006). Changes in *cis*-regulatory regions of *KNOX* promoters are responsible for *KNOX* expression in the *C. hirsuta* leaf (Hay and Tsiantis, 2006), suggesting that the compound *C. hirsuta* leaf form may have arisen through *cis*-regulatory changes in *KNOX* genes.

KNOX genes are components of interlinking feedback loops that control leaflet development in *C. hirsuta* leaves. A negative feedback loop is thought to operate between *KNOX* genes and auxin in the *C. hirsuta* leaf to pattern leaflet development (Barkoulas *et al.*, 2008). Auxin activity maxima form along the margin of the rachis at sites of leaflet development, whereas *KNOX* expression is excluded from these regions in a manner similar to leaf initiation at the SAM (Barkoulas *et al.*, 2008; Heisler *et al.*, 2005). Since exogenous auxin application represses the transcription of *ChSTM*, it is thought that the formation of

auxin activity maxima excludes *KNOX* expression from the margin, thereby allowing the outgrowth of leaflets (Barkoulas *et al.*, 2008). Furthermore in Chapter 4, I described a negative feedback loop between *CUC*, PIN1 and auxin where *CUC* promotes PIN1 convergence point formation, whilst high auxin activity represses *CUC* expression (Figure 4.14).

Transgenic *C. hirsuta* plants with reduced *ChCUC* levels produce simplified leaves with reduced levels of *ChSTM* expression, and *ChCUC* genes are required for the development of higher-order leaflets in transgenic *C. hirsuta* plants overexpressing *KNOX* genes (Blein *et al.*, 2008). Thus, it appears that a positive feedback loop between *ChCUC* and *ChSTM* genes promotes leaflet development in *C. hirsuta* leaves (Blein *et al.*, 2008).

Recently, clonal analysis revealed that each leaflet is derived from two to four cells originating at the margin of the rachis in *C. hirsuta* (Barkoulas *et al.*, 2008). This suggests that the cells comprising the leaflet arise from new cell divisions rather than the recruitment of existing cells. In support of this, foci of cell division are also associated with leaflet development in *C. hirsuta* and are thought to be generated by auxin activity maxima since loss-of-auxin transport reduces levels of cell division (Barkoulas *et al.*, 2008). *KNOX* genes also appear to influence cell division in the *C. hirsuta* leaf as they prevent differentiation (Hay and Tsiantis, 2006). Since *ChCUC* genes are thought to regulate *KNOX* expression, they may regulate leaflet development by influencing cell division.

Interestingly, ectopic *KNOX* expression in *A. thaliana* leaves leads to the production of deeply lobed leaf margins reminiscent of leaflets (Chuck *et al.*, 1996; Hay *et al.*, 2006). However, it is unclear whether these structures arise in a manner similar to leaflet development in *C. hirsuta*. Furthermore, it is not known whether interlinking feedback loops

involving *CUC*, *PIN1* and *KNOX* described for leaflet development in *C. hirsuta* also regulate lobe development in *A. thaliana* leaves with ectopic *KNOX* expression.

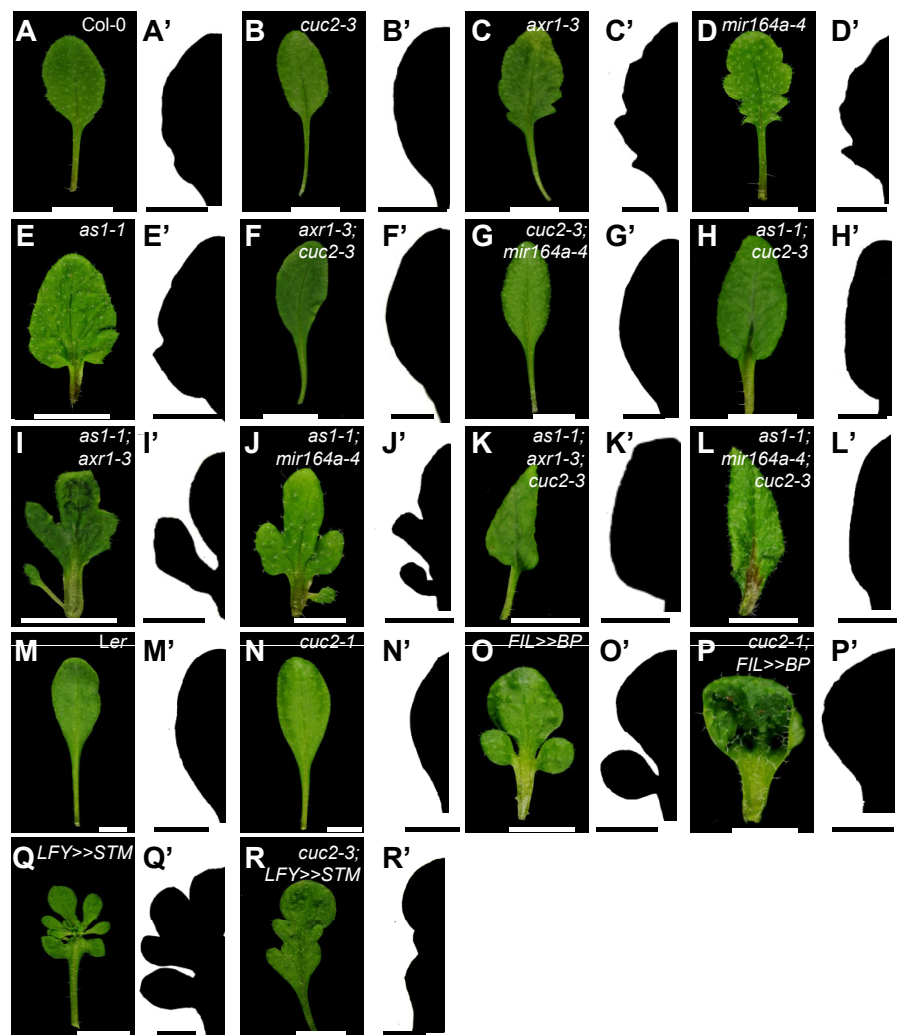
In this chapter, I investigate regulatory interactions between *PIN1*, *CUC* and *KNOX* during *KNOX*-induced lobe development in *A. thaliana* to acquire an in-depth understanding of how differences in *KNOX* expression influence leaf geometries. I demonstrate that *CUC2* and polar auxin transport are required for ectopic *KNOX* expression. Conversely, I show that ectopic *KNOX* expression up-regulates *CUC2* expression in *A. thaliana* leaves. These findings suggest that interlinking feedback loops between *KNOX*, *CUC* and *PIN1* that occur in leaflet development in *C. hirsuta* also occur in association with *KNOX*-induced lobe development in *A. thaliana*. Interestingly, whilst *ChCUC* gene activity promotes *ChBP* transcription in the *C. hirsuta* rachis, I show that *CUC* genes do not promote *ChBP* transcription in *A. thaliana* leaves. This suggests that a *cis*-regulatory change may have occurred in the *ChBP* promoter to alter regulatory interactions between *ChCUC* and *KNOX* genes. Finally, I demonstrate that *ChCUC* genes may promote leaflet outgrowth by influencing levels and patterns of cell division.

5.2 Results

5.2.1 *CUC2* is required for *KNOX*-dependent lobe development in *A. thaliana* leaves

KNOX gene expression is excluded from the simple leaves of *A. thaliana* (Byrne *et al.*, 2000; Ori *et al.*, 2000). However, *A. thaliana* leaves ectopically expressing *KNOX* genes develop deeply lobed margins reminiscent of leaflets (Chuck *et al.*, 1996; Hay *et al.*, 2006; Koyama *et al.*, 2010). *as1;axr1* and *as1;mir164a* double mutants produce leaves with deeply lobed margins and transcriptionally misexpress *BP* in the sinus regions between lobes (Hay *et al.*, 2006; Koyama *et al.*, 2010). Additionally, *FIL*>>*BP* and *LFY*>>*STM* transgenic plants

Figure 5.1. *CUC2* is required for lobe development in *A. thaliana* leaves ectopically expressing *KNOX* genes. (A-R) Fifth rosette leaf and half silhouette of fifth rosette leaf of Col-0 (A, A'), *cuc2-3* (B, B'), *axr1-3* (C, C'), *mir164a-4* (D, D'), *asl-1* (E, E'), *axr1-1;cuc2-3* (F, F'), *cuc2-3;mir164a-4* (G, G'), *asl-1;cuc2-3* (H, H'), *asl-1;axr1-3* (I, I'), *asl-1;mir164a-4* (J, J'), *asl-1;axr1-3;cuc2-3* (K, K'), *asl-1;cuc2-3;mir164a-4* (L, L'), *Ler* (M, M'), *cuc2-1* (N, N'), *FIL>>BP* (O, O'), *cuc2-1;FIL>>BP* (P, P'), *LFY>>STM* (Q, Q') and *cuc2-3;LFY>>STM* (R, R'). Scale bars: 1cm A-R; 0.5cm A'-R'.



post-transcriptionally misexpress *KNOX* genes specifically in leaves, and produce leaves with deeply lobed margins reminiscent of leaflets (Hay *et al.*, 2006; D. Weigel, unpublished). Together, these data suggest that *KNOX* gene activity is sufficient to promote leaf dissection in *A. thaliana* leaves.

To investigate whether *CUC2* is required for ectopic *KNOX*-dependent lobe development in *A. thaliana* leaves, I analysed the effect of loss-of-*CUC2* function on *asl;axr1* and *asl;mir164a* leaves, which transcriptionally misexpress *BP* (Hay *et al.*, 2006; Koyama *et al.*, 2010). Both *asl;axr1;cuc2* and *asl;cuc2;mir164a* triple mutants produce leaves with smooth margins with significantly lower levels of leaf dissection compared to *asl;axr1* and *asl;mir164a* double mutants, respectively (Figure 5.1 A-L; Table 5.1; *t*-test $p < 0.05$). This suggests that *CUC2* is required for lobe development associated with ectopic *KNOX* expression.

Table 5.1 *CUC2* is required for high dissection indices in mutants with reduced auxin signalling, *AS1* and *MIR164A* activities

Genotype	(Perimeter ²)/(4 π x area) $\pm$ standard error
Col-0	4.46 $\pm$ 0.03
<i>axr1-3</i>	4.71 $\pm$ 0.03
<i>asl-1</i>	6.38 $\pm$ 0.35
<i>mir164a-4</i>	6.93 $\pm$ 0.15
<i>cuc2-3</i>	4.17 $\pm$ 0.04
<i>axr1-3;cuc2-3</i>	3.81 $\pm$ 0.03
<i>asl-1;cuc2-3</i>	2.56 $\pm$ 0.12
<i>cuc2-3;mir164a-4</i>	3.29 $\pm$ 0.10
<i>asl-1;axr1-3</i>	6.82 $\pm$ 0.22
<i>asl-1;axr1-3;cuc2-3</i>	2.61 $\pm$ 0.16
<i>asl-1;mir164a-4</i>	7.30 $\pm$ 0.30
<i>asl-1;cuc2-3;mir164a-4</i>	1.95 $\pm$ 0.06

To investigate whether *CUC2* is required for lobe development in transgenic plants that post-transcriptionally misexpress *BP* and *STM* in their leaves, I assessed the effect of loss-of-*CUC2* function in *FIL*>>*BP* and *LFY*>>*STM* plants. *FIL*>>*BP;cuc2* plants produce

leaves with smooth margins, similar to the *cuc2* single mutant (Figure 5.1 A-B, M-P), and *LFY>>STM;cuc2* plants produce leaves with smoother margins compared to *LFY>>STM* plants (Figure 5.1 A-B, Q-R). This suggests that *CUC2* function is required for lobe development when *KNOX* genes are post-transcriptionally misexpressed in leaves.

5.2.2 *CUC2* expression is upregulated and broadened in *A. thaliana* leaves misexpressing *KNOX* genes

Since *CUC2* appears to be required for the development of lobes when *KNOX* genes are misexpressed in *A. thaliana* leaves, I hypothesised that *CUC2* may be upregulated in these leaves. To test this, I observed *CUC2::CUC2:VENUS* expression in *FIL>>BP* and *LFY>>STM* leaves. During early wild-type leaf development, *CUC2::CUC2:VENUS* is expressed in continuous strips along the margin (Figure 5.2 A, I). *CUC2::CUC2:VENUS* is then downregulated at sites of serration development (Figure 5.2 B, J) and is restricted to the distal and proximal sides of the developing serration (Figure 5.2 C-D, K-L). In contrast, *CUC2::CUC2:VENUS* is expressed in continuous strips along the *FIL>>BP* margin that are expanded along the medio-lateral axis compared to wild type (Figure 5.2 E). Once the *FIL>>BP* leaves initiate lobes, *CUC2::CUC2:VENUS* expression broadens along the proximo-distal axis compared to wild type (Figure 5.2 F-G), and the extended expression at the distal side of the lobe persists for longer than in wild type (Figure 5.2 H). Similarly, in *LFY>>STM* leaves, *CUC2::CUC2:VENUS* is initially expressed in continuous strips along the margin (Figure 5.2 M). Moreover, once *LFY>>STM* lobes initiate, *CUC2::CUC2:VENUS* expression expands along the medio-lateral and proximo-distal axes (Figure 5.2 N-O), and the broadened proximo-distal expression persists into later leaf development (Figure 5.2 P).

To determine whether transcriptional misexpression of *BP* in *A. thaliana* leaves also upregulates *CUC2* expression, I analysed *CUC2::CUC2:VENUS* expression in *asl;axr1* and

Figure 5.2 *CUC2* expression is broadened in *KNOX*-misexpressing *A. thaliana* leaves.

(A-D) Confocal micrographs of *CUC2::CUC2:VENUS* expression (yellow) in *Ler* seventh (A), sixth (B), fifth (C) and fourth rosette leaf (D). **(E-H)** Confocal micrographs of *CUC2::CUC2:VENUS* expression (yellow) in *FIL>>BP* seventh (E), sixth (F), fifth (G) and fourth rosette leaf (H). **(I-L)** Confocal micrographs of *CUC2::CUC2:VENUS* expression (yellow) in Col-0 seventh (I), sixth (J), fifth (K) and fourth rosette leaf (L). **(M-P)** Confocal micrographs of *CUC2::CUC2:VENUS* expression (yellow) in *LFY>>STM* seventh (M), sixth (N), fifth (O) and fourth rosette leaf (P). Scale bars: 50µm A-P.

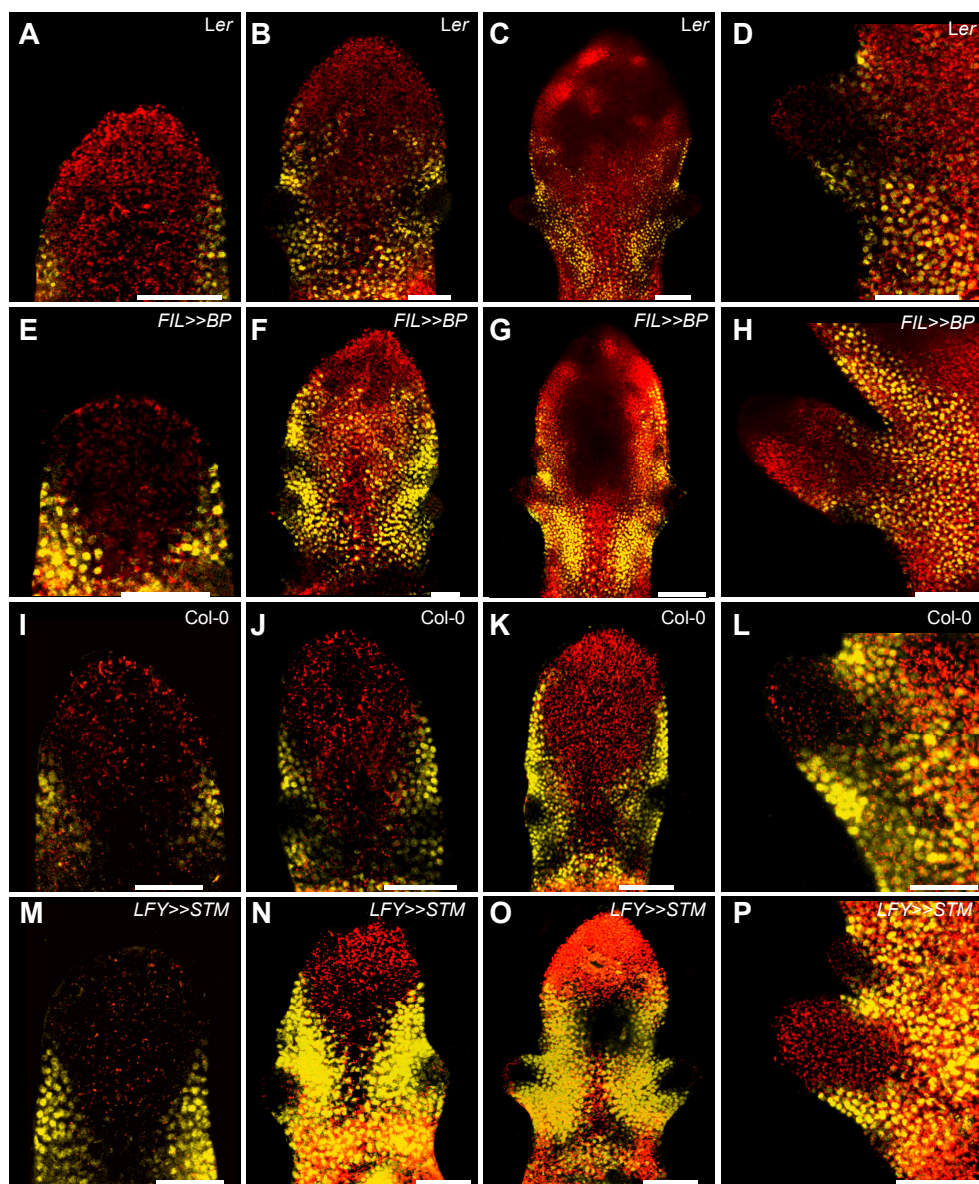
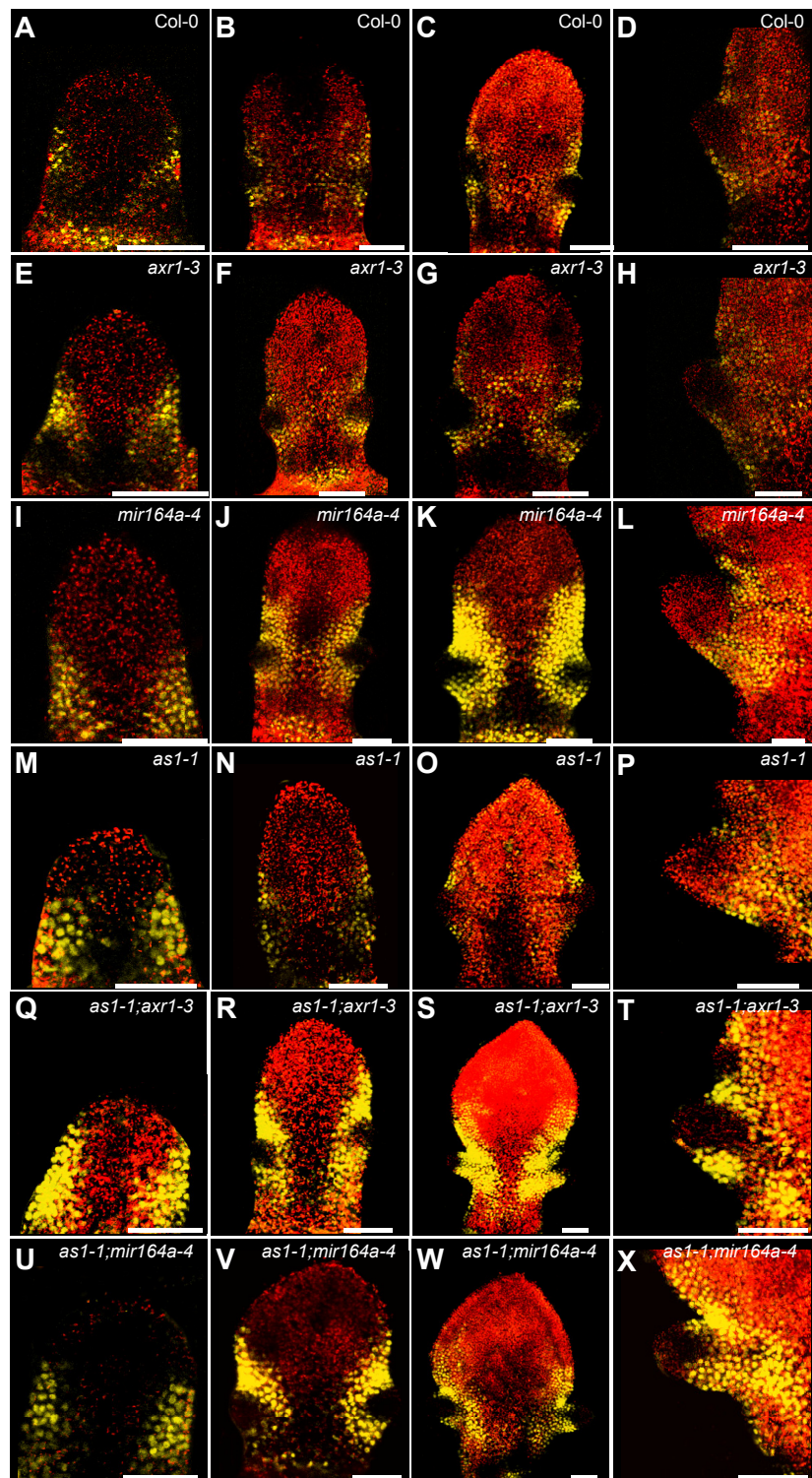


Figure 5.3 Loss-of-*AXR1* and *MIR164A* function in *as1* mutant leaves causes broadened *CUC2* expression. (A-D) Confocal micrographs of *CUC2::CUC2:VENUS* expression (yellow) in Col-0 seventh (A), sixth (B) fifth (C) and in close-up of fourth (D) rosette leaf. (E-H) Confocal micrographs of *CUC2::CUC2:VENUS* expression (yellow) in *axr1-3* seventh (E), sixth (F) fifth (G) and in close-up of fourth (H) rosette leaf. (I-L) Confocal micrographs of *CUC2::CUC2:VENUS* expression (yellow) in *mir164a-4* seventh (I), sixth (J) fifth (K) and in close-up of fourth (L) rosette leaf. (M-P) Confocal micrographs of *CUC2::CUC2:VENUS* expression (yellow) in *as1-1* seventh (M), sixth (N) fifth (O) and in close-up of fourth (P) rosette leaf. (Q-T) Confocal micrographs of *CUC2::CUC2:VENUS* expression (yellow) in *as1-1;axr1-3* seventh (Q), sixth (R) fifth (S) and in close-up of fourth (T) rosette leaf. (U-X) Confocal micrographs of *CUC2::CUC2:VENUS* expression (yellow) in *as1-1;mir164a-4* seventh (U), sixth (V) fifth (W) and in close-up of fourth (X) rosette leaf. Scale bars: 50µm A-X.



as1;mir164a mutants. *CUC2::CUC2:VENUS* is initially expressed in continuous strips at the margins of wild-type leaves, before being downregulated at sites of serration development (Figure 5.3 A-D). The same expression pattern occurs in the *axr1*, *mir164a* and *as1* single mutants (Figure 5.3 E-P), although levels of expression are increased in *axr1* and *mir164a* leaves compared to wild type (Figure 5.3 E-L). In *as1;axr1* and *as1;mir164a* double mutants, *CUC2::CUC2:VENUS* expression is initially broadened along the proximo-distal axis, before expanding along the medio-lateral axis once lobe initiation occurs (Figure 5.3 Q-X). This suggests that ectopic *KNOX* expression in *A. thaliana* leaves increases *CUC2::CUC2:VENUS* expression, which is responsible for the development of lobes reminiscent of leaflets.

5.2.3 Ectopic *KNOX* expression post-transcriptionally promotes *CUC2* expression in *A. thaliana* leaves

Since I showed that *KNOX* misexpression increases *CUC2* protein levels in the previous section, I next sought to determine whether ectopic *KNOX* expression promotes *CUC2* transcription. To investigate this, I analysed the expression pattern of a *CUC2::GUS* transcriptional reporter in *as1;axr1* double mutant leaves. In wild type, *CUC2::GUS* is initially expressed at the base of leaves (Figure 5.4 A). *CUC2::GUS* is then expressed in continuous strips along the leaf margin (Figure 5.4 B) before being downregulated at sites of serration development (Figure 5.4 C-D). As serration development is an iterative process, *CUC2::GUS* is downregulated in a basipetal sequence along the proximo-distal axis of the leaf (Figure 5.4 E). *CUC2::GUS* has a similar expression pattern in the leaves of *as1* and *axr1* single mutants (Figure 5.4 F-O), and in the leaves of *as1;axr1* double mutants (Figure 5.4 P-T). Crucially, *CUC2::GUS* expression is not broadened along the proximo-distal axis or the medio-lateral axis of the *as1;axr1* leaf, in contrast to *CUC2::CUC2:VENUS* expression. This suggests that ectopic *BP* expression in *A. thaliana* leaves does not upregulate *CUC2* transcription.

Figure 5.4 *CUC2* transcription is not altered in *axr1;asl* mutant leaves. (A-X) Eighth (A, F, K, P), seventh (B, G, L, Q), sixth (C, H, M, R), fifth (D, I, N, S) and fourth rosette leaves (E, J, O, T) stained for *CUC2::GUS* in Col-0 (A-E), *axr1-3* (F-J), *asl-1* (K-O) and *asl-1;axr1-3* (P-T). Scale bars: 50µm A-T.

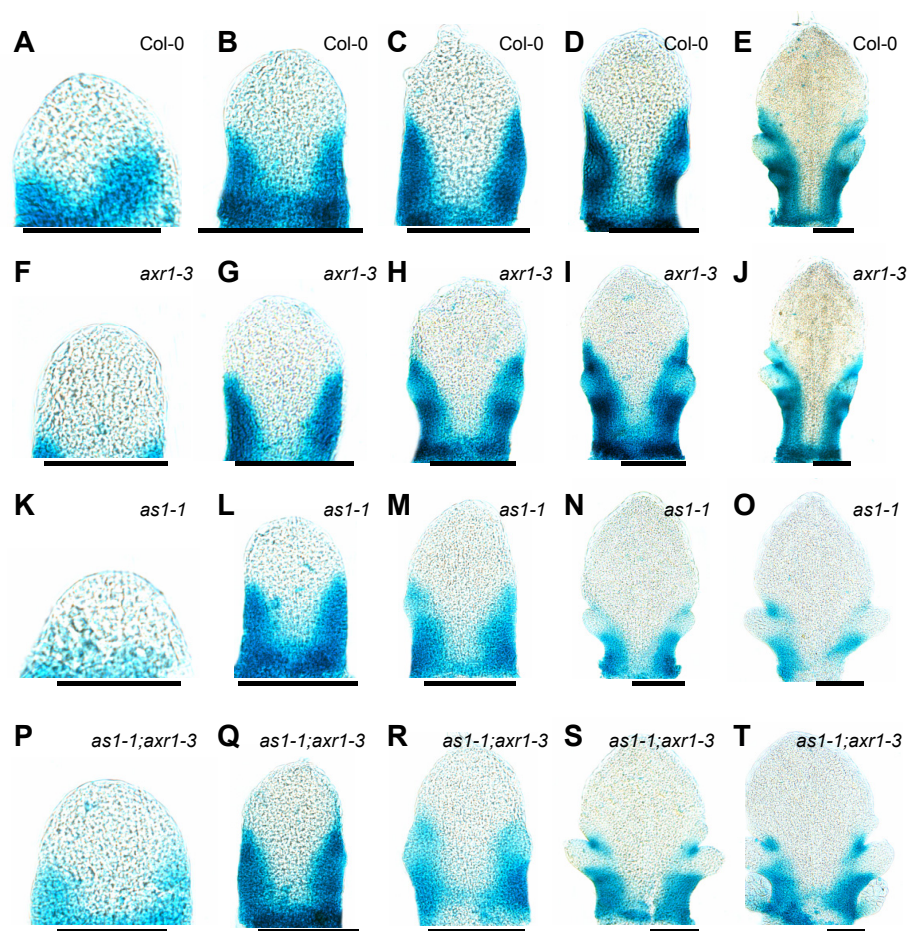
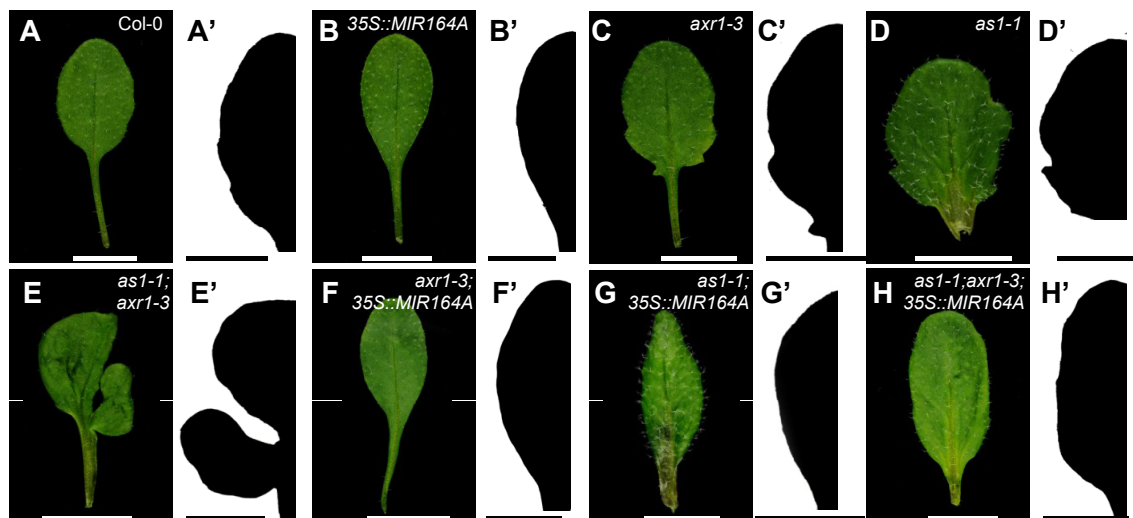


Figure 5.5 Overexpression of *MIR164A* is sufficient to suppress lobe formation in *axr1-1;asl-1* leaves. (A-H) Fifth rosette leaf and half silhouette of fifth rosette leaf of Col-0 (A, A'), *35S::MIR164A* (B, B'), *axr1-3* (C, C'), *asl-1* (D, D'), *asl-1;axr1-3* (E, E'), *axr1-3;35S::MIR164A* (F, F'), *asl-1;35S::MIR164A* (G, G') and *asl-1;axr1-3;35S::MIR164A* (H, H'). Scale bars: 1cm A-H; 0.5cm A'-H'.



I reasoned that if *KNOX* increases *CUC2* protein levels but is not transcriptionally upregulated by *KNOX*, then *CUC2* may be post-transcriptionally regulated by *KNOX*. Since *CUC2* transcripts are targeted for degradation by *mir164* (Laufs *et al.*, 2004; Mallory *et al.*, 2004), I hypothesised that *KNOX* misexpression in leaves may downregulate *MIR164A* expression to post-transcriptionally increase *CUC2* expression. To test this, I analysed the effect of overexpressing *MIR164A* in *as1;axr1* leaves. Whilst wild-type plants produce leaves with serrated margins (Figure 5.5 A), *35S::MIR164A* plants produce leaves with smooth margins (Figure 5.5 B); similarly, expressing *35S::MIR164A* in *as1*, *axr1* and in *as1;axr1* mutants causes smooth margin leaves to develop (Figure 5.5 C-H). This suggests that low levels of *MIR164A* expression are required for lobe formation in these *KNOX* misexpressing leaves. Thus, it is possible that *KNOX* genes upregulate *CUC2* expression at the post-transcriptional level by repressing *MIR164A* expression.

5.2.4 *CUC2* is required for *BP* misexpression at the margins of *A. thaliana* leaves

CUC genes are required for *KNOX* gene expression in the compound leaves of *C. hirsuta* (Blein *et al.*, 2008), and *CUC2* is required for *KNOX* gene expression in the *A. thaliana* SAM (Aida *et al.*, 1999). To investigate whether *CUC2* is required for *BP* misexpression in *as1;axr1* and *as1;mir164a* leaves, I examined *BP::GUS* expression in late-stage leaves. *BP::GUS* is not expressed in wild-type, *cuc2* and *mir164a* leaves (Figure 5.6 A-B, D), but is expressed at the tips of serrations in *axr1* (Figure 5.6 C), and at the tips of lobes and in the midvein of *as1* (Figure 5.6 E). *BP::GUS* is not expressed at the margins of *axr1;cuc2*, *mir164a;cuc2* or *as1;cuc2* double mutant leaves (Figure 5.6 F-H), suggesting that *CUC2* is required for *BP* expression in the tips of *axr1* and *as1* outgrowths. *BP::GUS* is expressed in the sinuses and tips of lobes, and in the petioles, of *as1;axr1* and *as1;mir164a* double mutant leaves (Figure 5.6 I-J). However, *BP::GUS* is not detected at the margins of *as1;axr1;cuc2* and *as1;cuc2;mir164a* triple mutant leaves (Figure 5.6 K-L). This suggests

Figure 5.6 *CUC2* function is required for *BP* misexpression in *A. thaliana* leaves. (A-L)

Fourth rosette leaves stained for *BP::GUS* expression in Col-0 (A), *cuc2-3* (B), *axr1-3* (C), *mir164a-4* (D), *asl-1* (E), *axr1-3;cuc2-3* (F), *cuc2-3;mir164a-4* (G), *asl-1;cuc2-3* (H), *asl-1;axr1-3* (I), *asl-1;mir164a-4* (J), *asl-1;axr1-3;cuc2-3* (K) and *asl-1;cuc2-3;mir164a-4* (L). Arrowheads point to ectopic *BP::GUS* expression in sinus regions. Scale bars: 1 cm A-L.

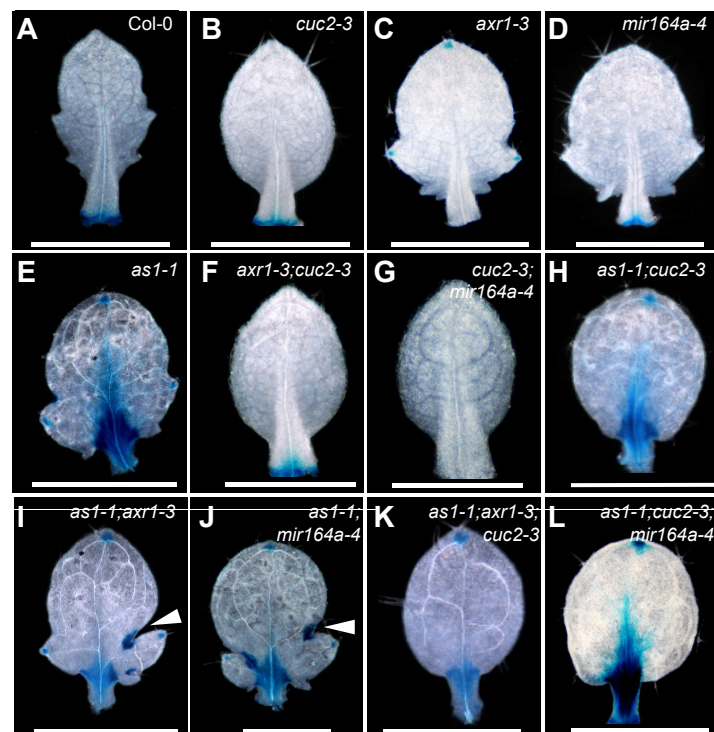
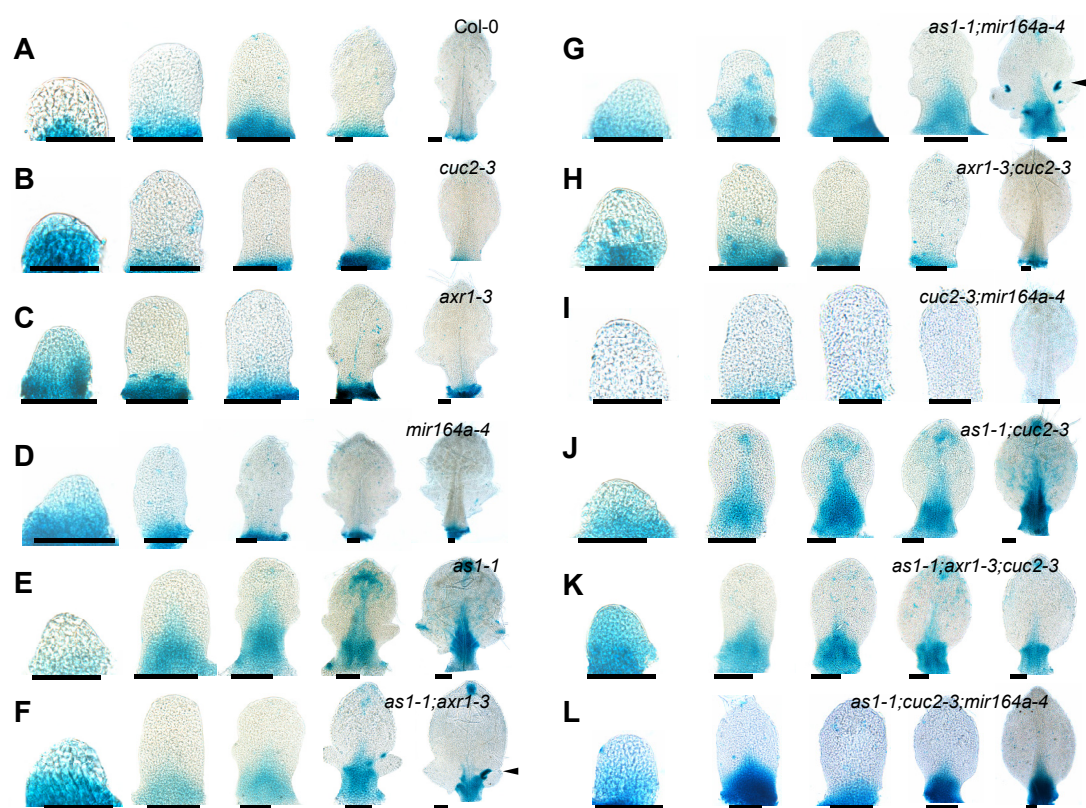


Figure 5.7 *CUC2* is required for *BP* misexpression during early leaf development. (A-K)

Ninth, eighth, seventh, sixth and fifth rosette leaf, respectively, stained for *BP::GUS* in Col-0 (A), *cuc2-3* (B), *axr1-3* (C), *mir164a-4* (D), *asl-1* (E), *asl-1;axr1-3* (F), *asl-1;mir164a-4* (G), *axr1-3;cuc2-3* (H), *cuc2-3;mir164a-4* (I), *asl-1;cuc2-3* (J), *asl-1;axr1-3;cuc2-3* (K) and *asl-1;cuc2-3;mir164a-4* (L). Arrowheads point to *BP::GUS* expression in sinus regions. Scale bars: 100µm A-L.



that *CUC2* is required for *BP* misexpression in late leaf development in the mutant backgrounds. Interestingly, *BP::GUS* is expressed in the petioles of *asl;axr1;cuc2* and *asl;cuc2;mir164a* leaves, suggesting that *CUC2* is not required to promote *BP* misexpression in the petioles of the mutant backgrounds.

To determine whether *CUC2* is required for *BP* misexpression during early stages of *asl;axr1* and *asl;mir164a* leaf development, I examined *BP::GUS* expression patterns. *BP::GUS* is not expressed at the margins of *asl;axr1;cuc2* or *asl;cuc2;mir164a* triple mutant leaves during leaf development (Figure 5.7 A-L), confirming that *CUC2* is required for *BP* misexpression throughout leaf development.

5.2.5 Polar auxin transport is required for *BP* misexpression at the margins of *A. thaliana* leaves

Polar auxin transport is required for leaflet development in *C. hirsuta* leaves (Barkoulas *et al.*, 2008) and for serration development in *A. thaliana* leaves (Hay *et al.*, 2006). To address whether polar auxin transport is required for lobe development in *KNOX*-misexpressing leaves, I treated *asl;axr1* and *asl;mir164a* double mutants with NPA, a chemical that inhibits polar auxin transport. Control DMSO-treated *axr1*, *mir164a*, and *asl* single mutants, and *asl;axr1* and *asl;mir164a* double mutants produce more deeply lobed leaves compared to wild type (Figure 5.8 A-F). In contrast, all NPA-treated genotypes produce leaves with smooth margins (Figure 5.8 G-L). This suggests that polar auxin transport is required for lobe development associated with ectopic *KNOX* expression in *A. thaliana* leaves.

In Chapter 3, I showed that reducing polar auxin transport causes the accumulation of auxin activity at leaf margins (Figure 3.9 A-H). Since auxin represses *KNOX* activity in the compound leaves of *C. hirsuta* (Barkoulas *et al.*, 2008) and in the *A. thaliana* SAM (Heisler

Figure 5.8 Polar auxin transport is required for lobe formation in mutants with elevated levels of *CUC2* and *BP*. Fifth rosette leaf and half silhouette of fifth rosette leaf of control DMSO-treated plants of Col-0 (A, A'), *axr1-3* (B, B'), *mir164a-4* (C, C'), *asl-1* (D, D'), *asl-1;axr1-3* (E, E'), *asl-1;mir164a-4* (F, F'). Fifth rosette leaf and half silhouette of fifth rosette leaf of 1μM NPA-treated plants of Col-0 (G, G'), *axr1-3* (H, H'), *mir164a-4* (I, I'), *asl-1* (J, J'), *asl-1;axr1-3* (K, K'), *asl-1;mir164a-4* (L, L'). Scale bars: 1cm A-L; 0.5cm A'-L'.

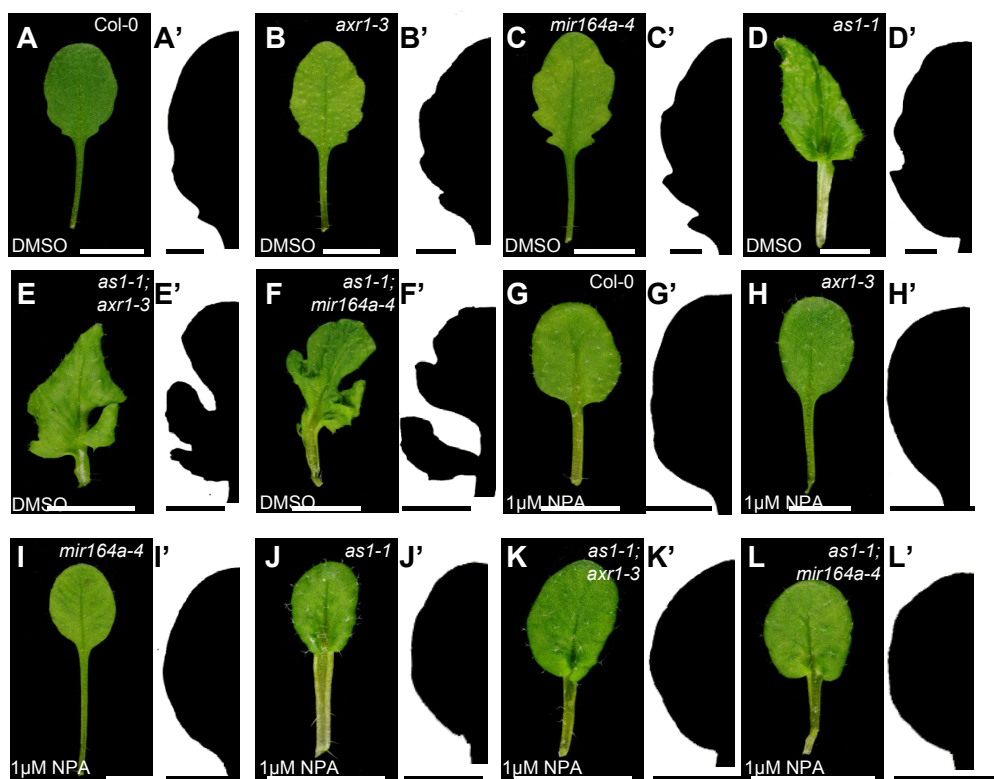
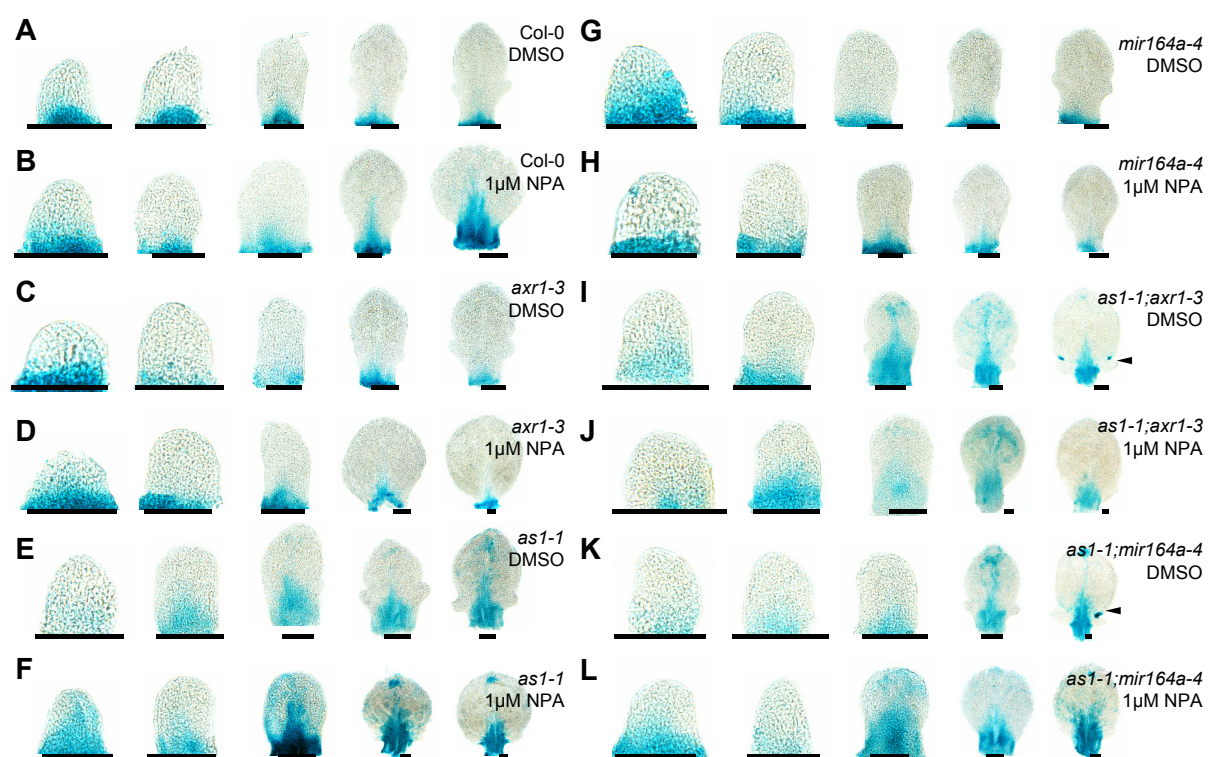


Figure 5.9 Polar auxin transport is required for *BP* misexpression at the margins of leaves. Eighth, seventh, sixth, and fifth rosette leaf, respectively, stained for *BP::GUS* in control DMSO-treated Col-0 (A), 5μM NPA-treated Col-0 (B), control DMSO-treated *axr1-3* (C), 5μM-NPA-treated *axr1-3* (D), control DMSO-treated *as1-1* (E), 5μM NPA-treated *as1-1* (F), control DMSO-treated *mir164a-4* (G), 5μM NPA-treated *mir164a-4* (H), control DMSO-treated *as1-1;axr1-3* (I), 5μM NPA-treated *as1-1;axr1-3* (J), control DMSO-treated *as1-1;mir164a-4* (K) and 5μM NPA-treated *as1-1;mir164a-4* (L). Arrowheads point to ectopic *BP::GUS* expression in sinus regions. Scale bars: 100μm A-L.



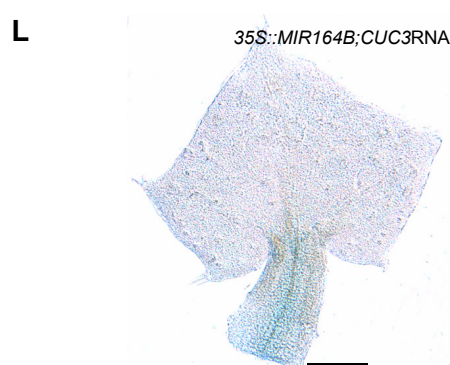
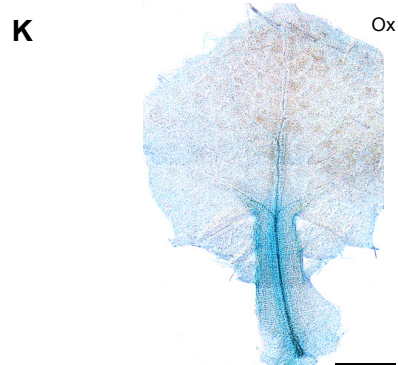
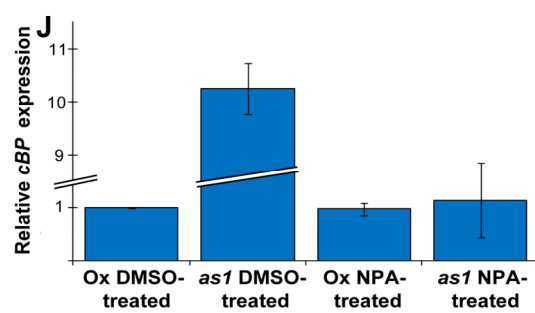
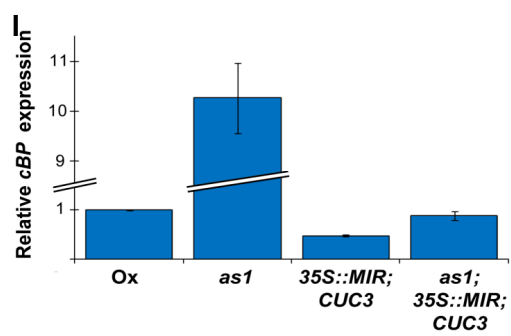
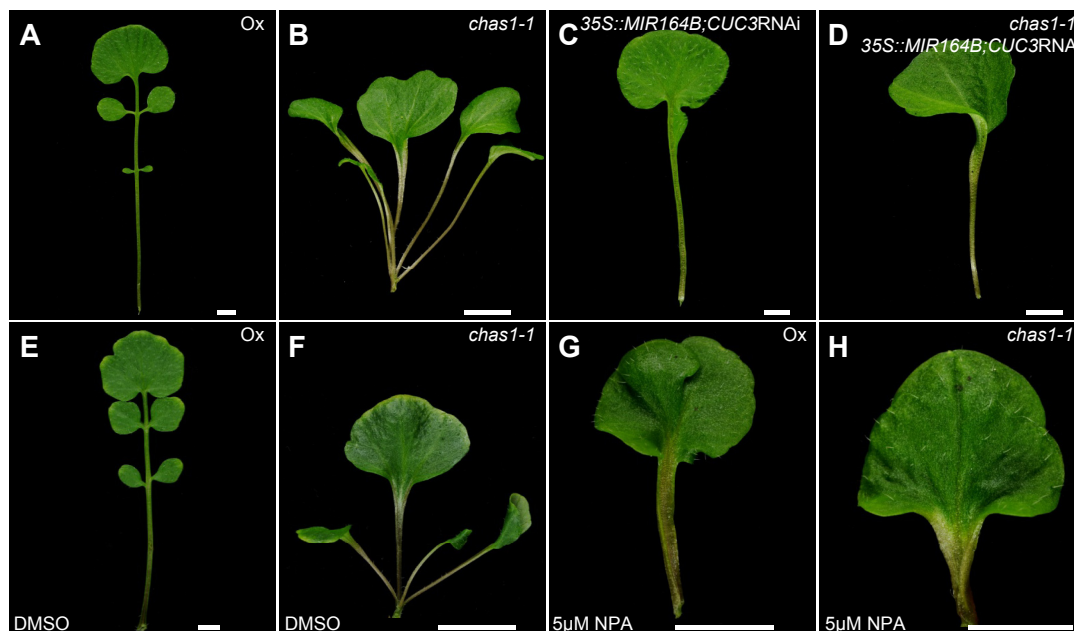
et al., 2005), I hypothesised that reducing polar auxin transport inhibits lobe development by repressing *BP* expression. To investigate this, I analysed *BP::GUS* expression in *asl;axr1* and *asl;mir164a* double mutant leaves in response to reducing polar auxin transport. NPA treatment prevents *BP::GUS* expression at the margins of *asl;axr1* and *asl;mir164a* double mutant leaves (Figure 5.9 A-L). This suggests that polar auxin transport is required for *BP::GUS* expression in the sinuses of *A. thaliana* leaves. This is most likely due to the accumulation of auxin activity at leaf margins, which may repress *KNOX* gene expression, consistent with previous reports.

5.2.6 *ChCUC* genes and polar auxin transport are required for leaflet development and *ChBP* misexpression in *chas1* leaves

In *C. hirsuta*, *chas1* mutants have increased levels of *ChBP* and produce leaves with higher-order leaflets and a compressed proximo-distal axis compared to wild type (Hay and Tsiantis, 2006). Since I showed that *CUC2* is required for the deeply lobed phenotypes of *A. thaliana* leaves that ectopically express *BP* (Figure 5.8), I hypothesised that *ChCUC* genes may be required for leaflet development in *chas1* leaves. To investigate this, I reduced *ChCUC* activity in *chas1* plants and the resulting leaf morphologies were examined. *chas1;35S::MIR164B;CUC3RNAi* produce simplified leaves similar to *35S::MIR164B;CUC3RNAi* leaves (Figure 5.10 A-D), suggesting that *ChCUC* activity is required for leaflet development in *C. hirsuta* mutants with increased *ChBP* expression.

Since I showed that polar auxin transport is required for the deeply lobed phenotypes of *A. thaliana* leaves that ectopically express *BP*, I hypothesised that polar auxin transport may be required for leaflet development in *chas1* leaves. To investigate this, I grew *chas1* plants on 1µM NPA. Whereas control DMSO-treated *chas1* plants produce leaflets along a compressed proximo-distal axis compared to wild type (Figure 5.10 E-F), NPA-treated wild-type and *chas1* plants produce simplified leaves (Figure 5.10 G-H). This suggests that polar

Figure 5.10 *CUC* function and polar auxin transport are required for leaflet formation and elevated levels of *ChBP* in *chas1* leaves. **(A-D)** Fifth rosette leaf of Ox (A), *chas1-1* (B), *35S::MIR164B;CUC3RNAi* (C) and *chas1-1;35S::MIR164B;CUC3RNAi* (D). **(E-H)** Fifth rosette leaf of control DMSO-treated Ox (E) and *chas1-1* (F), and fifth rosette leaf of 5μM NPA-treated Ox (G) and *chas1* (H). **(I)** Quantification of levels of *ChBP* transcript in Ox, *chas1-1* (*as1*), *35S::MIR164B;CUC3RNAi* (*35S::MIR;CUC3*) and *chas1-1;35S::MIR164B;CUC3RNAi* (*as1;35S::MIR;CUC3*); average derived from the mean of three biological replicates; error bars represent standard error of the mean. **(J)** Quantification of levels of *ChBP* transcript in control DMSO-treated Ox leaves (Ox DMSO-treated), control DMSO-treated *chas1-1* leaves (*as1* DMSO-treated), 5μM NPA-treated Ox leaves (Ox NPA-treated) and 5μM NPA-treated *chas1-1* leaves (*as1* NPA-treated); average derived from the mean of three biological replicates; error bars represent standard error of the mean. **(K-L)** Fifth rosette leaf stained for *ChBP>>GUS* activity in Ox (K) and *35S::MIR164B;CUC3RNAi* (L). Scale bars: 1cm A-H; 500μm K-L.



auxin transport is required for the production of leaflets associated with elevated *ChBP* levels.

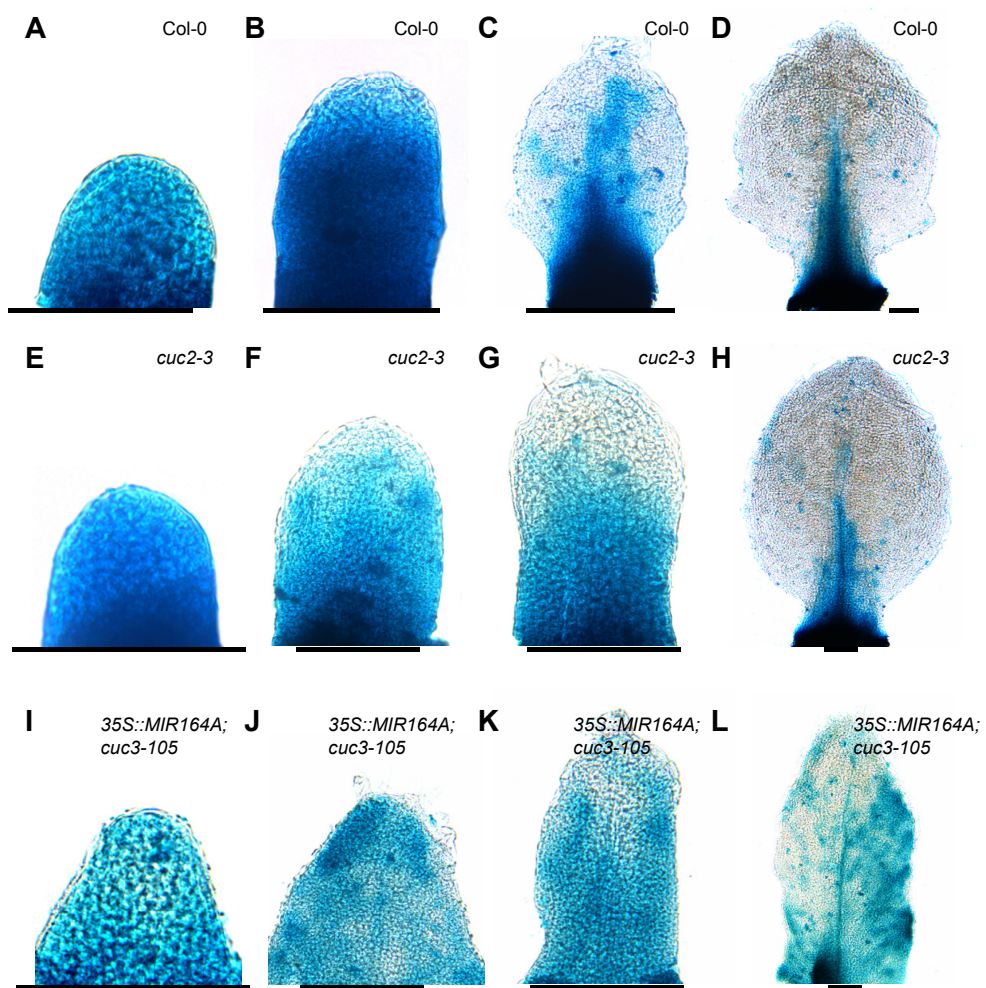
Since *ChCUC* genes and polar auxin transport are required for leaflet development in *chas1*, I next hypothesised that they may promote *ChBP* expression. To test this, I performed quantitative RT-PCR which demonstrated that levels of *ChBP* transcript are significantly lower in *chas1;35S::MIR164B;CUC3RNAi* compared to *chas1* (Figure 5.10 I). This suggests that *ChCUC* gene activity is required for elevated levels of *ChBP* in *chas1*. Furthermore, levels of *ChBP* transcripts are significantly lower in *35S::MIR164B;CUC3RNAi* compared to wild type (Figure 5.10 I), and *ChBP::GUS* expression is reduced in the rachis of *35S::MIR164B;CUC3RNAi* leaves compared to wild-type leaves (Figure 5.10 K-L). This suggests that *ChCUC* genes may be required for *ChBP* expression in wild-type leaves. Quantitative RT-PCR also shows that levels of *ChBP* are significantly lower in NPA-treated *chas1* compared to control DMSO-treated *chas1* (Figure 5.10 J), suggesting that polar auxin transport is required for increased levels of *ChBP* in *chas1* leaves. However, levels of *ChBP* transcripts are not affected in NPA-treated wild-type leaves compared to control DMSO-treated wild-type leaves (Figure 5.10 J), suggesting that polar auxin transport is not required for *ChBP* expression in wild-type leaves.

5.2.7 *CUC* genes do not promote *ChBP* transcription in *A. thaliana* leaves

A *cis*-regulatory change has been shown to promote *ChBP* expression in *C. hirsuta* leaves (Hay and Tsiantis, 2006). In this chapter, I showed that *CUC2* is required for ectopic misexpression of *BP* in *A. thaliana* leaves and *ChCUC* genes were shown to promote *ChBP* expression in *C. hirsuta* leaves. I next investigated whether *CUC* genes are required for *ChBP* transcription in *A. thaliana* leaves. In early wild-type leaf development, *ChBP::GUS* is expressed throughout the leaf (Figure 5.11 A), but *ChBP::GUS* is repressed at the leaf tip once the leaf is approximately 200µm in length, coinciding with initiation of the first pair of

Figure 5.11 *CUC* genes do not regulate *ChBP* transcription in *A. thaliana* leaves. (A-D)

Fifth rosette leaf stained for *ChBP::GUS* expression in Col-0 leaves approximately 100µm in length (A), 200µm in length (B), 300µm in length (C) and 400µm in length (D). **(E-H)** Fifth rosette leaf stained for *ChBP::GUS* expression in *cuc2-3* leaves approximately 100µm in length (E), 200µm in length (F), 300µm in length (G) and 400µm in length (H). **(I-L)** Fifth rosette leaf stained for *ChBP::GUS* expression in *35S::MIR164A;cuc3-105* leaves approximately 100µm in length (I), 200µm in length (J), 300µm in length (K) and 400µm in length (L). Scale bars: 100µm A-L.



serrations (Figure 5.11 B). Once the leaf is approximately 300µm in length, *ChBP::GUS* expression is restricted to the central domain of the leaf (Figure 5.11 C), and this expression persists during later leaf development (Figure 5.11 D).

Interestingly, I observed similar patterns of *ChBP::GUS* in *cuc2* and *35S::MIR164A;cuc3* leaves. *ChBP::GUS* is expressed throughout *cuc2* and *35S::MIR164A;cuc3* leaves during early leaf development (Figure 5.11 E, I). Although *ChBP::GUS* expression is repressed at the tips of *cuc2* leaves approximately 200µm in length (Figure 5.11 F), *ChBP::GUS* expression remains throughout *35S::MIR164A;cuc3* leaves (Figure 5.11 J). Once *cuc2* leaves are approximately 300µm in length, *ChBP::GUS* expression begins to be restricted to the central part of the leaf (Figure 5.11 G), whilst *ChBP::GUS* expression in *35S::MIR164A;cuc3* leaves begins to be repressed at the tip of the leaf (Figure 5.11 K). In later stages of *cuc2* leaf development, *ChBP::GUS* expression is similar to wild type as expression is restricted to the central and basal domains of the leaf (Figure 5.11 H). In later stages of *35S::MIR164A;cuc3* leaf development, *ChBP::GUS* is mostly restricted to the central and basal domains of the leaf, but also remains at the margins (Figure 5.11 L).

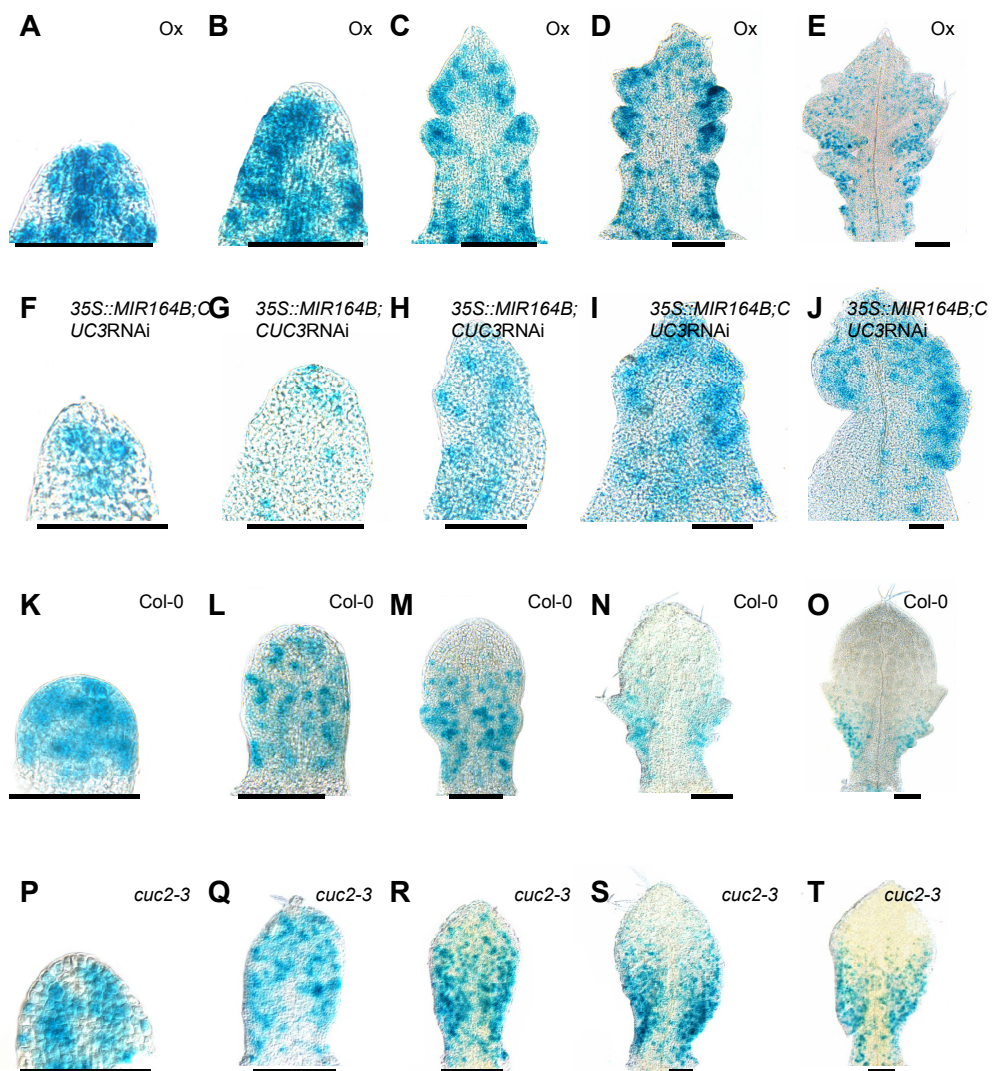
Although *ChBP::GUS* is expressed at the margins of *35S::MIR164A;cuc3* leaves and not in wild type, this is most likely due to the aberrant development of the medio-lateral axis in *35S::MIR164A;cuc3* leaves. Taken together, these observations suggest that loss-of-*CUC* function in *A. thaliana* does not lead to loss of *ChBP::GUS* expression in *A. thaliana* leaves. This is in contrast to *C. hirsuta* where loss-of-*CUC* function leads to repression of *ChBP* transcription in the rachis. This suggests that *A. thaliana* *CUC* activities may not act on the *cis*-regulatory elements of the *ChBP* promoter, unlike in *C. hirsuta*.

5.2.8 *CUC* genes regulate cell division in *C. hirsuta* leaves but not in *A. thaliana* leaves

KNOX genes have been shown to repress the transition from cell division to cell differentiation in *C. hirsuta* leaves (Hay and Tsiantis, 2006). Since my results in this chapter, and the results of other studies, suggest that *ChCUC* gene activity promotes *KNOX* expression (Blein *et al.*, 2008), I hypothesised that *ChCUC* genes may regulate leaflet development by regulating cell division. To investigate this, I analysed *CYCBI;1::GUS* expression in leaves with reduced *CUC* activity. As wild-type leaves initiate from the SAM, *CYCBI;1::GUS* is expressed throughout the leaf (Figure 5.12 A), and this expression persists as the leaf grows to approximately 150µm in length (Figure 5.12 B). Once the leaf is approximately 200µm in length, *CYCBI;1::GUS* expression is downregulated in the central domain of the leaf, but remains in the terminal and initiating lateral leaflets (Figure 5.12 C). As the leaf grows to approximately 300µm in length, *CYCBI;1::GUS* expression remains in the terminal and lateral leaflets (Figure 5.12 D). Once the leaf is approximately 500µm in length, *CYCBI;1::GUS* expression is repressed in the rachis and becomes less intense within the leaflets (Figure 5.12 E).

In contrast, low *CYCBI;1::GUS* expression is detected in initiating *35S::MIR164B;CUC3RNAi* leaves (Figure 5.12 F), which persists as the leaf grows to approximately 150µm in length (Figure 5.12 G). Once the leaf is approximately 200µm in length, *CYCBI;1::GUS* expression remains throughout the leaf (Figure 5.12 H) and only begins to be repressed in the central domain when leaves are approximately 300µm in length (Figure 5.12 I). Once leaves are approximately 500µm in length, *CYCBI;1::GUS* expression is repressed in the central domain but occurs as a continuous ring around the laminar margin (Figure 5.12 J). These data suggest that *ChCUC* genes promote cell division during early

Figure 5.12 *CUC* genes regulate cell division in *C. hirsuta* but not *A. thaliana* leaves. (A-E) Fifth rosette leaves of Ox approximately 100µm in length (A), 150µm in length (B), 200µm in length (C), 300µm in length (D) and 500µm in length (E) stained for *CYCB1;1::GUS* expression. **(F-J)** Fifth rosette leaves of *35S::MIR164B;CUC3RNAi* approximately 100µm in length (F), 150µm in length (G), 200µm in length (H), 300µm in length (I) and 500µm in length (J) stained for *CYCB1;1::GUS* expression. **(K-O)** Fifth rosette leaves of Col-0 approximately 100µm in length (K), 150µm in length (L), 200µm in length (M), 300µm in length (N) and 500µm in length (O) stained for *CYCB1;1::GUS* expression. **(P-T)** Fifth rosette leaves of *cuc2-3* approximately 100µm in length (P), 150µm in length (Q), 200µm in length (R), 300µm in length (S) and 500µm in length (T) stained for *CYCB1;1::GUS* expression. Scale bars: 100µm A-T.



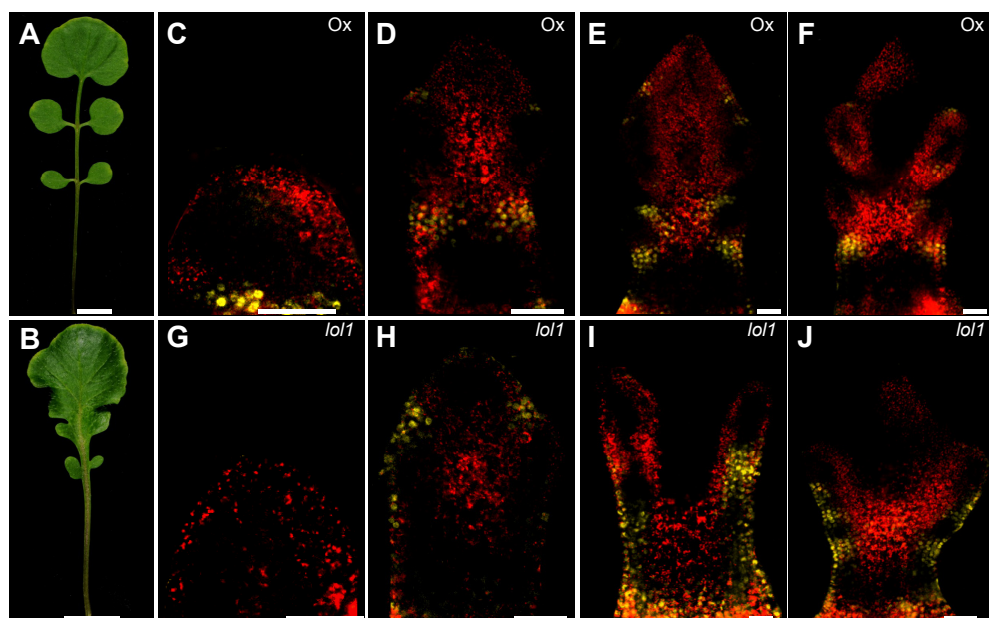
stages of *C. hirsuta* leaf development, and are required for generating foci of cell division associated with leaflet outgrowth.

To investigate whether *CUC* genes influence the distribution of cell division in the simple leaves of *A. thaliana*, I analysed *CYCB1;1::GUS* expression in *cuc2* leaves compared to wild type. As wild-type leaves initiate from the SAM, *CYCB1;1::GUS* is expressed throughout the leaf (Figure 5.12 K), and this expression pattern is maintained as the leaf grows to approximately 150µm in length (Figure 5.12 L). However, once the leaf is approximately 200µm in length, *CYCB1;1::GUS* expression is lost from the leaf tip (Figure 5.12 M), and at approximately 300µm in length, *CYCB1;1::GUS* expression is repressed in the upper half and central domain of the leaf (Figure 5.12 N). In later leaf development, *CYCB1;1::GUS* expression is restricted to the margins of the lower half of the leaf (Figure 5.12 O). *CYCB1;1::GUS* has a similar pattern of expression in *cuc2* leaves as expression is initially throughout leaves (Figure 5.12 P-Q) and becomes repressed in the leaf tip when the leaf is approximately 200µm in length (Figure 5.12 R). Expression also becomes restricted in the basipetal direction leaving expression in leaves at the margins at the base of the leaf (Figure 5.12 S-T). These data suggest that *CUC2* does not regulate cell division in the *A. thaliana* leaf despite promoting serration initiation.

5.2.9 *LOL1* regulates leaflet development and *CUC2* expression

Although interspersed peaks of *CUC2* expression are generated along the margins of both *A. thaliana* and *C. hirsuta* leaves, initial *CUC2* expression patterns vary strikingly. *CUC2* is expressed in continuous strips along the margins of initiating *A. thaliana* leaves, whereas *CUC2* expression is absent from the margins of initiating *C. hirsuta* leaves and becomes expressed in high activity maxima prior to leaflet outgrowth. Since these differences in *CUC2* expression correlate with differences in margin morphology between the two species, I hypothesised that upstream regulators of *ChCUC* gene expression may be critical

Figure 5.13 *LOL1* is required for leaflet development in *C. hirsuta* and regulates *CUC2* expression. (A-B) Fifth rosette leaf of Ox (A) and *lol1* (B). **(C-F)** Confocal micrograph images of *CUC2::CUC2:VENUS* expression (yellow) in Ox fifth rosette leaf approximately 75µm in length (C), 150µm in length (D), 250µm in length (E) and 400µm in length (F). **(G-J)** Confocal micrograph images of *CUC2::CUC2:VENUS* expression (yellow) in *lol1* fifth rosette leaf approximately 75µm in length (G), 150µm in length (H), 250µm in length (I), 400µm in length (J). Scale bars: 1cm A-B; 50µm C-J.



for leaflet development in *C. hirsuta*. *loss of leaflets1 (loli)* is a *C. hirsuta* mutant isolated from an EMS mutagenesis screen for mutants that produce leaves with lobe-like outgrowths instead of leaflets (Figure 5.13 A-B; mutagenesis screen performed by P. Sarchet). To investigate whether *CUC* expression is perturbed during *loli* leaf development, *CUC2::CUC2:VENUS* expression was observed in *loli* mutants in comparison with wild type. *CUC2::CUC2:VENUS* is expressed at the base of initiating wild-type leaves (Figure 5.13 C), and is then expressed at the junction between the terminal leaflet and the rachis of leaves approximately 150µm in length (Figure 5.13 D). *CUC2::CUC2:VENUS* is then expressed at the proximal side of the second pair of initiating leaflets when the leaf is 250µm-350µm in length (Figure 5.13 E-F).

In contrast, *CUC2::CUC2:VENUS* expression is absent from initiating *loli* leaves (Figure 5.13 G), but is expressed in maxima at the margins of the upper quarter of leaves approximately 150µm in length (Figure 5.13 H). As the *loli* leaf grows to approximately 250µm in length, *CUC2::CUC2:VENUS* is expressed in strips along the margin of the rachis (Figure 5.13 I). *CUC2::CUC2:VENUS* is then downregulated in specific foci once the leaf is approximately 500µm in length, which is also when lobes develop (Figure 5.13 J). These data suggest that *LOLI* restricts *CUC2* expression to the distal side of leaflets and prevents *CUC2* expression in strips along the margin in wild-type *C. hirsuta* leaves. This correlates with the role of *LOLI* in promoting leaflet outgrowth as opposed to lobe outgrowth in *C. hirsuta* leaves.

5.3 Discussion

5.3.1 Interlinking feedback loops between *CUC*, *KNOX* and auxin regulate lobe development in *A. thaliana* leaves ectopically expressing *KNOX* in the leaf

A fundamental question in developmental biology is to what extent the conservation and divergence of genetic mechanisms results in divergent leaf morphologies. In Chapters 3 and 4, I showed that a conserved negative feedback loop between auxin and *CUC* genes regulates serration development in *A. thaliana* and leaflet development in *C. hirsuta* (Figure 5.14 A-B, D-E). *CUC* and *PIN1* functions are required for the initiation of serration development in *A. thaliana* and leaflet development in *C. hirsuta* (Table 5.2; Figures 3.1, 4.2, Nikovics *et al.*, 2006; Blein *et al.*, 2008). Additionally, *CUC* genes promote the formation of *PIN1* convergence points in serration and leaflet development (Figures 3.6, 4.6), whilst auxin feeds back to repress *CUC2* expression (Table 5.2; Figures 3.9, 3.10, 4.8). Furthermore, interspersed peaks of *CUC2* and auxin activity likely pattern serration development in *A. thaliana* and leaflet development in *C. hirsuta* (Table 5.2; Figures 3.2, 4.3), despite early differences in the relative timing of *CUC2*, *PIN1* and auxin expression patterns (Table 5.3; Figure 3.2-3.4; Figures 4.3-4.4). These data suggest that a conserved negative feedback loop between *CUC2*, *PIN1* and auxin patterns both serration and leaflet development.

A critical species specific difference between *A. thaliana* and *C. hirsuta* is that *KNOX* genes are necessary and sufficient for leaflet development in *C. hirsuta* leaves, whereas *KNOX* genes are not expressed in *A. thaliana* leaves (Table 5.3; Hay and Tsiantis, 2006). There are, therefore, two additional feedback loops that operate in the *C. hirsuta* leaf to pattern leaflet development as *KNOX* genes form a positive and negative feedback loop with *ChCUC* genes and auxin, respectively (Table 5.2; Barkoulas *et al.*, 2008; Blein *et al.*, 2008). Interestingly, ectopic *KNOX* expression in *A. thaliana* leaves leads to the development of deeply lobed

Figure 5.14 Genetic mechanisms controlling serration, lobe and leaflet development. (A)

Cartoon of an initiating *C. hirsuta* leaflet. *CUC* (red oval) and *KNOX* (purple rectangle) genes promote the formation of an auxin activity focus (blue circle), which feeds back to repress *CUC* and *KNOX* expression. However, a positive feedback loop exists between *CUC* and *KNOX* genes. Auxin promotes cell division (yellow oval) and *KNOX* genes delay the transition from cell division to differentiation in the *C. hirsuta* leaf, whereas *CUC* genes act to delimit cell division to foci associated with leaflet outgrowth. **(B)** Cartoon of an initiating *A. thaliana* serration. *CUC2* (red oval) is expressed in the sinuses between serrations and promotes the formation of an auxin activity focus (blue circle) at the tips of serrations. Auxin feeds back to repress *CUC2* transcription. Cell division is not regulated by this feedback loop to control serration outgrowth. **(C)** Cartoon of an initiating *A. thaliana* lobe in a *KNOX*-misexpressing background. *CUC2* (red oval) and *KNOX* (purple rectangle) genes promote the formation of an auxin activity focus (blue circle), which feeds back to repress *CUC2* and *KNOX* expression. A positive feedback loop operates between *CUC2* and *KNOX* genes. Cell division (yellow oval) may have a role in lobe development. **(D-F)** Cartoon of a rosette leaf of *C. hirsuta* (D), *A. thaliana* (E) and a *KNOX*-misexpressing *A. thaliana* background (F).

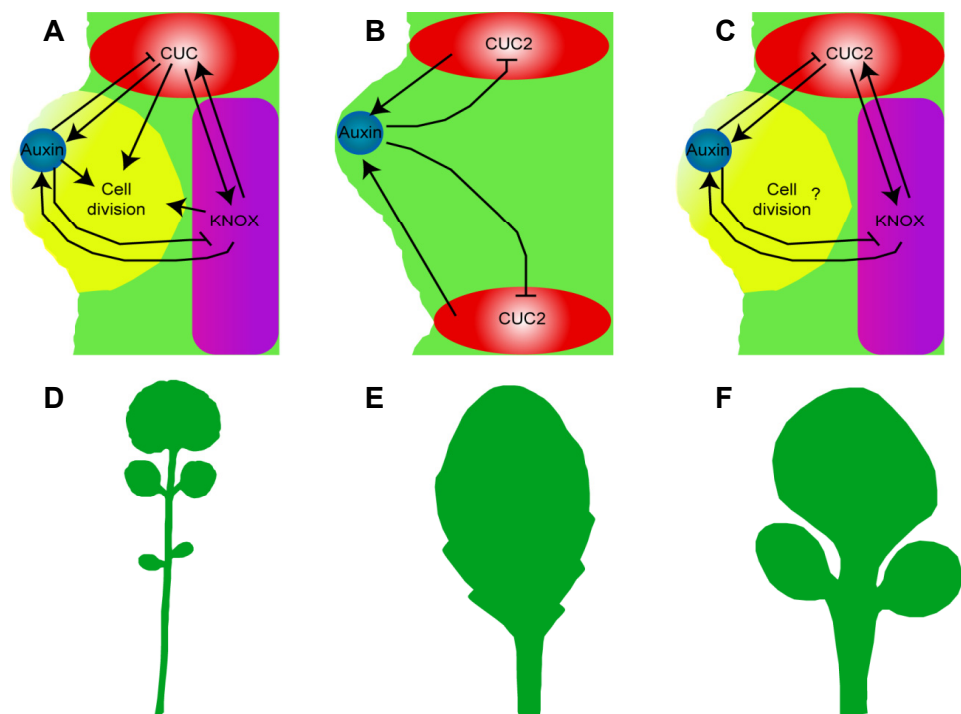


Table 5.2 Similarities between the genetic pathways regulating leaf development in *A. thaliana* and *C. hirsuta*.

<i>A. thaliana</i>	<i>C. hirsuta</i>	References
<i>CUC2</i> is required for serration initiation	<i>CUC</i> genes are required for leaflet initiation	This study; Nikovics <i>et al.</i> , 2006; Blein <i>et al.</i> , 2008
Interspersed peaks of <i>CUC2</i> and auxin activity pattern serration development	Interspersed peaks of <i>CUC2</i> and auxin activity pattern leaflet development	This study
<i>PIN1</i> is required for serration initiation	<i>PIN1</i> is required for leaflet initiation	This study; Hay <i>et al.</i> , 2006; Barkoulas <i>et al.</i> , 2008
<i>CUC2</i> promotes <i>PIN1</i> convergence point formation	<i>CUC</i> genes promote <i>PIN1</i> convergence point formation	This study
Auxin represses <i>CUC2</i> expression	Auxin represses <i>CUC2</i> expression	This study
<i>CUC2</i> is required for ectopic <i>KNOX</i> expression in leaves	<i>CUC</i> genes promote <i>KNOX</i> expression in leaves	This study; Blein <i>et al.</i> , 2008
Ectopic <i>KNOX</i> expression in leaves promotes <i>CUC2</i> expression	<i>KNOX</i> promotes <i>CUC</i> expression in leaves	This study; Blein <i>et al.</i> , 2008
Polar auxin transport is required for ectopic <i>KNOX</i> expression	Polar auxin transport is required for <i>KNOX</i> expression	This study; Barkoulas <i>et al.</i> , 2008

margin outgrowths resembling leaflets (Chuck *et al.*, 1996; Hay *et al.*, 2006). Thus, this led me to investigate whether ectopic *KNOX* expression establishes feedback loops with auxin and *CUC2* to generate deeply lobed margins reminiscent of leaflets in *A. thaliana*.

In this chapter, I showed that ectopically expressing *KNOX* genes in *A. thaliana* leaves upregulates *CUC2* expression, whereas *CUC2* is required for ectopic *KNOX* transcription (Table 5.2; Figure 5.2-5.7). This suggests that a positive feedback loop forms between *KNOX* and *CUC2* when *KNOX* genes are ectopically expressed in the *A. thaliana* leaf. Furthermore, this feedback loop is required for leaflet-like lobe development as loss-of-

CUC2 function prevents lobe development in *KNOX*-misexpressing backgrounds (Figure 5.1). I additionally showed that polar auxin transport is required for ectopic *KNOX* expression in *A. thaliana* leaves (Table 5.2; Figure 5.9). Since *KNOX* expression has also been shown to promote the formation of PIN1 convergence points (Hasson *et al.*, 2011; Hay *et al.*, 2006), this suggests that a negative feedback loop exists between *KNOX* and auxin when *KNOX* genes are ectopically expressed in *A. thaliana* leaves. I also demonstrated that reducing polar auxin transport prevents the formation of lobes in backgrounds misexpressing *KNOX* genes in the *A. thaliana* leaf (Figure 5.8), suggesting that the negative feedback loop between auxin and *KNOX* is required to pattern leaflet-like lobe development. Taken together, these data also suggest that interlinking feedback loops involving auxin, *CUC2* and *KNOX* are essential for generating more complex marginal structures such as lobes and leaflets (Figure 5.14 C, F).

Table 5.3 Species specific differences between the genetic pathways regulating leaf development in *A. thaliana* and *C. hirsuta*.

<i>A. thaliana</i>	<i>C. hirsuta</i>	References
<i>CUC2</i> does not regulate <i>CYCBI;1::GUS</i> expression in leaves	<i>CUC</i> genes are required to delimit <i>CYCBI;1::GUS</i> expression to leaflets	This study
In early leaf development, <i>CUC2</i> is expressed in continuous strips along the margin	In early leaf development, <i>CUC2</i> is absent from leaves	This study; Nikovics <i>et al.</i> , 2006; Blein <i>et al.</i> , 2008
<i>KNOX</i> genes are not expressed in leaves	<i>KNOX</i> genes are necessary and sufficient for leaflet development	Hay and Tsiantis, 2006
Ectopic <i>KNOX</i> expression in leaves prolongs duration of PIN1 convergence points	<i>KNOX</i> promotes auxin activity maxima formation in leaves	Hay <i>et al.</i> , 2006; Barkoulas <i>et al.</i> , 2008

In this chapter, I showed that *ChCUC* genes are required to delimit cell division in the *C. hirsuta* leaf to control leaflet development (Table 5.3; Figure 5.12). Previous studies showed that auxin promotes cell division and *KNOX* genes delay the transition from cell division to differentiation in the *C. hirsuta* leaf (Barkoulas *et al.*, 2008; Blein *et al.*, 2008; Hay and Tsiantis 2006). This suggests that the interlinking feedback loops between *CUC*, *KNOX* and auxin control growth by regulating cell division in *C. hirsuta* leaves. Additionally, foci of cell division correlate with sites of leaflet development, and clonal analyses have demonstrated that leaflets form from the active division of two-four cells at the rachis of the leaf (Barkoulas *et al.*, 2008; Blein *et al.*, 2008). This suggests that cell division is an important regulator of leaf morphology in *C. hirsuta*. In contrast, I showed that cell division is not regulated by *CUC2* in the *A. thaliana* leaf and, furthermore, cell divisions do not correlate with serration development in wild-type leaves (Donnelly *et al.*, 1999).

Thus, it could be hypothesised that the ectopic expression of *KNOX* genes in *A. thaliana* leaves causes the development of leaflet-like lobes due to an activation of three interlinking feedback loops that regulate the duration of cell division and marginal outgrowths in a similar manner to *C. hirsuta*. However, PIN1 convergence points form at sites of serration development along the margin of the lamina in *KNOX*-misexpressing backgrounds, and persist for longer during lobe outgrowth in *KNOX*-misexpressing backgrounds compared with wild-type serration outgrowth (Hay *et al.*, 2006; Table 5.3). This correlates with prolonged localised growth and the formation of leaflet-like lobes in *KNOX*-misexpressing backgrounds (Hay *et al.*, 2006). In contrast, PIN1 convergence points form at the margin of the rachis in *C. hirsuta* leaves, and not at the margins of lamina (Barkoulas *et al.*, 2008). These data suggest that although conserved interlinking feedback loops between *CUC2*, PIN1, auxin and *KNOX* pattern the development of lobes produced in *KNOX*-

misexpressing backgrounds in *A. thaliana* and leaflets in *C. hirsuta*, these gene regulatory networks differentially regulate growth between the two species.

5.3.2 *ChCUC* genes regulate cell division to control leaflet development

In this chapter, I showed that transgenic *C. hirsuta* plants with reduced *ChCUC* activity have reduced levels of cell division in early stages of leaf development and continuous strips of cell division surrounding the margins of the terminal leaflet in later development (Figure 5.12 A-J). This is in contrast to wild-type leaves where cell division foci are associated with leaflet development (Barkoulas *et al.*, 2008). These data suggest that *ChCUC* genes regulate cell division activity during early leaf development. PIN1 convergence points generate foci of auxin activity that also promote cell division along the *C. hirsuta* rachis (Barkoulas *et al.*, 2008). Since I showed in Chapter 4 that *ChCUC* genes promote the formation of PIN1 convergence points and auxin activity foci (Figures 4.5-4.6), it is possible that *ChCUC* genes regulate cell divisions via auxin. However, studies in *A. thaliana* have shown that a downstream target of *CUC2* is the receptor kinase *ERECTA* which regulates cell division (Larue *et al.*, 2009). Thus, whilst it is not known whether *ChCUC* genes regulate *ERECTA* to control cell division and morphology in the *C. hirsuta* leaf, this remains an interesting possibility.

Continuous strips of *CYCBI;1::GUS* expression around the margin of the terminal leaflet of transgenic plants with reduced *ChCUC* activity suggests that *ChCUC* genes are also required to delimit cell division to leaflets. Thus, *ChCUC* genes appear important in locally repressing growth. Regulation of PIN1 convergence points may also explain this role of *ChCUC* by generating auxin activity foci at the tips of leaflets in a complementary domain of expression to *ChCUC* genes. In this way, *ChCUC* genes concomitantly create an auxin minimum between leaflets, resulting in comparatively fewer cell divisions. This *ChCUC*-dependent generation of auxin minima would parallel fruit development in *A. thaliana* as

auxin minima have been shown to be critical for the patterning of the valve margin separation layer (Sorefan *et al.*, 2009).

In contrast to *C. hirsuta*, *CUC* genes do not regulate cell division in the *A. thaliana* leaf (Figure 5.12 K-T), and cell division foci are not associated with serration development (Donnelly *et al.*, 1999), implying that another aspect of growth regulates serration development. This is most likely to be cell expansion since the marginal cells of the serration tip are more elongated compared with the cells of the sinus region of wild-type leaves (Kawamura *et al.*, 2010). However, as yet, there are no known targets of *CUC2* involved in regulating cell expansion.

5.3.3 *LOL1* regulates *CUC2* expression and leaflet development

In an unbiased forward genetics approach in *C. hirsuta*, we identified *lol1* mutants as producing lobe-like marginal structures as opposed to leaflets (Figure 5.13 A-B). Correlating with this, I showed that *CUC2* expression is aberrant in *lol1* mutant leaves, forming continuous strips of expression along the margin of the rachis in *lol1* mutants and becoming downregulated at sites of lobe outgrowth (Figure 5.13 G-J). This is in contrast to wild-type leaves where *CUC2* maxima form at the distal sides of leaflets (Figure 5.13 C-F). In this way, the expression pattern of *CUC2* in *lol1* resembles the *CUC2* expression pattern in wild-type *A. thaliana* leaves (Figure 5.4 A-E; Nikovics *et al.*, 2006). This suggests that *LOL1* may promote leaflet development by preventing the expression of *CUC2* in continuous strips along the leaf margin. *LOL1*, therefore, represents a tantalising target for species-specific regulatory differences between *C. hirsuta* and *A. thaliana* that contribute to generating divergent leaf forms via controlling *CUC* expression.

The product of the *LOL1* gene locus is unknown, however, it is likely to be an upstream regulator of *CUC2* expression. *ChCUC* genes have been shown to be required for

leaflet development in *STM*-overexpressing *C. hirsuta* leaves (Blein *et al.*, 2008), suggesting that *ChCUC* genes may act downstream of *STM*. Although null alleles of *STM* lack a SAM in *C. hirsuta* (Hay and Tsiantis, 2006), *loli* could be a weak allele of *STM*. Alternatively, the SWI/SNF chromatin remodelling ATPase *AtBRM* has been shown to act upstream of *CUC2* in *A. thaliana* SAMs (Kwon *et al.*, 2006), raising the possibility that *LOLI* may encode the orthologue of *AtBRM*. Alternatively, *LOLI* may regulate the activity of *BOP* genes. In *A. thaliana*, loss-of-*BOP* function causes the ectopic lamina tissue to develop along the petiole, giving phenotypes similar to *loli* leaves.

5.4 Conclusions

- Ectopic *KNOX* expression in *A. thaliana* leaves establishes a positive feedback loop between *KNOX* and *CUC2*, and a negative feedback loop between *KNOX* and auxin, that are required for the development of leaflet-like lobes.
- *ChCUC* genes regulate cell division to control leaflet development in *C. hirsuta* leaves, whereas *CUC* genes do not regulate cell division in the control of serration development in *A. thaliana*.
- *LOLI* is a novel regulator of *CUC2* expression and leaflet development in *C. hirsuta*.

**Chapter 6: Towards identifying a
novel component of the *CUC2*
developmental pathway via a
screen for genetic suppressors of
*cuc2***

Chapter 6: Towards identifying a novel component of the *CUC2* developmental pathway via a screen for suppressors of *cuc2*

6.1 Introduction

The genetic mechanisms regulating serration development were first studied over a century ago in nettle (Correns, 1909). However, work in the past ten years on the model species *A. thaliana* has begun to uncover key regulators of serration development. The NAC domain transcription factor *CUC2* plays a pivotal role in controlling both serration initiation and depth (Nikovics *et al.*, 2006). *cuc2* mutants produce leaves with smooth margins, suggesting that *CUC2* is required for serration initiation (Nikovics *et al.*, 2006). *CUC2* transcripts are targeted for degradation by the miRNA *miR164*, and *mir164a* mutants produce leaves with deeply serrated margins, suggesting that *CUC2* promotes serration development (Mallory *et al.*, 2004; Nikovics *et al.*, 2006; Sieber *et al.*, 2007). Moreover, increased levels of *CUC2* are responsible for the development of deeply serrated margins in *mir164a* as *cuc2;mir164a* double mutants produce leaves with smooth margins (Nikovics *et al.*, 2006). These phenotypes suggest that the ratio of *CUC2:miR164* determines the depth of serrations (Nikovics *et al.*, 2006).

CUC2 has recently been shown to promote the transcription of *CUC3*, which also encodes a NAC domain transcription factor (Hasson *et al.*, 2011). *CUC3* regulates serration depth as loss-of-function *cuc3* mutants produce leaves with less deeply serrated margins compared to wild type, and *CUC3* is transcribed in sinus regions after serration outgrowths become visible (Hasson *et al.*, 2011). However, expressing *CUC3* under the *CUC2* promoter

does not rescue serration development in *cuc2* leaves, suggesting that CUC3 protein function cannot compensate for CUC2 function in serration initiation (Hasson *et al.*, 2011).

A recent study found evidence suggesting that *CUC2* regulates *ERECTA* to control serration depth (Larue *et al.*, 2009). Semi-dominant gain-of-function *cuc2-ID* mutants produce leaves with deeply serrated margins, and loss-of-*ERECTA* function suppresses this phenotype (Larue *et al.*, 2009). Since *ERECTA* regulates cell division, it was proposed that *CUC2* may regulate serration development by controlling cell division in the *A. thaliana* leaf. However, the expression patterns of cell division markers suggest that foci of cell divisions are not associated with serration development, meaning that control of cell division may not be relevant to wild-type serration development (Donnelly *et al.*, 1999). Furthermore, suppression of the *cuc2-ID* phenotype by *erecta* mutations indicates that *ERECTA* function is required for a gain-of-*CUC2* function in the leaf, not necessarily that *CUC2* regulates *ERECTA* under wild-type conditions to control serration development. Thus, validated components mediating the effects of *CUC2* in controlling serration development remain elusive.

In this chapter, to determine how *CUC2* regulates serration development, I performed an EMS-mutagenesis screen to identify novel components of the *CUC2* pathway. I identified fifteen mutants which suppressed the smooth leaf margin phenotype of *cuc2*, and further characterised *cuc2 suppressor1* (*cus1*). I show that loss-of-*CUS1* function does not enhance the serrated margins of *mir164a* leaves. This suggests that *CUS1* may act downstream of *CUC2* and *MIR164* to regulate serration development. However, I show that *CUC3* is likely to act in a separate pathway to *CUS1* to regulate serration depth in leaves. Finally, I demonstrate that loss-of-*CUS1* restores PIN1 convergence point and auxin activity maxima formation in *cuc2* leaves. This suggests that *CUS1* acts to repress PIN1 convergence points in the control of serration initiation.

6.2 Results

6.2.1 Genetic screen identifies suppressors of the smooth leaf margin phenotype of *cuc2*

To identify suppressors of the *cuc2* leaf phenotype, I took an unbiased forward genetics approach. We EMS-mutagenised *cuc2* seed and I screened M2 populations derived from pools of five M1 plants to find suppressors of the smooth leaf margin phenotype of *cuc2*. Out of one hundred and sixty nine M2 populations, I identified fifteen suppressors (Figure 6.1, Figure 6.2) and classified these into four groups based on whole-plant phenotypes (Table 6.1).

Table 6.1 Phenotypic groupings of suppressors of the smooth leaf margin phenotype of *cuc2*.

Group	Mutant	Group descriptor
I	<i>cuc2;cus1, cuc2;cus2, cuc2;cus3, cuc2;cus4</i>	Serrations occur in regular pairs and are more deeply serrated than wild type.
II	<i>cuc2;cus5, cuc2;cus6, cuc2;cus7, cuc2;cus8</i>	Serrations occur in regular pairs but are less deeply serrated than wild type, and rescue of serration development occurs only in later leaves.
III	<i>cuc2;cus9, cuc2;cus10, cuc2;cus11</i>	Serrations are not as deep as wild type and do not occur in regular pairs. Rescue of serration development occurs only in later leaves.
IV	<i>cuc2;cus12, cuc2;cus13, cuc2;cus14, cuc2;cus15</i>	Leaves are smaller than wild type and serration positioning is irregular. Plants are small in stature.

Loss-of-*CUS1* appears to strongly suppress the smooth margin phenotype of *cuc2* mutant leaves (Figure 6.1 A, Q). All F1 individuals from a cross between *cus1* and Col-0 have wild-type leaf development, indicating that *cus1* is a recessive mutation, and as such the *cus1* phenotype represents an absence of its wild-type function. In addition, *cus1* is fully fertile, allowing genetic and expression analyses to elucidate how *CUS1* functions in the control of serration development. Thus, *cus1* is an ideal candidate with which to perform

Figure 6.1 Leaf phenotypes of suppressors of *cuc2*. (A-D) Fifth rosette leaf and half silhouette of fifth rosette leaf of Group I suppressors, including *cuc2-3;cus1* (A, A'), *cuc2-3;cus2* (B, B'), *cuc2-3;cus3* (C, C'), and *cuc2-3;cus4* (D, D'). (E-H) Fifth rosette leaf and half silhouette of fifth rosette leaf of Group II suppressors, including *cuc2-3;cus5* (E, E'), *cuc2-3;cus6* (F, F'), *cuc2-3;cus7* (G, G'), and *cuc2-3;cus8* (H, H'). (I-K) Fifth rosette leaf and half silhouette of fifth rosette leaf of Group III suppressors, including *cuc2-3;cus9* (I, I'), *cuc2-3;cus10* (J, J') and *cuc2-3;cus11* (K, K'). (L-O) Fifth rosette leaf and half silhouette of fifth rosette leaf of Group IV suppressors, including *cuc2-3;cus12* (L, L'), *cuc2-3;cus13* (M, M'), *cuc2-3;cus14* (N, N'), and *cuc2-3;cus15* (O, O'). (P-Q) Fifth rosette leaf and half silhouette of fifth rosette leaf of Col-0 (P, P') and *cuc2-3* (Q, Q'). Scale bars: 1cm: A-Q; 0.5cm A'-Q'.

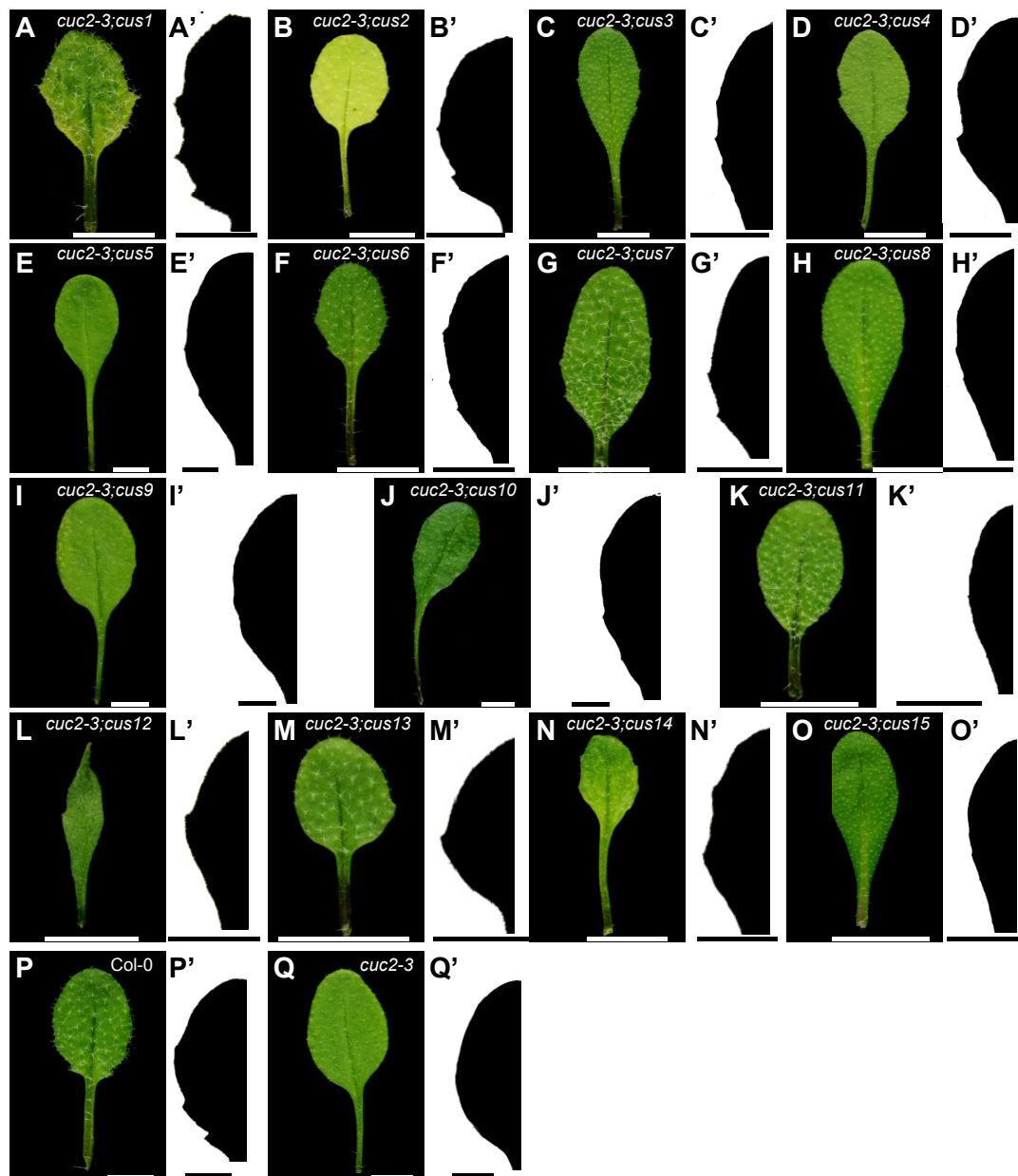
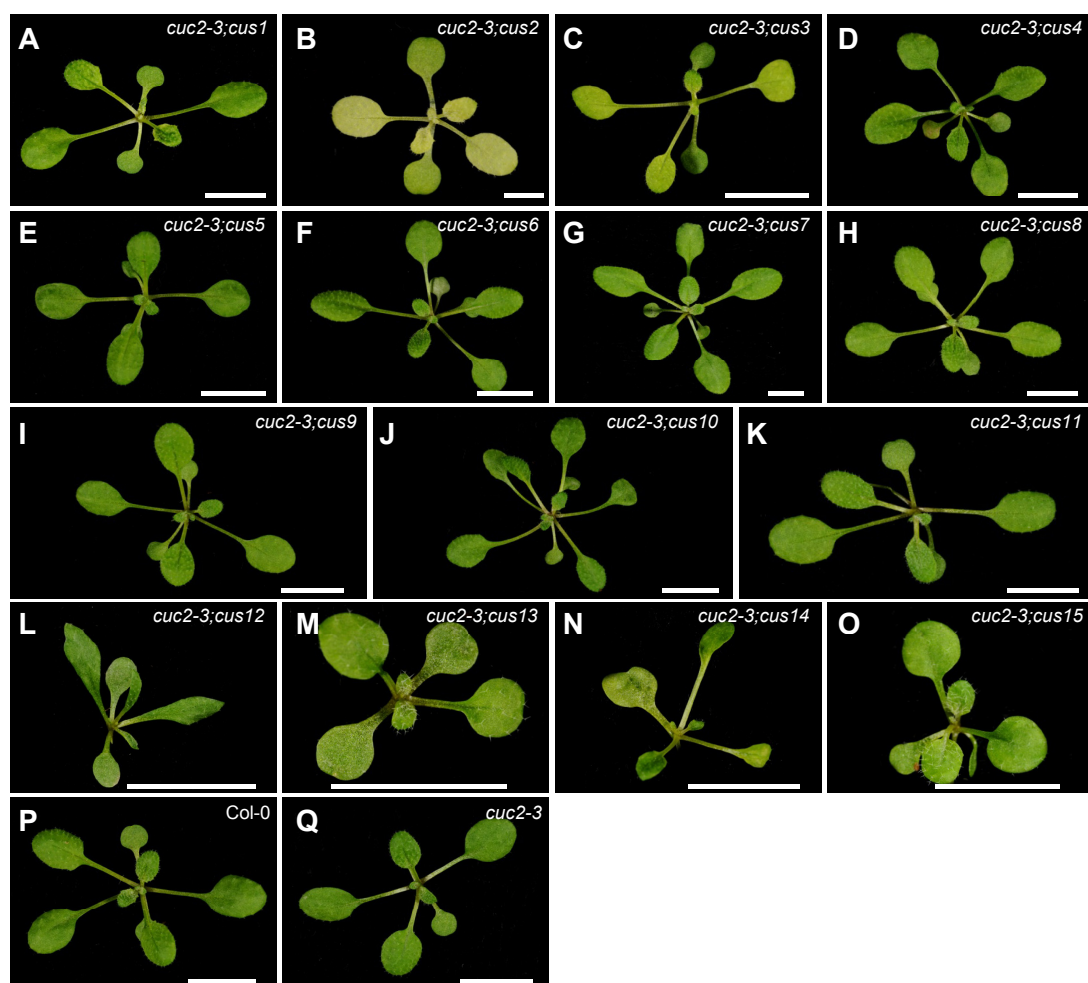


Figure 6.2 Whole plant phenotypes of *cuc2* suppressors. (A-D) Whole plant photographs of Group I suppressors, including *cuc2-3;cus1* (A), *cuc2-3;cus2* (B), *cuc2-3;cus3* (C) and *cuc2-3;cus4* (D). (E-H) Whole plant photographs of Group II suppressors, including *cuc2-3;cus5* (E), *cuc2-3;cus6* (F), *cuc2-3;cus7* (G) and *cuc2-3;cus8* (H). (I-K) Whole plant photographs of Group III suppressors, including *cuc2-3;cus9* (I), *cuc2-3;cus10* (J) and *cuc2-3;cus11* (K). (L-O) Whole plant photographs of Group IV suppressors, including *cuc2-3;cus12* (L), *cuc2-3;cus13* (M), *cuc2-3;cus14* (N) and *cuc2-3;cus15* (O). (P-Q) Whole plant photographs of Col-0 (P) and *cuc2 3* (Q). Scale bars: 1cm A-Q.



genetic and molecular analyses to determine how *CUC2* regulates serration development. For these reasons, I used *cus1* mutants for all subsequent genetic, molecular and map-based cloning investigations in this chapter.

6.2.2 *CUS1* likely acts downstream of *CUC2* to control serration development

Although *cus1* suppresses the smooth leaf margin phenotype of *cuc2* mutants, there are two possible genetic interactions between *cus1* and *cuc2* which may account for suppression of the *cuc2* leaf phenotype by *cus1*. Firstly, *cus1* could be epistatic to *cuc2*, suggesting that *CUC2* and *CUS1* act in a linear genetic pathway with *CUS1* downstream of *CUC2*. Secondly, *cus1* could act additively to *cuc2* whereby *CUS1* functions in a separate pathway from *CUC2* to inhibit serration development. To determine whether *cus1* is likely to be epistatic or additive to *cuc2*, I quantified serration number and leaf dissection. *cuc2;cus1* leaves produce significantly higher numbers of serrations compared to *cuc2* leaves (Figure 6.3 A-E; *t*-test $p < 0.001$), but there is no significant difference in the number of serrations produced between *cus1* and *cuc2;cus1* leaves (Figure 6.3 A-E). In addition, *cuc2;cus1* leaves have a significantly higher leaf dissection index compared to *cuc2* leaves (Figure 6.3 A-D, F; *t*-test $p < 0.05$), whereas there is no significant difference between the leaf dissection indices of *cuc2;cus1* and *cus1* leaves (Figure 6.3 A-D, F). These quantitative analyses suggest that *cus1* is likely epistatic to *cuc2*; however, as it is not known whether *cus1* is a null mutation, epistasis cannot be directly assumed.

6.2.3 Loss-of-*CUS1* function restores serration initiation in *cuc2* leaves

Although *CUS1* appears to act in the same genetic pathway as *CUC2*, it is not clear whether loss-of-*CUS1* function restores serration initiation to the same periodicity as wild type. To investigate this, I characterised *cuc2;cus1* leaf development using SEM. Wild-type leaves initiate as rounded structures with smooth margins (Figure 6.4 A), and grow primarily along the proximo-distal axis when leaves are approximately 150µm in length (Figure 6.4 B).

Figure 6.3 *CUS1* is likely acts downstream of *CUC2* to control serration development.

(A-D) Heteroblastic leaf series from first rosette leaf to eighth rosette leaf of Col-0 (A), *cuc2-3* (B), *cus1* (C) and *cuc2-3;cus1* (D). **(E)** Quantification of average number of serration in Col-0, *cuc2-3*, *cus1* and *cuc2-3;cus1*; error bars represent standard error of the mean, where n=15. **(F)** Quantification of leaf dissection index of Col-0, *cuc2-3*, *cus1* and *cuc2-3;cus1*; error bars represent standard error of the mean, where n=15. Scale bars: 1cm A-D.

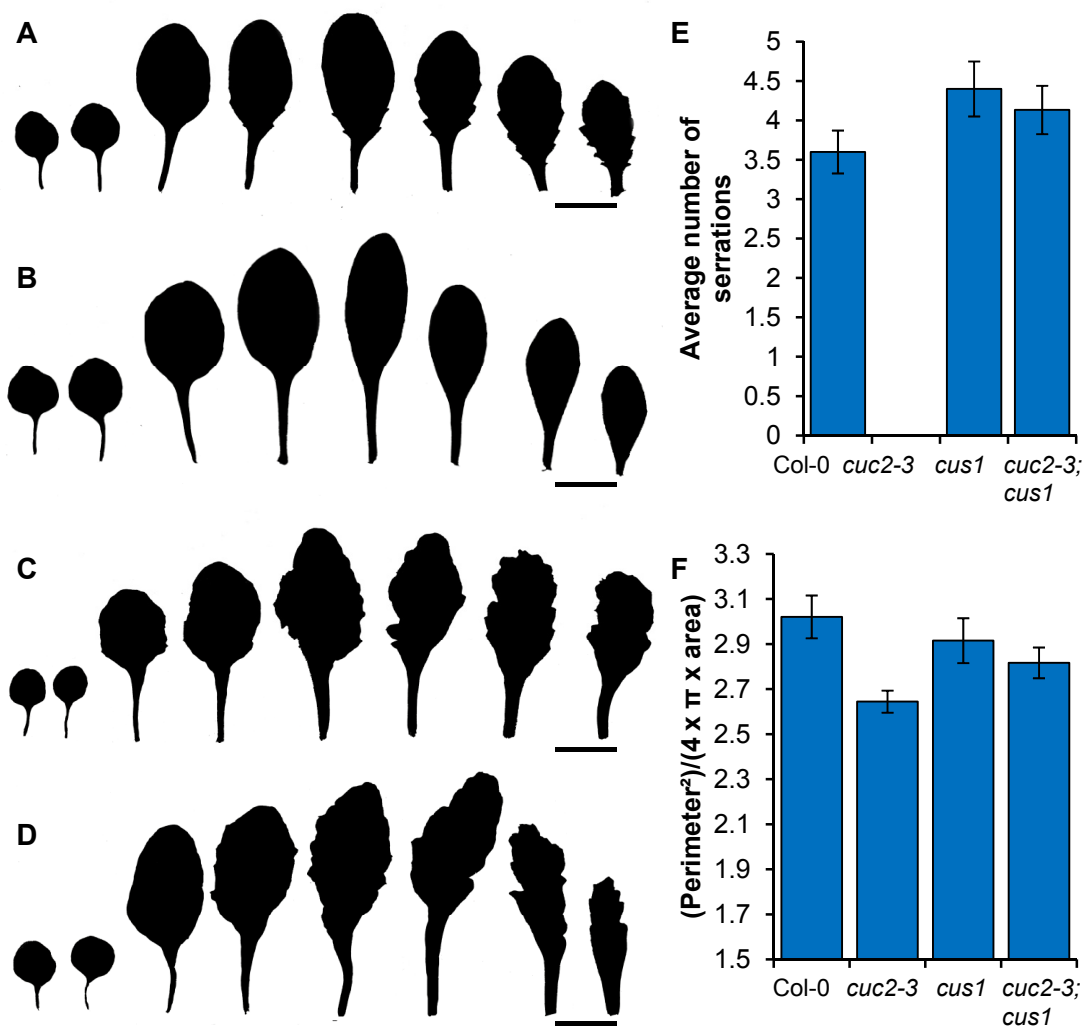
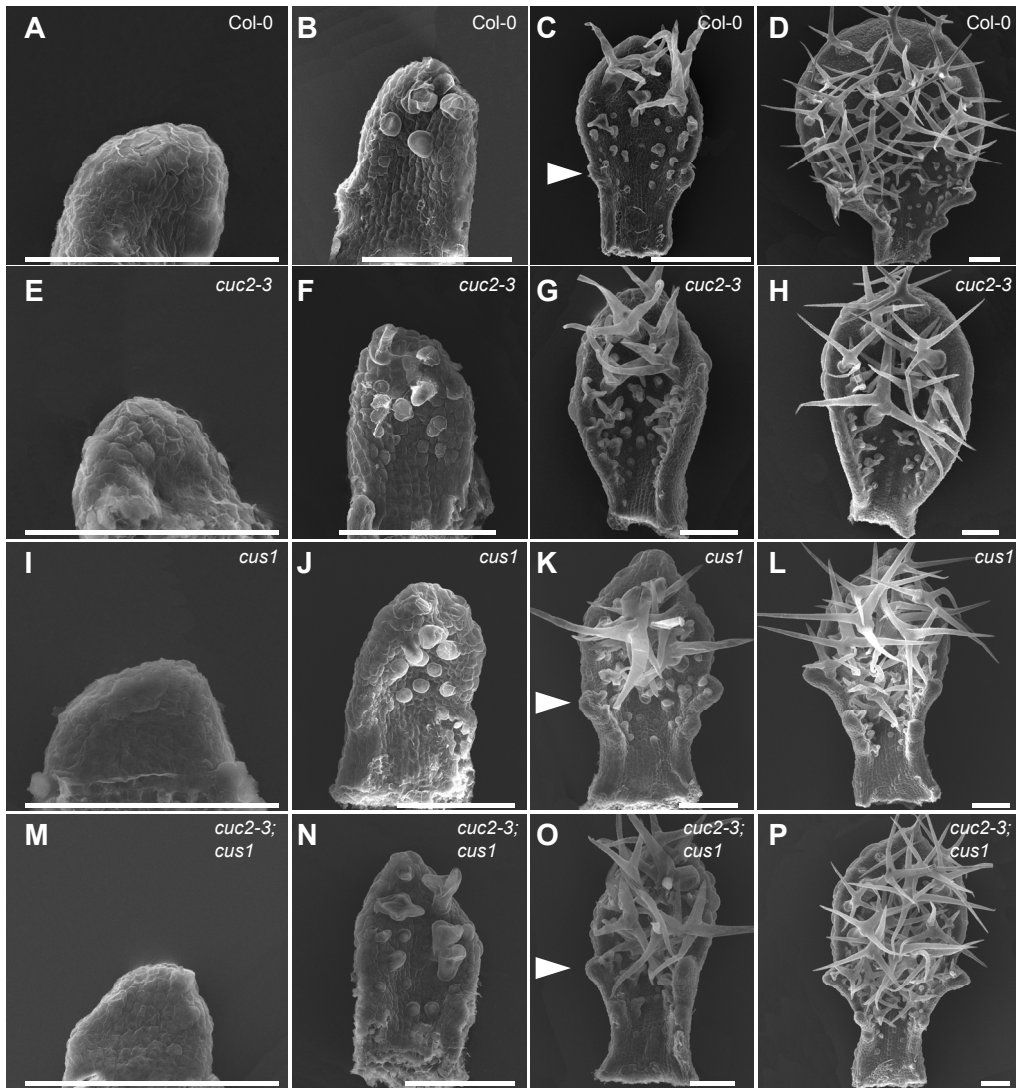


Figure 6.4 Loss-of-*cus1* function restores serration initiation in *cuc2* leaves. (A-D) Scanning electron micrograph of Col-0 fifth rosette leaf approximately 100µm in length (A), 150µm in length (B), 250µm in length (C) and 500µm in length (D). **(E-H)** Scanning electron micrograph of *cuc2-3* fifth rosette leaf approximately 100µm in length (E), 150µm in length (F), 250µm in length (G) and 500µm in length (H). **(I-L)** Scanning electron micrograph of *cus1* fifth rosette leaf approximately 100µm in length (I), 150µm in length (J), 250µm in length (K) and 500µm in length (L). **(M-P)** Scanning electron micrograph of *cuc2-3;cus1* fifth rosette leaf approximately 100µm in length (M), 150µm in length (N), 250µm in length (O) and 500µm in length (P). Scale bars: 100µm A-P.



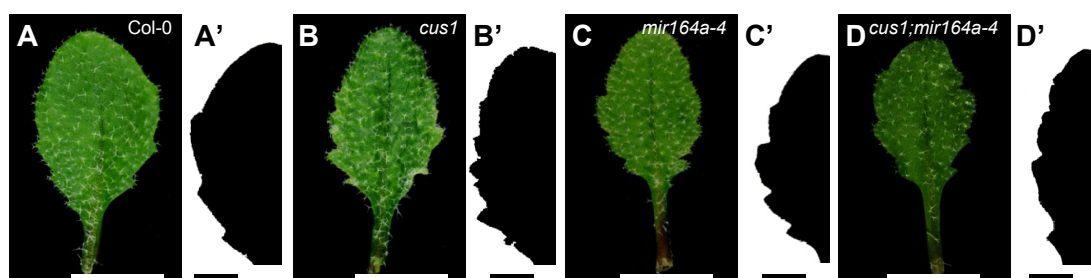
Once the leaf is approximately 250µm in length, the first pair of serrations becomes visible (Figure 6.4 C), and the second pair of serrations becomes visible when the leaf is approximately 500µm in length (Figure 6.4 D). In contrast, *cuc2* leaves do not produce serrations during early or late stages of leaf development (Figure 6.4 E-H). However, both *cus1* and *cuc2;cus1* leaves initiate as rounded structures (Figure 6.4 I-J, M-N), and produce their first pair of serrations when the leaf is approximately 250µm in length (Figure 6.4 K, O). The second pair of serrations also form when *cus1* and *cuc2;cus1* leaves are approximately 500µm in length (Figure 6.4 L, P). These data, therefore, suggest that serration initiation in *cus1* and *cuc2;cus1* leaves occurs at the same developmental time and with the same periodicity as in wild-type leaves.

6.2.4 Loss-of-*CUS1* function does not enhance serration development in *mir164a* leaves

In Chapter 3, I showed that auxin represses the transcription of *CUC2* (Figure 3.10), and *CUC2* has also been shown to promote its own transcription in an auto-feedback loop (Hasson *et al.*, 2011). *CUC2* is also regulated at the post-transcriptional level as *CUC2* transcripts contain a miRNA binding site that *miR164* binds to, causing the degradation of the *CUC2* transcript (Mallory *et al.*, 2004; Nikovics *et al.*, 2006; Sieber *et al.*, 2007). To investigate whether *miR164*-dependent regulation of *CUC2* controls *CUS1* function, I analysed the leaf margin phenotypes of *cus1;mir164a*. Both *cus1* and *mir164a* single mutants produce more deeply serrated leaf margins than wild type (Figure 6.5 A-C), however, *cus1;mir164a* leaves are not more deeply serrated than either single parent (Figure 6.5 B-D). This suggests that *MIR164A* and *CUS1* likely act in the same genetic pathway to control serration development.

Figure 6.5 *CUS1* likely acts downstream of *MIR164A* to control serration development.

(A-D) Fifth rosette leaf and half silhouette of fifth rosette leaf of Col-0 (A, A'), *cus1* (B, B'), *mir164a-4* (C, C'), *cus1;mir164a-4* (D, D'). Scale bars: 1cm A-D; 0.5cm A'-D'.



6.2.5 *CUC2* and *CUC3* act in a separate pathway to *CUS1* to regulate serration development

It was recently shown that *CUC3* regulates serration development as *cuc3* leaves are less serrated compared to wild type, and *CUC3* transcription is promoted by *CUC2* (Hasson *et al.*, 2011). To investigate whether *CUC3* acts in the same pathway as *CUS1* to regulate serration development, I analysed genetic interactions between *CUC2*, *CUC3* and *CUS1*. Although *cuc3* leaves are slightly less deeply serrated compared to wild type (Figure 6.6 A-E), *cuc3;cus1* leaves are as deeply serrated as *cus1* single mutants (Figure 6.6 B, F). These phenotypes suggest that *CUC3* is not required for serration development in *cus1* leaves. However, *cuc2;cuc3;cus1* triple mutants produce leaves with less deeply serrated margins compared to *cus1* single mutants and more deeply serrated margins compared to *cuc2;cuc3* double mutants (Figure 6.6 A-H). These phenotypes suggest that *CUC2* and *CUC3* together act independently of *CUS1* to regulate serration development.

6.2.6 Polar auxin transport is required for serration development in *cus1*

In Chapter 5, I showed that reducing polar auxin transport represses lobe development in *as1;axr1* and *as1;mir164a* double mutants (Figure 5.8). To investigate whether polar auxin transport is also required for serration development in *cus1* leaves, I treated *cus1* plants with NPA. Whereas wild-type plants grown under control conditions produce leaves with serrated margins, wild-type plants grown on 0.1 μ M and 0.5 μ M NPA produce leaves with smooth margins (Figure 6.7 A-C). However, *cus1* plants grown on 0.1 μ M and 0.5 μ M NPA also produce leaves with smooth margins, contrasting with the deeply serrated margins of *cus1* plants grown in control conditions (Figure 6.7 D-F). This suggests that polar auxin transport is required for serration development in *cus1* leaves.

To further investigate whether PIN1-mediated auxin transport is required for serration development in *cus1* leaves, I analysed the leaf margin phenotypes of *cus1;pin1* double

Figure 6.6 *CUC2* and *CUC3* likely act in separate pathways to *CUS1*. (A-J) Fifth rosette leaf and half silhouette of fifth rosette leaf of Col-0 (A, A'), *cus1* (B, B'), *cuc2-3* (C, C'), *cuc2-3;cus1* (D, D'), *cuc3-105* (E, E'), *cuc3-105;cus1* (F, F'), *cuc2-3;cuc3-105* (G, G') and *cuc2-3;cuc3-105;cus1* (H, H'). Scale bars: 1cm A-H; 0.5cm A'-H'.

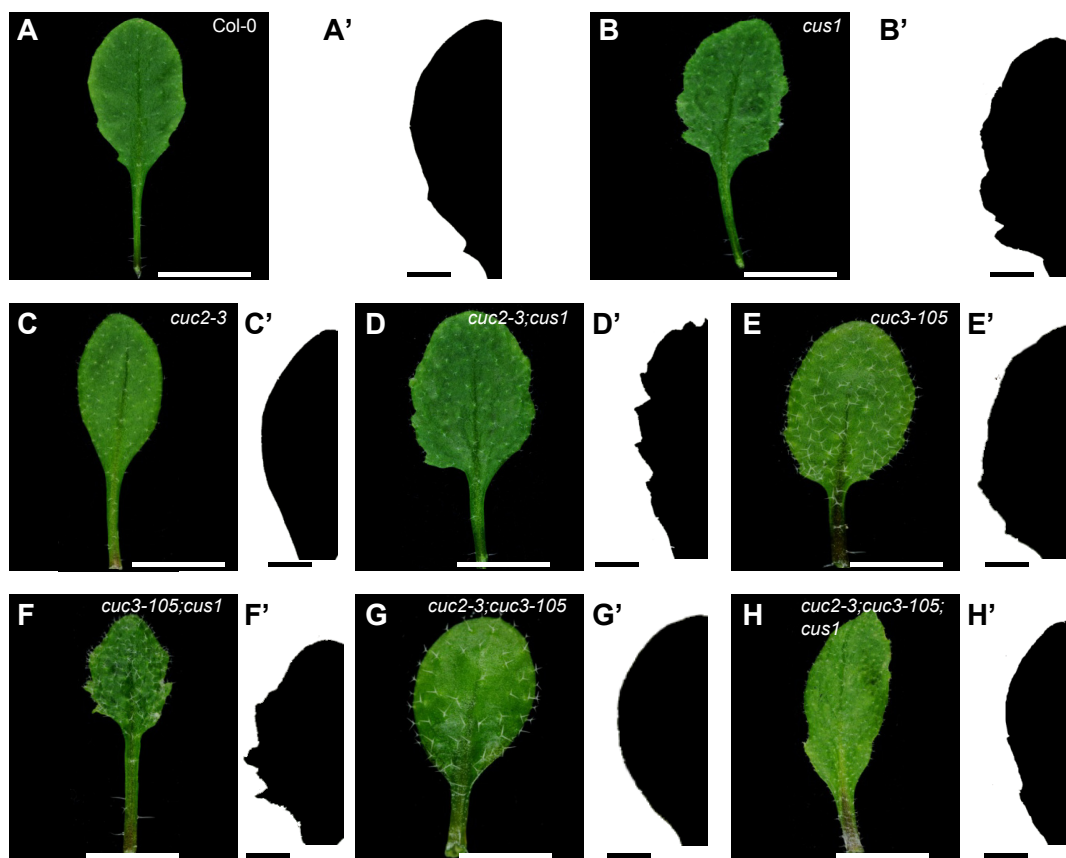
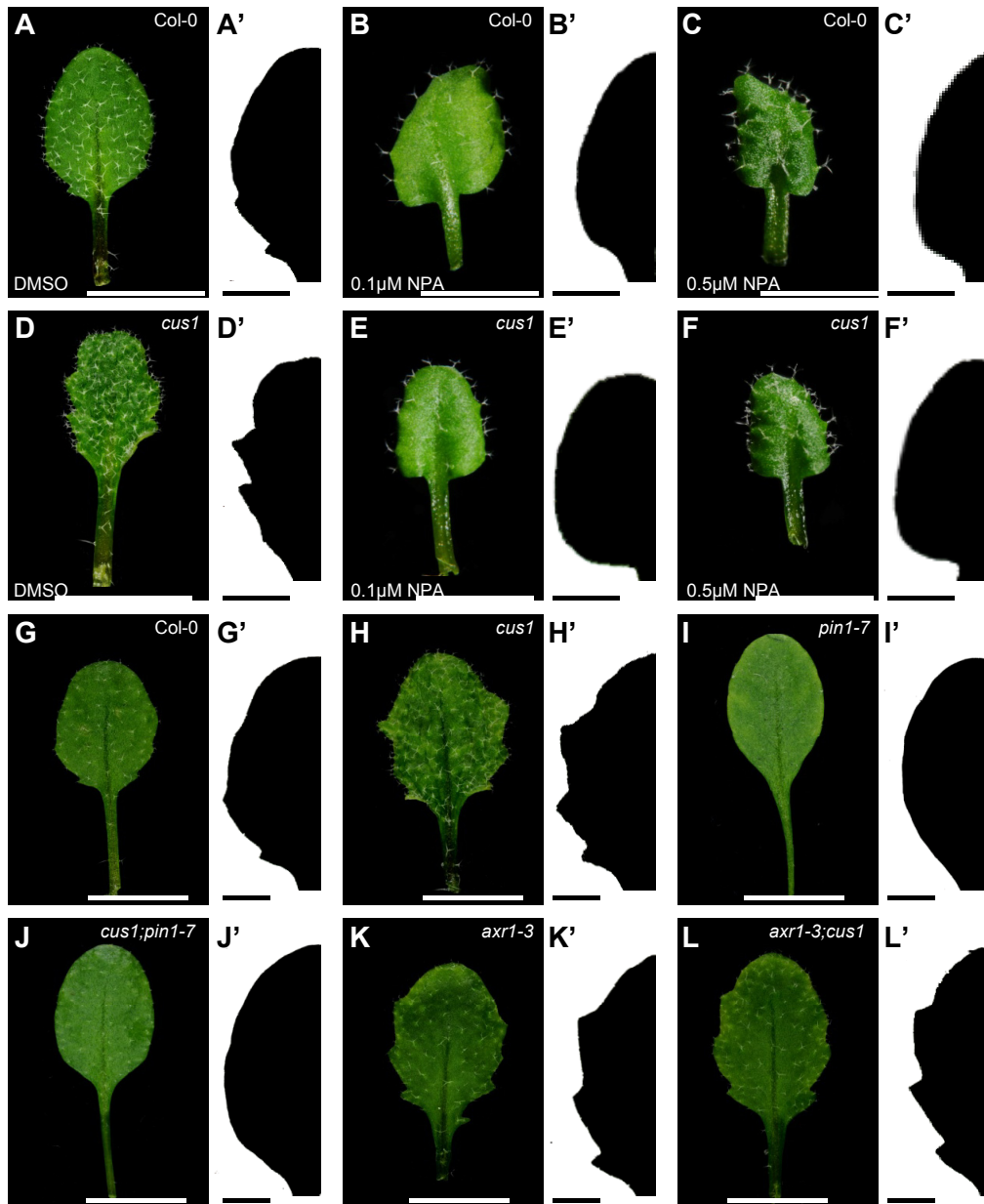


Figure 6.7 *CUS1* regulates auxin transport to control serration development. (A-C) Fifth rosette leaf and half silhouette of fifth rosette leaf of control DMSO-treated Col-0 plants (A, A'), 0.1µM NPA-treated Col-0 plants (B, B') and 0.5µM NPA-treated Col-0 plants (C, C'). (D-F) Fifth rosette leaf and half silhouette of fifth rosette leaf of control DMSO-treated *cus1* plants (D, D'), 0.1µM NPA-treated plants (E, E') and 0.5µM NPA-treated plants (F, F'). (G-L) Fifth rosette leaf and half silhouette of fifth rosette leaf of Col-0 (G, G'), *cus1* (H, H'), *pin1-7* (I, I'), *cus1;pin1-7* (J, J'), *axr1-3* (K, K'), *axr1-3;cus1* (L, L'). Scale bars: 1cm A-L; 0.5cm A'-L'.

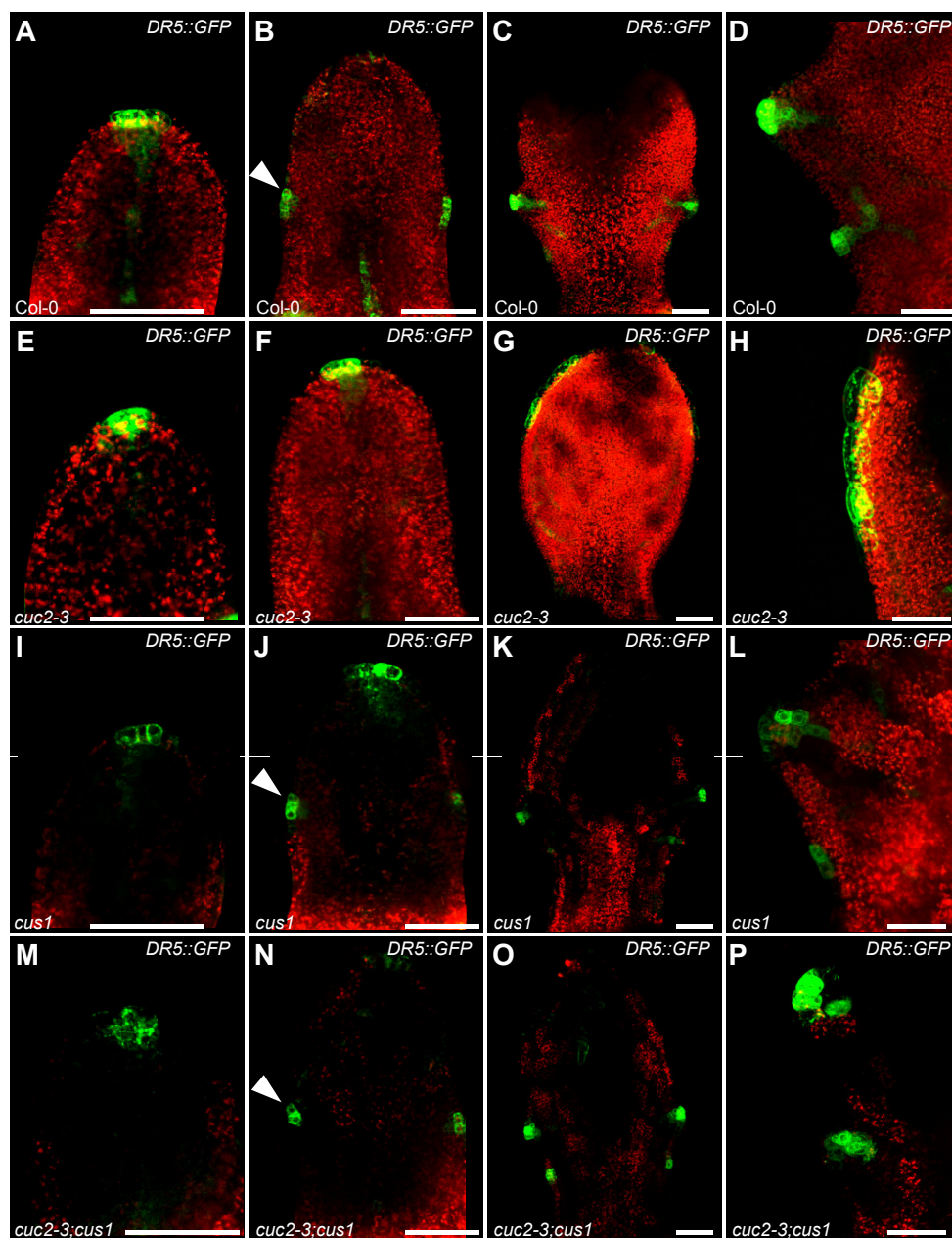


mutants. Whilst *cus1* single mutants produce leaves with deeply serrated margins, both *pin1* single mutants and *cus1;pin1* double mutants produce leaves with smooth margins (Figure 6.7 G-J). This indicates that *PIN1* is required for serration development in *cus1* leaves. To determine whether auxin signalling operates in the same genetic pathway as *CUS1*, I examined the leaf margin phenotypes of *axr1;cus1* double mutants. *axr1* and *cus1* mutants both produce more deeply serrated leaf margins compared to wild type (Figure 6.7 G-H, K), however, the leaf margins of *axr1;cus1* are not more deeply serrated than either parent (Figure 6.7 H, K-L). This indicates that auxin signalling acts in the same genetic pathway as *CUS1*. Since I showed in Chapter 3 that auxin signalling represses *CUC2* expression (Figure 3.11), it is likely that auxin signalling may regulate *CUS1* expression via repression of *CUC2* expression.

6.2.7 *CUS1* regulates the formation of auxin activity foci and PIN1 convergence points at the leaf margin

In Chapter 3, I showed that auxin activity foci fail to form at the margins of *cuc2* leaves (Figure 3.5), correlating with the smooth margin phenotypes of *cuc2* leaves. As loss-of-*CUS1* function restores serration development in *cuc2* leaves, I hypothesised that loss-of-*CUS1* function rescues formation of auxin activity foci in *cuc2* leaves. To test this, I analysed *DR5::GFP* expression in *cuc2;cus1* leaves. *DR5::GFP* is initially expressed at the tips of wild-type leaves (Figure 6.8 A). Once the leaf grows to approximately 200µm in length, *DR5::GFP* is expressed in maxima at the leaf margin, marking the sites of initiation of the first pair of serrations (Figure 6.8 B). As the leaf continue to grow, reaching approximately 500µm in length, subsequent *DR5::GFP* foci form in a basipetal sequence along the margin, marking the initiation of further serrations (Figure 6.8 C-D). In contrast, *DR5::GFP* is expressed at the tips of initiating *cuc2* leaves (Figure 6.8 E), but *DR5::GFP* maxima fail to form at the margins of *cuc2* leaves approximately 200µm in length (Figure 6.8 F). Once the

Figure 6.8 *CUS1* regulates auxin activity maxima formation at the margins of *A. thaliana* leaves. (A-D) Confocal micrograph of *DR5::GFP* expression (green) in fifth rosette leaf of Col-0 approximately 100µm in length (A), 200µm in length (B), 500µm in length (C) and close-up of leaf 500µm in length (D). (E-H) Confocal micrograph of *DR5::GFP* expression (green) in fifth rosette leaf of *cuc2-3* approximately 100µm in length (E), 200µm in length (F), 500µm in length (G) and close-up of leaf 500µm in length (H). (I-L) Confocal micrograph of *DR5::GFP* expression (green) in fifth rosette leaf of *cus1* approximately 100µm in length (I), 200µm in length (J), 500µm in length (K) and close-up of leaf 500µm in length (L). (M-P) Confocal micrograph of *DR5::GFP* expression (green) in fifth rosette leaf of *cuc2-3;cus1* approximately 100µm in length (M), 200µm in length (N), 500µm in length (O) and close-up of leaf 500µm in length (P). Scale bars: 50µm A-P.

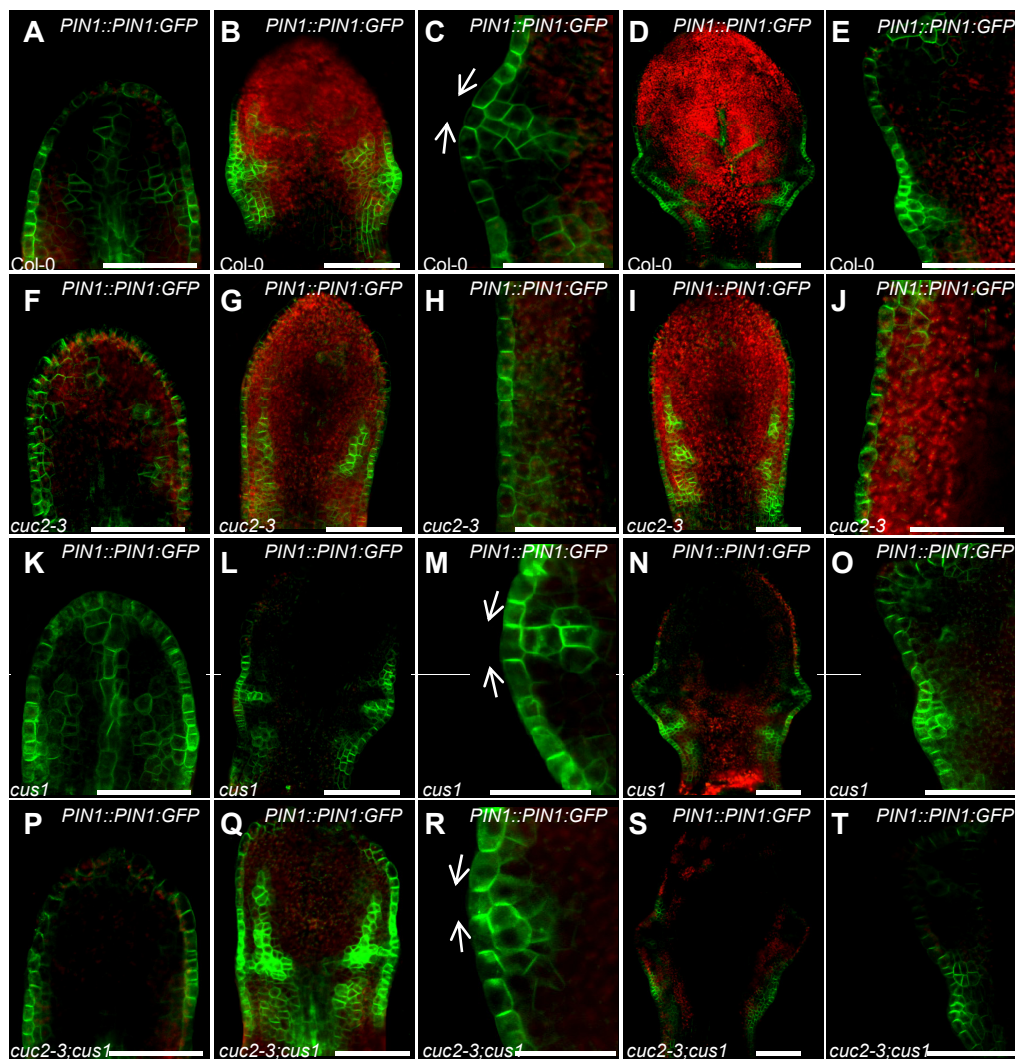


cuc2 is approximately 500µm in length, *DR5::GFP* is expressed in diffuse strips around the margin (Figure 6.8 G-H).

DR5::GFP is expressed at the tips of initiating *cus1* and *cuc2;cus1* leaves (Figure 6.8 I, M). Once *cus1* and *cuc2;cus1* leaves are approximately 200µm in length, *DR5::GFP* expression maxima form at the margin, defining the first pair of serrations (Figure 6.8 J, N). Furthermore, as *cus1* and *cuc2;cus1* leaves grow to approximately 500µm in length, subsequent *DR5::GFP* expression maxima form at the leaf margins in a basipetal sequence, thereby specifying sites of further serration development (Figure 6.8 K-L, O-P). This suggests that loss-of-*CUS1* function in *cuc2* leaves allows the formation of auxin activity foci at the *cuc2* leaf margin at the same developmental time as wild type, thereby restoring serration development.

In Chapter 3, I showed that absence of auxin activity maxima from the margins of *cuc2* leaves correlates with an absence PIN1 convergence points (Figure 3.6). I, therefore, hypothesised that loss-of-*CUS1* function in *cuc2* leaves causes the formation of auxin activity maxima by generating PIN1 convergence points. To test this, I analysed *PIN1::PIN1:GFP* expression in *cuc2;cus1* leaves. *PIN1::PIN1:GFP* is expressed at the margins and in the central vasculature of initiating wild-type leaves (Figure 6.9 A). Once leaves are approximately 200µm in length, *PIN1::PIN1:GFP* forms convergence points at the leaf margin (Figure 6.9 B-C). Subsequent *PIN1::PIN1:GFP* convergence points form in a basipetal sequence along the margin once the leaf is approximately 500µm in length (Figure 6.9 D-E). In contrast, *PIN1::PIN1:GFP* convergence points fail to form at the margins of *cuc2* leaves during early and late stages of leaf development (Figure 6.9 F-J). However, *PIN1::PIN1:GFP* is expressed at the margins and in the central vasculature of initiating *cus1* and *cuc2;cus1* leaves (Figure 6.9 K, P). Once *cus1* and *cuc2;cus1* leaves are approximately 200µm in length, *PIN1::PIN1:GFP* convergence points form at the margins (Figure 6.9 L-M,

Figure 6.9 *CUS1* regulates PIN1 convergence point formation at the *A. thaliana* leaf margin. (A-E) Confocal micrograph images of *PIN1::PIN1:GFP* expression (green) in fifth rosette leaf of Col-0 approximately 150µm in length (A), 200µm in length (B), close-up of leaf 200µm in length (C), 500µm in length (D) and close-up of leaf 500µm in length (E). **(F-J)** Confocal micrograph images of *PIN1::PIN1:GFP* expression (green) in fifth rosette leaf of *cuc2-3* approximately 150µm in length (F), 200µm in length (G), close-up of leaf 200µm in length (H), 500µm in length (I) and close-up of leaf 500µm in length (J). **(K-O)** Confocal micrograph images of *PIN1::PIN1:GFP* expression (green) in fifth rosette leaf of *cus1* approximately 150µm in length (K), 200µm in length (L), close-up of leaf 200µm in length (M), 500µm in length (N) and close-up of leaf 500µm in length (O). **(P-T)** Confocal micrograph images of *PIN1::PIN1:GFP* expression (green) in fifth rosette leaf of *cuc2-3;cus1* approximately 150µm in length (P), 200µm in length (Q), close-up of leaf 200µm in length (R), 500µm in length (S) and close-up of leaf 500µm in length (T). Scale bar: 50µm A-T.



Q-R). Subsequent *PIN1::PIN1:GFP* convergence points form in a basipetal sequence along the margins of *cus1* and *cuc2;cus1* once the leaves are approximately 500µm in length (Figure 6.9 N-O, S-T).

These data suggest that *CUS1* prevents PIN1 convergence points forming at the margins of *cuc2* leaves, and that loss-of-*CUS1* function is required to re-establish PIN1 convergence point formation in *cuc2* leaves. These data also suggest that auxin activity foci form in *cuc2;cus1* leaves due to the re-establishment of PIN1 convergence points.

6.2.8 Loss-of-*CUS1* function does not enhance lobe development in *as1* leaves

Many mutants with deeply serrated margins, such as *axr1*, *mir164a* and *se*, act synergistically with *as1*; this causes double mutants between serrated mutants and *as1* to produce leaves with extremely lobed leaf margins associated with ectopic *BP* expression at the margins (Hasson *et al.*, 2011; Hay *et al.*, 2006). To investigate whether *cus1* also acts synergistically with *as1*, I analysed the leaf phenotypes of *as1;cus1*. *cus1* single mutants produce leaves with more serrated margins compared wild type, whereas *as1* mutants produce leaves with lobed margins and shortened proximo-distal axes (Figure 6.10 A-C). *as1;cus1* double mutants produce leaves with similar phenotypes to *as1* and leaf dissection is not enhanced with respect to *as1* leaves (Figure 6.10 A-D). This suggests that *AS1* and *CUS1* likely act in the same genetic pathway to control leaf margin morphogenesis, and that *CUS1* function in controlling leaf margin development differs to that of many other serrated leaf mutants.

6.2.9 The *CUS1* locus lies within a 380kbp region on the southern arm of chromosome II

In an attempt to identify the mutated locus in *cus1*, I performed map-based cloning. Rough mapping shows that the *cus1* mutation lies on the southern arm of chromosome II

Figure 6.10 Genetic interaction between *CUS1* and *ASI*. (A-D) Fifth rosette and half rosette leaf of Col-0 (A, A'), *cus1* (B, B'), *asl-1* (C, C') and *asl-1;cus1* (D, D'). Scale bars: 1cm A-D; 0.5cm A'-D'.

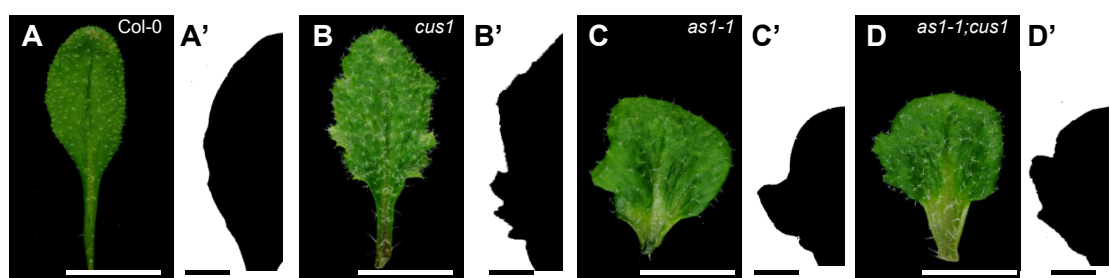
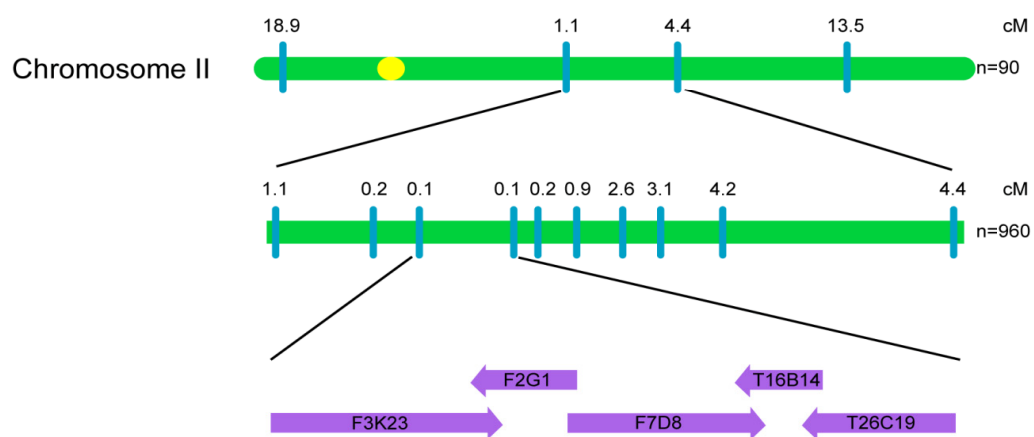


Figure 6.11 Map-based cloning of *cusI*. Schematic representation of linkage of markers to the *CUSI* locus. Green bars represent chromosome II, blue bars represent markers and purple arrows represent bacterial artificial chromosomes (BACs). cM represents genetic distance in centimorgans. n represents the number of chromosomes.



within a 5.5cM interval (Figure 6.11). Subsequent fine-mapping demonstrates that the *cus1* mutation lies within a 0.2cM interval spanning a 380kbp region that includes 145 genes and is covered by five BACs (Figure 6.11). However, the identity of the *CUS1* locus currently remains unknown.

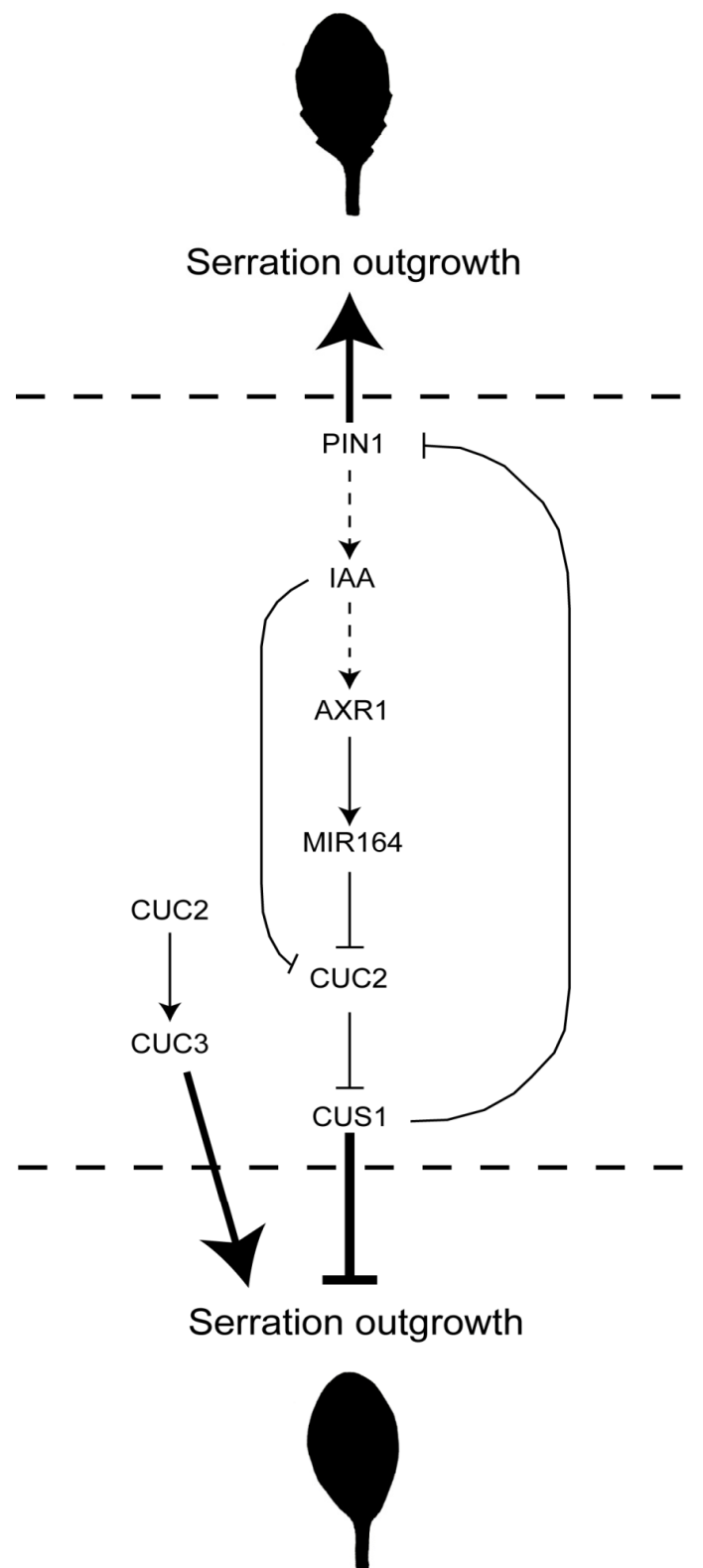
6.3 Discussion

6.3.1 *CUS1* likely acts downstream of *CUC2* to regulate serration outgrowth

Genetic analyses in this chapter indicate that *CUS1* likely acts downstream of *CUC2* as recessive *cus1* mutations restore serration development in *cuc2* leaves (Figure 6.3). It is also likely that the wild-type function of *CUS1* is to repress serration initiation as *cus1* leaves produce significantly more serrations than wild type (Figure 6.3 E). However, serration initiation begins at the same developmental stage in *cus1* and wild-type leaves, suggesting that either the rate of serration initiation or the duration of serration initiation is altered in *cus1* (Figure 6.4). Although serration initiation is not associated with foci of cell divisions (Figure 5.12 K-O; Donnelly *et al.*, 1999), delaying the progression of the cell cycle arrest front can correlate with the production of more serrated leaf margins, as seen in the *jaw-ID* mutant (Nath *et al.*, 2003). However, the progression of the cell cycle arrest front was shown to be unaffected in *cuc2* mutant leaves in this study (Figure 5.12 K-T), making it unlikely that the duration of serration initiation is affected in *cus1* leaves. This suggests that the rate of serration initiation is more likely to be affected in *cus1* leaves, however, future experiments should clarify this.

Although *CUS1* likely acts in the same genetic pathway as *CUC2* to control serration initiation, the combined actions of *CUC2* and *CUC3* appear to act independently of *CUS1* to regulate serration development. This is because loss-of-*CUC2* and *CUC3* function partially represses serration development in *cus1* leaves (Figure 6.6 A-H). *CUC3* acts late during

Figure 6.12 Gene regulatory network including *CUS1* in the control of serration development. PIN1 promotes serration outgrowth by promoting auxin (IAA) activity, which represses *CUC2* transcription. Auxin also acts via *AXR1* to promote *MIR164A* transcription, leading to the post-transcriptional repression of *CUC2*. *CUC2* regulates serration outgrowth by repressing *CUS1* function, and by independently regulating *CUC3* transcription. *CUC2* also acts to promote serration outgrowth by promoting the formation of PIN1 convergence points.



serration development as it is transcribed after visible serration outgrowth (Hasson *et al.*, 2011). Furthermore, loss-of-function *cuc3* mutants produce leaves with less deeply serrated margins compared to wild type, without affecting serration number (Hasson *et al.*, 2011). *CUC3* transcription is promoted by *CUC2* (Hasson *et al.*, 2011), and the balance between the ratio of *CUC2:miR164* has been shown to regulate serration depth (Nikovics *et al.*, 2006). Thus, it is possible that *CUC2* and *CUC3* act in a separate pathway to *CUS1* either due to a difference in the developmental time at which they act in serration development or due to differences in their spatial expression patterns. Future comparisons of the spatio-temporal dynamics of *CUC2*, *CUC3* and *CUS1* expression patterns will help to address these possibilities.

6.3.2 *CUS1* regulates serration initiation by repressing PIN1 convergence points

In this chapter, I showed that loss-of-*CUS1* function restores serration development in *cuc2* leaves by allowing PIN1 convergence points and auxin activity maxima to form at the leaf margin (Figures 6.8-6.9). This, therefore, suggests that *CUS1* represses PIN1 convergence point formation in wild-type leaves (Figure 6.12). PIN1 convergence points form at the margins of wild-type leaves due to a reversal in PIN1 polarity (Hay *et al.*, 2006). PIN polarity can be regulated by the phosphorylation status of the PIN protein, regulation of clathrin-dependent endocytosis or cell wall composition (Dhonukshe *et al.*, 2007; Feraru *et al.*, 2011). PID, WAG1 and WAG2 are membrane-associated AGC3 kinases that phosphorylate serine residues in PIN proteins to promote an apical PIN subcellular localisation (Dhonukshe *et al.*, 2010; Friml *et al.*, 2004; Michniewicz *et al.*, 2007). However, PPK2A dephosphorylates PIN proteins, thereby promoting the basal subcellular localisation of PIN proteins (Michniewicz *et al.*, 2007). Thus, *CUS1* may be involved in the phosphorylation dynamics of PIN1, possibly by indirectly or directly regulating the activity

of an AGC3 kinase or a dephosphorylase to prevent reversals in PIN polarity and PIN1 convergence point formation.

Additionally, PIN proteins cycle between the plasma membrane and internal vesicles, and are endocytosed by a clathrin-dependent mechanism (Dhonukshe *et al.*, 2007; Geldner *et al.*, 2001). ABP1 is an endoplasmic reticulum localised protein that prevents clathrin-dependent endocytosis of PIN proteins in response to binding auxin (Effendi *et al.*, 2011; Paciorek *et al.*, 2005; Robert *et al.*, 2010). Thus, a plausible explanation is that *CUS1* could regulate an aspect of ABP1-dependent regulation to prevent PIN1 convergence points from forming. Alternatively, mutants with reduced activity of an enzyme involved in synthesising cellulose, the main component of the cell wall, have symmetrically localised PIN proteins, suggesting that the composition of the cell wall is fundamental in controlling PIN polarity (Feraru *et al.*, 2011). Thus, perhaps *CUS1* is involved in regulating cell wall biosynthesis.

6.4 Conclusions

- *CUS1* is a novel component of the *CUC2* developmental pathway that controls serration development in *A. thaliana*.
- Loss-of-*CUS1* function in *cuc2* leaves is required to re-establish PIN1 convergence points and serration development.
- *CUS1* regulates serration initiation and is unlikely to regulate later stages of serration development since it acts in a separate pathway to *CUC3*.

Chapter 7: General discussion

Chapter 7: General discussion

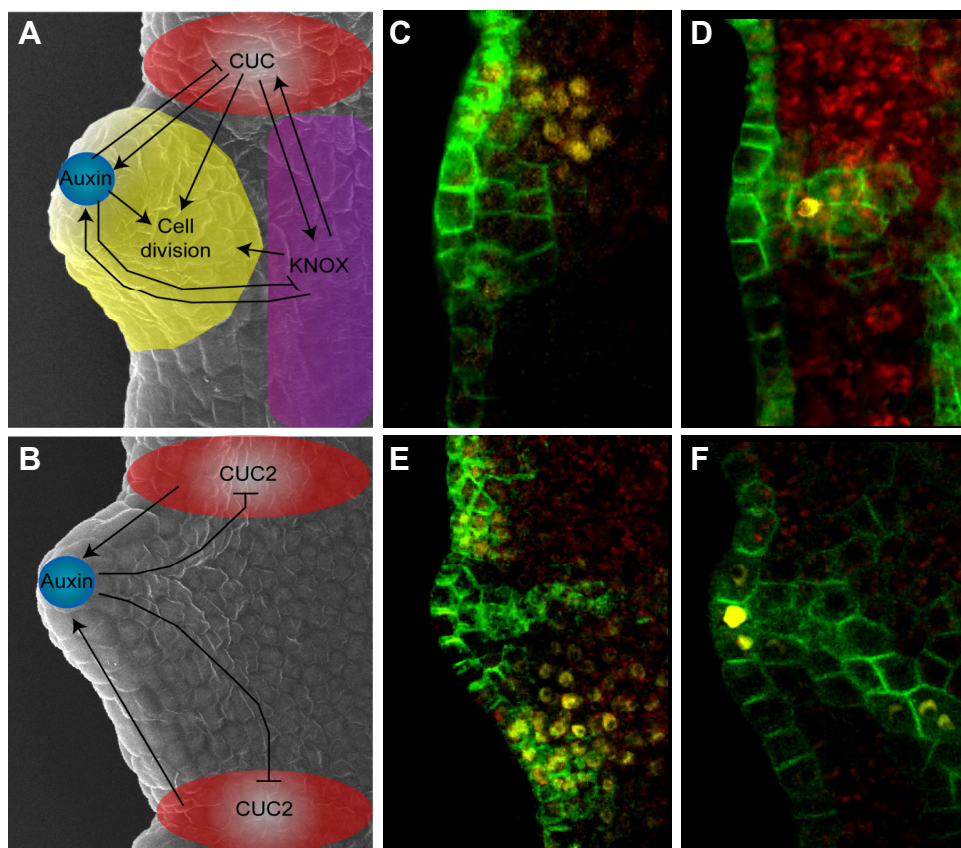
7.1 Interlinking feedback loops regulate simple and compound leaf morphology

All multicellular organisms undergo the process of development whereby a single fertilised egg divides and differentiates to create a complex, organised form. A key question that, therefore, arises in developmental biology is how morphology is generated. The model species *A. thaliana* produces leaves with marginal protrusions termed serrations, whereas the closely-related species *C. hirsuta* produces compound leaves that are divided into individual units termed leaflets. In this study, I investigated the gene regulatory networks controlling serration and leaflet development to determine the genetic basis of leaf morphogenesis.

Molecular genetic analyses suggest that three interlinking feedback loops operate within the *C. hirsuta* leaf to control leaflet development (Figure 7.1 A). In Chapter 4, I showed that *ChCUC* genes promote the formation of PIN1 convergence points and auxin activity maxima, whilst auxin represses *CUC2* expression (Figures 4.5-4.6). These genetic interactions suggest that a negative feedback loop exists between *CUC*, PIN1 and auxin to promote leaflet development. In a second negative feedback loop, auxin represses *KNOX* transcription whilst *KNOX* activity is required for PIN1 convergence point formation (Barkoulas *et al.*, 2008). Finally, a positive feedback loop exists where *ChCUC* and *KNOX* genes mutually promote their transcription (Figure 5.10 K-L; Blein *et al.*, 2008).

Interestingly, part of this gene regulatory network employed in leaflet development also operates in serration development (Figure 7.1 B). In Chapter 3, I demonstrated that *CUC2* is required to promote PIN1 convergence point formation (Figure 3.6), whereas auxin

Figure 7.1 Interlinking feedback loops control leaflet and serration development. (A) Scanning electron micrograph close-up of a *C. hirsuta* leaflet. Auxin activity foci (blue circle) form at the tips of leaflets where they promote cell division (red), and repress both *ChCUC* (yellow oval) and *KNOX* (purple rectangle) expression. *ChCUC* and *KNOX* genes both feed back to promote the formation of auxin activity foci. A positive feedback loop exists between *ChCUC* and *KNOX* genes. *KNOX* genes act to delay the transition from cell division to cell differentiation in the leaf, whereas *ChCUC* genes act to delimit cell division to foci associated with leaflet development. **(B)** Scanning electron micrograph close-up of an *A. thaliana* serration. *CUC2* (yellow oval) is expressed in the sinuses between serrations where it promotes the formation of auxin activity foci (blue circle). Auxin then feeds back to repress *CUC2* expression. **(C)** Confocal micrograph image of colocalised *PIN1::PIN1:GFP* (green) and *CUC2::CUC2:VENUS* (yellow) expression in a *C. hirsuta* leaflet. The expression of *CUC2::CUC2:VENUS* in the distal sinus promotes the formation of a *PIN1::PIN1:GFP* convergence point. **(D)** Confocal micrograph image of colocalised *PIN1::PIN1:GFP* (green) and *DR5::VENUS* (yellow) expression in a *C. hirsuta* leaflet. A focus of subepidermal *DR5::VENUS* expression occurs after the formation of a *PIN1::PIN1:GFP* point. **(E)** Confocal micrograph image of colocalised *PIN1::PIN1:GFP* (green) and *CUC2::CUC2:VENUS* (yellow) expression in the serration of an *A. thaliana* leaf. The occurrence of a *PIN1::PIN1:GFP* convergence point coincides with the downregulation of *CUC2::CUC2:VENUS* expression. **(F)** Confocal micrograph image of colocalised *PIN1::PIN1:GFP* (green) and *DR5::VENUS* (yellow) expression in an *A. thaliana* serration. A focus of *DR5::VENUS* expression occurs at the same time as a *PIN1::PIN1:GFP* convergence point forms.



can repress *CUC2* transcription (Figure 3.10). This, therefore, suggests that a negative feedback loop exists between *CUC2*, PIN1 and auxin to promote serration development. Additionally, PIN1 and auxin interact in a positive feedback loop as PIN1 convergence points promote the formation of auxin activity maxima, and auxin application promotes *PIN1* transcription (Hay *et al.*, 2006; Scarpella *et al.*, 2006; Vieten *et al.*, 2005). Thus, two interlinking feedback loops between *CUC2*, PIN1 and auxin appear to regulate serration development in *A. thaliana* leaves. Moreover, computational modelling confirms that these molecular interactions are sufficient to generate the interspersed peaks of *CUC2* and auxin activity observed along the *A. thaliana* leaf margin (modelling performed by A. Runions in Bilsborough *et al.*, 2011).

Intriguingly, although the negative feedback loop between *CUC2*, PIN1 and auxin has been conserved in leaflet and serration development, there are differences in the early spatio-temporal dynamics of *CUC2*, PIN1 and auxin activity foci expression patterns. In *A. thaliana*, the formation of PIN1 convergence points is temporally linked to the formation of auxin activity maxima and downregulation of *CUC2* expression (Figure 7.1 C-D). In contrast, the formation of *CUC2* expression foci at the margins of the *C. hirsuta* rachis precedes the formation of PIN1 convergence points. Furthermore, there is temporal delay between PIN1 convergence point and auxin activity maximum formation (Figure 7.1 E-F). Despite these early differences in expression, interspersed peaks of *CUC2* and auxin activity are generated along the *A. thaliana* and *C. hirsuta* leaf margins. Nevertheless, it will be interesting to determine whether early spatio-temporal differences in *CUC*, PIN1 and auxin activity expression patterns contribute to differences in leaf morphology between the two species.

KNOX genes are expressed in the *C. hirsuta* leaf where they are necessary and sufficient for leaflet development, however, they are not expressed in *A. thaliana* leaves (Hay and Tsiantis, 2006; Heisler *et al.*, 2005). Thus, the negative feedback loop between auxin and

KNOX, and the positive feedback loop between *CUC* and *KNOX* that are present in *C. hirsuta* leaves are absent from *A. thaliana* leaves. Interestingly, in Chapter 5 I showed that the three interlinking feedback loops between *CUC*, *KNOX*, PIN1 and auxin are re-established in *A. thaliana* leaves when *KNOX* genes are ectopically expressed in leaves (Figure 5.14 A-F). Since *KNOX* expression is associated with the development of deeply lobed margins reminiscent of leaflets (Chuck *et al.*, 1996; Hay *et al.*, 2006), this raises the question of how these three feedback loops regulate growth dynamics.

Clonal analyses indicate that between two to four cells are recruited from the margin of the *C. hirsuta* rachis to generate a leaflet via cell division (Barkoulas *et al.*, 2008). These data suggest that the cells comprising the leaflet are derived from new cell divisions rather than the recruitment of existing cells, and arise as new lateral axes of growth. Auxin activity foci promote cell division and *KNOX* genes delay the transition from cell division to differentiation (Barkoulas *et al.*, 2008; Hay and Tsiantis, 2006) and in Chapter 5 I showed that *ChCUC* genes are required to delimit cell division foci (Figure 5.12 A-J). Thus, it seems that interlinking feedback loops between *ChCUC*, *KNOX* and auxin influence cell division to regulate leaflet growth and development.

In contrast, cell divisions are not correlated with serration development in *A. thaliana* leaves (Donnelly *et al.*, 1999). This suggests that whilst the feedback loop between *CUC* genes and auxin is required to establish sites of serration initiation in *A. thaliana*, it is not sufficient to drive foci of cell division. Since *KNOX* genes are not expressed in *A. thaliana* leaves, it is possible that *KNOX* genes may be the critical component in establishing lateral axes of proliferation associated with leaflet development. This idea is consistent with the proposed role of *STM* in preventing the transition from cell division to differentiation in *C. hirsuta* leaves (Hay and Tsiantis, 2006). Thus, it is possible that the positive feedback loop between *KNOX* and *CUC* on the one hand potentiates the ability of *CUC* to polarise growth

via auxin, and potentiates the ability of *STM* to keep cells in an undifferentiated state. In this way, leaf cells would remain competent to respond to leaf differentiating factors.

In a recent study, *CUC* genes were shown to regulate leaflet development in a broad range of independently evolved compound leafed angiosperms (Blein *et al.*, 2008). In addition, *CUC* genes were also shown to regulate serration development at the margins of leaflets and serration development in simple leaves (Blein *et al.*, 2008; Nikovics *et al.*, 2006). These findings led to the hypothesis that serrations and leaflets are generated by similar mechanisms involving *CUC* genes. Auxin is also required for leaflet development in a range of compound leafed species such as *C. hirsuta*, tomato and pea (Barkoulas *et al.*, 2008; DeMason and Chawla, 2004; Koenig *et al.*, 2009), and for serration development in simple *A. thaliana* leaves (Hay *et al.*, 2006). Thus, it is possible that the negative feedback loop observed between *CUC*, PIN1 and auxin in *A. thaliana* and *C. hirsuta* in this study could be responsible for the development of serrations, lobes and leaflets in all leaves. Furthermore, integrating additional components such as *KNOX* genes could lead to changes in growth and overall morphology as small differences in the strength of genetic interactions may generate morphological diversity.

7.2 Conserved genetic mechanisms regulate lobe and leaflet development

There are endless forms in nature and, incredibly, all this morphological diversity originates from a single universal common ancestor (Darwin, 1859). Differences in leaf shape were first noted in antiquity (Theophrastus, 1916), and paleobotanical data suggest that the ancestral angiosperm produced simple leaves (Doyle and Endress 2000). Molecular studies have since shown that compound leaves have evolved on several independent occasions from simple leafed ancestors (Bharathan *et al.*, 2002; Doyle and Endress, 2000). However,

compound leaf forms have also evolved back into simple leaves, as exemplified by the model species *A. thaliana* which evolved from a compound leafed ancestor (Piazza *et al.*, 2010).

A decade ago, a strong correlation between *KNOX* expression in leaves and the development of compound leaves was made (Bharathan *et al.*, 2002). More recently it was shown that *KNOX* genes are expressed in the compound leaves of *C. hirsuta* where they are both necessary and sufficient for leaflet development along the rachis (Hay and Tsiantis, 2006). In contrast, *KNOX* expression is excluded from the simple leaves of *A. thaliana*, but ectopic *KNOX* expression in *A. thaliana* leaves causes the formation of deep lobes along the margin of the blade that are reminiscent of leaflets (Chuck *et al.*, 1996). These data led to the hypothesis that lobes arise from prolonged growth of serrations on leaf blades.

Subsequent investigations of *A. thaliana* backgrounds ectopically expressing *KNOX* genes in leaves demonstrated that lobes develop from sites of serration development associated with temporal persistence of PIN1 convergence points and auxin activity maxima (Hay *et al.*, 2006). In Chapter 5, I showed that ectopic *KNOX* expression causes the re-establishment of three interlinking feedback loops between *CUC*, *KNOX*, PIN1 and auxin that are present in *C. hirsuta* leaves (Figure 5.14 A-F). These include negative feedback loop between *CUC*, PIN1 and auxin, a negative feedback loop between *KNOX*, PIN1 and auxin, and a positive feedback loop between *KNOX* and *CUC*. Thus, *KNOX* appears to be a species-specific determinant of leaf dissection by implementing conserved genetic mechanisms between *CUC*, *KNOX* and auxin.

Endogenous *KNOX* expression delays the transition from cell division to cell differentiation in *C. hirsuta* leaves and ectopic *KNOX* expression promotes growth of lobes in *A. thaliana* leaves (Hay and Tsiantis, 2006). However, lobe growth is restricted to the blade of the *A. thaliana* leaf, whereas leaflets arise *de novo* along the rachis of the *C. hirsuta* leaf

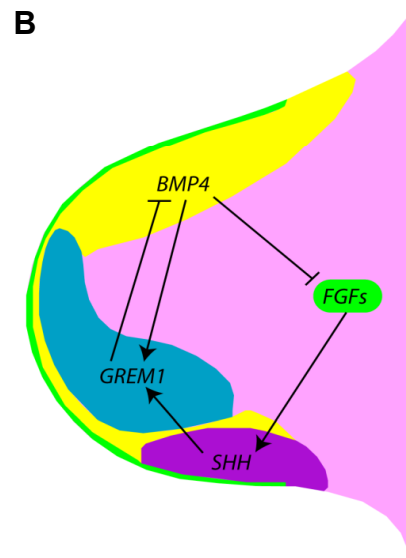
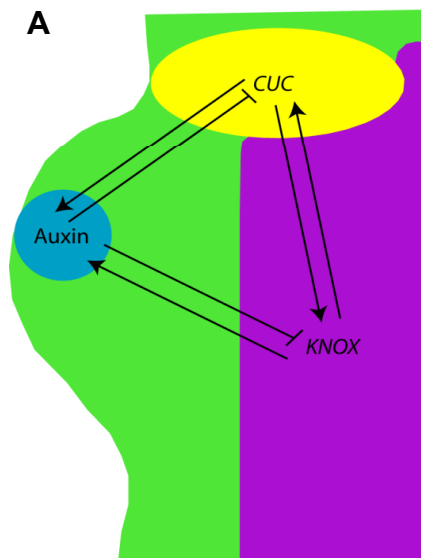
(Hay and Tsiantis, 2006). This suggests that additional factors in the *C. hirsuta* leaf are required for outgrowths to develop along the rachis. In this study, I showed that a *C. hirsuta* mutant named *loli* produces a lobed terminal leaflet in contrast to compound leaves with lateral leaflets in wild type (Figure 5.13). Interestingly, *bop1;bop2* mutants in *A. thaliana* produce ectopic lamina outgrowths along the petiole (Hepworth *et al.*, 2005), giving a phenotype similar to *loli* leaves. *BOP1* and *BOP2* control cell proliferation by repressing *JAG* and *NUB* expression (Dinneny *et al.*, 2006; Dinneny *et al.*, 2004; Ohno *et al.*, 2004). Thus, a tantalising prospect is that species-specific changes in the regulation of BOP activity may have contributed to differences in leaf growth and morphology between *A. thaliana* and *C. hirsuta*, and this may be revealed in *loli* mutants.

7.3 Interlinking feedback loops regulate outgrowths in plant and animal systems

Studies of gene regulatory networks controlling morphogenesis have revealed that interlinking positive and negative feedback loops may be important for patterning morphogenesis in both plants and animals by creating interspersed peaks of activity. In this study, I showed that the interlinking of three feedback loops regulates leaflet development in *C. hirsuta* leaves (Figure 7.2 A). In Chapter 4, I demonstrated that a negative feedback loop between *CUC*, PIN1 and auxin controls leaflet growth and development likely by creating interspersed peaks of *CUC2* and auxin activity along the margin of the *C. hirsuta* leaf. A second negative feedback loop was shown to exist between auxin and *KNOX* (Barkoulas *et al.*, 2008). Since *KNOX* genes prevent the transition from cell division to cell differentiation and auxin promotes cell division in the *C. hirsuta* leaf, this feedback loop, therefore, controls leaflet growth (Hay and Tsiantis, 2006). Finally, *KNOX* and *ChCUC* genes mutually promote the expression of each other in a positive feedback loop (Blein *et al.*, 2008), thereby influencing cell division within the leaflet.

Figure 7.2 Interlinking feedback loops regulate outgrowths in plants and animals. (A)

Cartoon depicting gene regulatory networks associated with leaflet development in *C. hirsuta*. In the first feedback loop, *ChCUC* genes (yellow oval) are expressed in the distal sinuses of leaflets and promote the formation of auxin activity foci (blue circle) at the tips of leaflets. Auxin feeds back to repress *ChCUC* expression. In a second feedback loop, auxin represses the expression of *KNOX* genes (purple rectangle), whereas *KNOX* genes feed back to promote the formation of auxin activity foci. In a final feedback loop, *KNOX* and *ChCUC* genes positively regulate each other. **(B)** Cartoon depicting limb bud development in mice and chick. In the first feedback loop, *FGFs* (green strip) promote the expression of *SHH* (purple oval), and consequently *GREM1* (blue strip). *GREM1* then represses the expression of *BMP4* (yellow strip), leading to the de-repression of *FGF* expression. In the second feedback loop, *GREM1* represses *BMP4* expression, but *BMP4* feeds back to positively regulate *GREM1* expression.



Over the past twenty years, studies of gene regulatory networks in mice and chick have revealed that two interlinking feedback loops regulate limb bud development (Figure 7.2 B). A positive feedback loop exists between *FIBROBLAST GROWTH FACTORS* (*FGFs*), *SONIC HEDGEHOG* (*SHH*), *GREMLIN1* (*GREM1*) and *BONE MORPHOGENETIC PROTEIN 4* (*BMP4*) to pattern the proximo-distal and antero-posterior axes of the limb bud, as well as controlling growth. *FGFs* are expressed in the most distal cells of the limb bud where they specify the proximo-distal axis of the bud, and maintain the expression of *SHH* (Lewandoski *et al.*, 2000; Mariani *et al.*, 2008; Sun *et al.*, 2002). *SHH* in turn promotes the expression of *GREM1* which patterns the antero-posterior axis and regulates cell division within the limb bud (Laufer *et al.*, 1994; Michos *et al.*, 2004; Niswander *et al.*, 1994). *GREM1* represses the activity of *BMP4*, thereby de-repressing *FGF* expression and completing the feed forward loop (Michos *et al.*, 2004). A negative feedback loop also exists between *GREM1* and *BMP4* to control limb bud development (Nissim *et al.*, 2006). Thus, it appears that interlinking positive and negative feedback loops pattern the proximo-distal and antero-posterior axes of the limb bud whilst also regulating growth within the limb bud.

Reaction-diffusion models suggest that an interaction between two components, an activator and an inhibitor, are able to create interspersed peaks of morphogenetic activities, allowing the periodic generation of outgrowths. Given that two components can generate iterations, a key question that arises is what the significance is of interlinking feedback loops in producing outgrowths. One hypothesis is that the coupling of positive and negative feedback loops can create a robust oscillating patterning mechanism (Brandman and Meyer, 2008). Positive feedback loops act to amplify input signals, thereby promoting downstream target processes. In contrast, negative feedback loops can restore the input signal back to a basal level, allowing new positive feedback loops to be established, and thus creating an oscillating patterning mechanism. In leaflet and limb bud development, the interlinking of

positive and negative feedback loops could, therefore, pattern the iterative formation of outgrowths along the proximo-distal axis by establishing an oscillating patterning mechanism.

The gene regulatory networks resulting from interlinking feedback loops appear to control morphogenesis with dynamic spatio-temporal gene expression patterns, and the read-out from such networks is the control of growth. A crucial goal of future research will be to investigate the precise timing of growth and whether evolutionary changes in interlinking feedback loops influencing patterning result in altered patterns of growth to generate different organismal forms.

References

- Abel, S. and Theologis, A. (1996) Early genes and auxin action. *Plant Physiology* **111**: 9-17.
- Adenot, X., Elmayan, T., Laussergues, D., Boutet, S., Bouche, N., Gascioli, V. and Vaucheret, H. (2006) DRB4-dependent TAS3 trans-acting siRNAs control leaf morphology through AGO7. *Current Biology* **16**: 927-932.
- Aida, M., Ishida, T., Fukaki, H., Fujisawa, H. and Tasaka, M. (1997) Genes involved in organ separation in Arabidopsis: an analysis of the cup-shaped cotyledon mutant. *The Plant Cell* **9**: 841-857.
- Aida, M., Ishida, T. and Tasaka, M. (1999) Shoot apical meristem and cotyledon formation during Arabidopsis embryogenesis: interaction among the CUP-SHAPED COTYLEDON and SHOOT MERISTEMLESS genes. *Development* **126**: 1563-1570.
- Aida, M., Vernoux, T., Furutani, M., Traas, J. and Tasaka, M. (2002) Roles of PINFORMED1 and MONOPTEROS in pattern formation of the apical region of the Arabidopsis embryo. *Development* **129**: 3965-3974.
- Allen, E., Xie, Z., Gustafson, A.M. and Carrington, J.C. (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell* **121**: 207-221.
- Aloni, R., Schwalm, K., Langhans, M. and Ullrich, C.I. (2003) Gradual shifts in sites of free-auxin production during leaf-primordium development and their role in vascular differentiation and leaf morphogenesis in Arabidopsis. *Planta* **216**: 841-853.
- Avery, G.S. (1933) Structure and development of the tobacco leaf. *American Journal of Botany* **20**: 565-592.
- Avivi, Y., Lev-Yadun, S., Morozova, N., Libs, L., Williams, L., Zhao, J., Varghese, G. and Grafi, G. (2000) *Clausa*, a tomato mutant with a wide range of phenotypic perturbations, displays a cell type-dependent expression of the homeobox gene LeT6/TKn2. *Plant Physiology* **124**: 541-552.
- Bai, Y., Falk, S., Schnittger, A., Jakoby, M.J. and Hulskamp, M. (2010) Tissue layer specific regulation of leaf length and width in Arabidopsis as revealed by the cell autonomous action of ANGUSTIFOLIA. *The Plant Journal* **61**: 191-199.
- Bailey, W. and Sinnott, E.W. (1915) A Botanical Index of Cretaceous and Tertiary Climates. *Science* **41**: 831-834.
- Bailey, W. and Sinnott, E.W. (1916) The Climatic Distribution of Certain Types of Angiosperm Leaves. *American Journal of Botany* **3**: 24-39.
- Baima, S., Nobili, F., Sessa, G., Lucchetti, S., Ruberti, I. and Morelli, G. (1995) The expression of the Athb-8 homeobox gene is restricted to provascular cells in Arabidopsis thaliana. *Development* **121**: 4171-4182.
- Bainbridge, K., Guyomarc'h, S., Bayer, E., Swarup, R., Bennett, M., Mandel, T. and Kuhlemeier, C. (2008) Auxin influx carriers stabilize phyllotactic patterning. *Genes & Development* **22**: 810-823.
- Baker-Brosh, K.F. and Peet, R.K. (1997) The ecological significance of lobed and toothed leaves in temperate forest trees. *Ecology* **78**: 1250-1255.
- Baker, C.C., Sieber, P., Wellmer, F. and Meyerowitz, E.M. (2005) The *early extra petals1* mutant uncovers a role for microRNA miR164c in regulating petal number in Arabidopsis. *Current Biology* **15**: 303-315.
- Ballas, N., Wong, L.M. and Theologis, A. (1993) Identification of the auxin-responsive element, *AuxRE*, in the primary indoleacetic acid-inducible gene, *PS-IAA4/5*, of pea (*Pisum sativum*). *Journal of Molecular Biology* **233**: 580-596.
- Barkoulas, M., Hay, A., Kougiumoutzi, E. and Tsiantis, M. (2008) A developmental framework for dissected leaf formation in the Arabidopsis relative Cardamine hirsuta. *Nature Genetics* **40**: 1136-1141.
- Bellaoui, M., Pidkowich, M.S., Samach, A., Kushalappa, K., Kohalmi, S.E., Modrusan, Z., Crosby, W.L. and Haughn, G.W. (2001) The Arabidopsis BELL1 and KNOX TALE

- homeodomain proteins interact through a domain conserved between plants and animals. *The Plant Cell* **13**: 2455-2470.
- Belles-Boix, E., Hamant, O., Witiak, S.M., Morin, H., Traas, J. and Pautot, V. (2006) KNAT6: an Arabidopsis homeobox gene involved in meristem activity and organ separation. *The Plant Cell* **18**: 1900-1907.
- Benjamins, R., Quint, A., Weijers, D., Hooykaas, P. and Offringa, R. (2001) The PINOID protein kinase regulates organ development in Arabidopsis by enhancing polar auxin transport. *Development* **128**: 4057-4067.
- Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G. and Friml, J. (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell* **115**: 591-602.
- Bennett, S.R.M., Alvarez, J., Bossinger, G. and Smyth, D.R. (1995) Morphogenesis in *pinoid* mutants of *Arabidopsis thaliana*. *The Plant Journal* **8**: 505-520.
- Bennett, T., Sieberer, T., Willett, B., Booker, J., Luschnig, C. and Leyser, O. (2006) The Arabidopsis MAX pathway controls shoot branching by regulating auxin transport. *Current Biology* **16**: 553-563.
- Berger, Y., Harpaz-Saad, S., Brand, A., Melnik, H., Sirding, N., Alvarez, J.P., Zinder, M., Samach, A., Eshed, Y. and Ori, N. (2009) The NAC-domain transcription factor GOBLET specifies leaflet boundaries in compound tomato leaves. *Development* **136**: 823-832.
- Bharathan, G., Goliber, T.E., Moore, C., Kessler, S., Pham, T. and Sinha, N.R. (2002) Homologies in leaf form inferred from *KNOX* gene expression during development. *Science* **296**: 1858-1860.
- Bhatt, A.M., Etchells, J.P., Canales, C., Lagodienko, A. and Dickinson, H. (2004) VAAMANA-a BEL1-like homeodomain protein, interacts with KNOX proteins BP and STM and regulates inflorescence stem growth in Arabidopsis. *Gene* **328**: 103-111.
- Bilborough, G.D., Runions, A., Barkoulas, M., Jenkins, H.J., Hasson, A., Galinha, C., Laufs, P., Hay, A., Prusinkiewicz, P. and Tsiantis, M. (2011) Model for the regulation of *Arabidopsis thaliana* leaf margin development. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 3424-3429.
- Bleckmann, A., Weidtkamp-Peters, S., Seidel, C.A. and Simon, R. (2010) Stem cell signaling in Arabidopsis requires CRN to localize CLV2 to the plasma membrane. *Plant Physiology* **152**: 166-176.
- Blein, T., Pulido, A., Vialette-Guiraud, A., Nikovics, K., Morin, H., Hay, A., Johansen, I.E., Tsiantis, M. and Laufs, P. (2008) A conserved molecular framework for compound leaf development. *Science* **322**: 1835-1839.
- Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K. and Scheres, B. (2005) The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. *Nature* **433**: 39-44.
- Bolduc, N. and Hake, S. (2009) The Maize transcription factor KNOTTED1 directly regulates the gibberellin catabolism gene *ga2ox1*. *The Plant Cell* **21**: 1647-1658.
- Bowman, J.L., Eshed, Y. and Baum, S.F. (2002) Establishment of polarity in angiosperm lateral organs. *Trends in Genetics* **18**: 134-141.
- Brand, U., Fletcher, J.C., Hobe, M., Meyerowitz, E.M. and Simon, R. (2000) Dependence of stem cell fate in Arabidopsis on a feedback loop regulated by CLV3 activity. *Science* **289**: 617-619.
- Brandman, O. and Meyer, T. (2008) Feedback loops shape cellular signals in space and time. *Science* **322**: 390-395.

- Bright, K.L. and Rausher, M.D. (2008) Natural selection on a leaf-shape polymorphism in the ivyleaf Morning Glory (*Ipomoea hederacea*). *Evolution* **62**: 1978-1990.
- Burnham, R.J., Pitman, N.C.A., Johnson, K.R. and Wilf, P. (2001) Habitat-related error in estimating temperatures from leaf margins in a humid tropical forest. *American Journal of Botany* **88**: 1096-1102.
- Byrne, M.E., Barley, R., Curtis, M., Arroyo, J.M., Dunham, M., Hudson, A. and Martienssen, R.A. (2000) Asymmetric leaves1 mediates leaf patterning and stem cell function in Arabidopsis. *Nature* **408**: 967-971.
- Byrne, M.E., Groover, A.T., Fontana, J.R. and Martienssen, R.A. (2003) Phyllotactic pattern and stem cell fate are determined by the Arabidopsis homeobox gene BELLRINGER. *Development* **130**: 3941-3950.
- Byrne, M.E., Simorowski, J. and Martienssen, R.A. (2002) ASYMMETRIC LEAVES1 reveals *knox* gene redundancy in Arabidopsis. *Development* **129**: 1957-1965.
- Chen, H., Banerjee, A.K. and Hannapel, D.J. (2004) The tandem complex of BEL and KNOX partners is required for transcriptional repression of GA20ox1. *The Plant Journal* **38**: 276-284.
- Cho, H.T. and Cosgrove, D.J. (2000) Altered expression of expansin modulates leaf growth and pedicel abscission in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 9783-9788.
- Choi, D., Lee, Y., Cho, H.T. and Kende, H. (2003) Regulation of expansin gene expression affects growth and development in transgenic rice plants. *The Plant Cell* **15**: 1386-1398.
- Christensen, S.K., Dagenais, N., Chory, J. and Weigel, D. (2000) Regulation of auxin response by the protein kinase PINOID. *Cell* **100**: 469-478.
- Chuck, G., Lincoln, C. and Hake, S. (1996) KNAT1 induces lobed leaves with ectopic meristems when overexpressed in Arabidopsis. *The Plant Cell* **8**: 1277-1289.
- Clark, S.E., Running, M.P. and Meyerowitz, E.M. (1993) CLAVATA1, a regulator of meristem and flower development in Arabidopsis. *Development* **119**: 397-418.
- Cnops, G., Jover-Gil, S., Peters, J.L., Neyt, P., De Block, S., Robles, P., Ponce, M.R., Gerats, T., Micol, J.L. and Van Lijsebettens, M. (2004) The *rotunda2* mutants identify a role for the LEUNIG gene in vegetative leaf morphogenesis. *Journal of Experimental Botany* **55**: 1529-1539.
- Coen, E., Rolland-Lagan, A.G., Matthews, M., Bangham, J.A. and Prusinkiewicz, P. (2004) The genetics of geometry. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 4728-4735.
- Cole, M., Nolte, C. and Werr, W. (2006) Nuclear import of the transcription factor SHOOT MERISTEMLESS depends on the heterodimerization with BLH proteins expressed in discrete subdomains of the shoot apical meristem of Arabidopsis thaliana. *Nucleic acids research* **34**: 1281-1292.
- Correns, C. (1909) Vererbungsversuche mit blass(gelb)grünen und buntblättrigen Sippen bei *Mirabilis Jalapa*, *Urtica pilulifera* und *Lunaria annua*. *Z. Indukt. Abstammungs-Verebungsl.* **1**: 291-329.
- Crick, F.H. (1968) The origin of the genetic code. *Journal of Molecular Biology* **38**: 367-379.
- Curtis, M.D. and Grossniklaus, U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiology* **133**: 462-469.
- Darwin, C. (1859) On the Origin of Species by Means of Natural Selection.
- Darwin, F. and Darwin, C. (1880) The Power and Movement in Plants. London, John Murray.

- De Veylder, L., Beeckman, T., Beemster, G.T., Krols, L., Terras, F., Landrieu, I., van der Schueren, E., Maes, S., Naudts, M. and Inze, D. (2001) Functional analysis of cyclin-dependent kinase inhibitors of Arabidopsis. *The Plant Cell* **13**: 1653-1668.
- Del Pozo, J.C. and Estelle, M. (1999) Function of the ubiquitin-proteasome pathway in auxin response. *Trends in Plant Science* **4**: 107-112.
- Del Pozo, J.C., Timpte, C., Tan, S., Callis, J. and Estelle, M. (1998) The ubiquitin-related protein RUB1 and auxin response in *Arabidopsis*. *Science* **280**: 1760-1763.
- DeMason, D.A. and Chawla, R. (2004) Roles for auxin during morphogenesis of the compound leaves of pea (*Pisum sativum*). *Planta* **218**: 435-448.
- DeMason, D.A. and Schmidt, R.J. (2001) Roles of the *uni* gene in shoot and leaf development of pea (*Pisum sativum*): phenotypic characterization and leaf development in the uni and uni-tac mutants. *International Journal of Plant Sciences* **162**: 1033-1051.
- Dharmasiri, N., Dharmasiri, S. and Estelle, M. (2005) The F-box protein TIR1 is an auxin receptor. *Nature* **435**: 441-445.
- Dharmasiri, N. and Estelle, M. (2004) Auxin signaling and regulated protein degradation. *Trends in Plant Science* **9**: 302-308.
- Dhonukshe, P., Aniento, F., Hwang, I., Robinson, D.G., Mravec, J., Stierhof, Y.D. and Friml, J. (2007) Clathrin-mediated constitutive endocytosis of PIN auxin efflux carriers in *Arabidopsis*. *Current Biology* **17**: 520-527.
- Dhonukshe, P., Huang, F., Galvan-Ampudia, C.S., Mähönen, A.P., Kleine-Vehn, J., Xu, J., Quint, A., Prasad, K., Friml, J., Scheres, B. and Offringa, R. (2010) Plasma membrane bound AGC3 kinases phosphorylate PIN auxin carriers at TPRXS(N/S) motifs to direct apical PIN recycling. *Development* **137**: 3245-3255.
- Dinneny, J.R., Weigel, D. and Yanofsky, M.F. (2006) NUBBIN and JAGGED define stamen and carpel shape in Arabidopsis. *Development* **133**: 1645-1655.
- Dinneny, J.R., Yadegari, R., Fischer, R.L., Yanofsky, M.F. and Weigel, D. (2004) The role of JAGGED in shaping lateral organs. *Development* **131**: 1101-1110.
- Dobzhansky, T. (1973) Nothing in biology makes sense except in the light of evolution. *The American Biology Teacher* **35**: 125-129.
- Dolan, L. and Poethig, R.S. (1998) Clonal analysis of leaf development in cotton. *American Journal of Botany* **85**: 315-321.
- Donnelly, P.M., Bonetta, D., Tsukaya, H., Dengler, R.E. and Dengler, N.G. (1999) Cell cycling and cell enlargement in developing leaves of Arabidopsis. *Developmental Biology* **215**: 407-419.
- Donner, T.J., Sherr, I. and Scarpella, E. (2009) Regulation of preprocambial cell state acquisition by auxin signaling in *Arabidopsis* leaves. *Development* **136**: 3235-3246.
- Douady, S. and Couder, Y. (1996) Phyllotaxis as a dynamical self organizing process. 1. The spiral modes resulting from time-periodic iterations. *Journal of Theoretical Biology* **178**: 255-274.
- Doyle, J.A. and Endress, P.K. (2000) Morphological phylogenetic analysis of basal angiosperms: comparison and combination with molecular data. *International Journal of Plant Sciences* **161**: S121-S153.
- Dunphy, W.G. (1994) The decision to enter mitosis. *Trends in Cell Biology* **4**: 202-207.
- Effendi, Y., Rietz, S., Fischer, U. and Scherer, G.F.E. (2011) The heterozygous *abp1/ABP1* insertional mutant has defects in functions requiring polar auxin transport and in regulation of early auxin-regulated genes. *The Plant Journal* **65**: 282-294.
- Elliott, R.C., Betzner, A.S., Huttner, E., Oakes, M.P., Tucker, W.Q., Gerentes, D., Perez, P. and Smyth, D.R. (1996) AINTEGUMENTA, an APETALA2-like gene of

- Arabidopsis with pleiotropic roles in ovule development and floral organ growth. *The Plant Cell* **8**: 155-168.
- Elmore, C.D. (1986) Mode of reproduction and inheritance of leaf shape in *Ipomoea hederacea*. *Weed Science* **34**: 391-395.
- Emery, J.F., Floyd, S.K., Alvarez, J., Eshed, Y., Hawker, N.P., Izhaki, A., Baum, S.F. and Bowman, J.L. (2003) Radial patterning of Arabidopsis shoots by class III HD-ZIP and KANADI genes. *Current Biology* **13**: 1768-1774.
- Eshed, Y., Baum, S.F. and Bowman, J.L. (1999) Distinct mechanisms promote polarity establishment in carpels of Arabidopsis. *Cell* **99**: 199-209.
- Eshed, Y., Baum, S.F., Perea, J.V. and Bowman, J.L. (2001) Establishment of polarity in lateral organs of plants. *Current Biology* **11**: 1251-1260.
- Eshed, Y., Izhaki, A., Baum, S.F., Floyd, S.K. and Bowman, J.L. (2004) Asymmetric leaf development and blade expansion in Arabidopsis are mediated by KANADI and YABBY activities. *Development* **131**: 2997-3006.
- Fahlgren, N., Montgomery, T.A., Howell, M.D., Allen, E., Dvorak, S.K., Alexander, A.L. and Carrington, J.C. (2006) Regulation of AUXIN RESPONSE FACTOR3 by TAS3 ta-siRNA affects developmental timing and patterning in Arabidopsis. *Current Biology* **16**: 939-944.
- Feild, T.S., Sage, T.L., Czerniak, C. and Iles, W.J.D. (2005) Hydathodal leaf teeth of *Chloranthus japonicus* (Chloranthaceae) prevent guttation-induced flooding of the mesophyll. *Plant, Cell and Environment* **28**: 1179-1190.
- Feraru, E., Feraru, M.I., Kleine-Vehn, J., Martiniere, A., Mouille, G., Vanneste, S., Vernhettes, S., Runions, J. and Friml, J. (2011) PIN polarity maintenance by the cell wall in Arabidopsis. *Current Biology* **21**: 338-343.
- Fleming, A.J., Mandel, T., McQueen-Mason, S. and Kuhlemeier, C. (1997) Induction of leaf primordia by the cell wall protein expansin. *Science* **276**: 1415-1418.
- Fletcher, J.C., Brand, U., Running, M.P., Simon, R. and Meyerowitz, E.M. (1999) Signaling of cell fate decisions by CLAVATA3 in Arabidopsis shoot meristems. *Science* **283**: 1911-1914.
- Frankel, N., Erezyilmaz, D.F., McGregor, A.P., Wang, S., Payre, F. and Stern, D.L. (2011) Morphological evolution caused by many subtle-effect substitutions in regulatory DNA. *Nature* **474**: 598-603.
- Friml, J., Yang, X., Michniewicz, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P.B., Ljung, K., Sandberg, G., Hooykaas, P.J., Palme, K. and Offringa, R. (2004) A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. *Science* **306**: 862-865.
- Furner, I.J. and Pumfrey, J.E. (1992) Cell fate in the shoot apical meristem of *Arabidopsis thaliana*. *Development* **115**: 755-764.
- Garcia, D., Collier, S.A., Byrne, M.E. and Martienssen, R.A. (2006) Specification of leaf polarity in Arabidopsis via the trans-acting siRNA pathway. *Current Biology* **16**: 933-938.
- Geldner, N., Friml, J., Stierhof, Y.D., Jurgens, G. and Palme, K. (2001) Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* **413**: 425-428.
- Gifford, M.L., Dean, S. and Ingram, G.C. (2003) The Arabidopsis ACR4 gene plays a role in cell layer organisation during ovule integument and sepal margin development. *Development* **130**: 4249-4258.
- Goldsmith, M.H.M. (1977) Polar transport of auxin. *Annual Review of Plant Physiology and Plant Molecular Biology* **28**: 439-478.
- Golz, J.F., Roccaro, M., Kuzoff, R. and Hudson, A. (2004) GRAMINIFOLIA promotes growth and polarity of Antirrhinum leaves. *Development* **131**: 3661-3670.

- Gordon, S.P., Chickarmane, V.S., Ohno, C. and Meyerowitz, E.M. (2009) Multiple feedback loops through cytokinin signaling control stem cell number within the Arabidopsis shoot meristem. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 16529-16534.
- Gourlay, C.W., Hofer, J.M. and Ellis, T.H. (2000) Pea compound leaf architecture is regulated by interactions among the genes UNIFOLIATA, cochleata, afilea, and tendril-lessn. *The Plant Cell* **12**: 1279-1294.
- Green, P.B., Steele, C.S. and Rennich, S.C. (1996) Phyllotactic patterns: A biophysical mechanism for their origin. *Annals of Botany* **77**: 515-527.
- Greenwood, D.R. (2005) Leaf form and the reconstruction of past climates. *New Phytologist* **166**: 355-357.
- Greenwood, D.R. (2005) Leaf margin analysis: taphonomic constraints. *Palaios* **20**: 498-505.
- Greenwood, D.R., Wilf, P., Wing, S.L. and Christophel, D.C. (2004) Paleotemperature estimation using leaf-margin analysis: Is Australia different? *Palaios* **19**: 129-142.
- Guo, H.S., Xie, Q., Fei, J.F. and Chua, N.H. (2005) MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for Arabidopsis lateral root development. *The Plant Cell* **17**: 1376-1386.
- Guo, M., Thomas, J., Collins, G. and Timmermans, M.C. (2008) Direct repression of KNOX loci by the ASYMMETRIC LEAVES1 complex of Arabidopsis. *The Plant Cell* **20**: 48-58.
- Hackbusch, J., Richter, K., Muller, J., Salamini, F. and Uhrig, J.F. (2005) A central role of Arabidopsis thaliana ovate family proteins in networking and subcellular localization of 3-aa loop extension homeodomain proteins. *Proceedings of the National Academy of Sciences United States of America* **102**: 4908-4912.
- Hagemann, W. and Gleissberg, S. (1996) Organogenetic capacity of leaves: the significance of marginal blastozones in angiosperms. *Plant Systematics and Evolution* **199**: 121-152.
- Hake, S., Smith, H.M., Holtan, H., Magnani, E., Mele, G. and Ramirez, J. (2004) The role of knox genes in plant development. *Annual Review of Cell and Developmental Biology* **20**: 125-151.
- Hamann, T., Benkova, E., Baurle, I., Kientz, M. and Jurgens, G. (2002) The Arabidopsis BODENLOS gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning. *Genes & Development* **16**: 1610-1615.
- Hamann, T., Mayer, U. and Jurgens, G. (1999) The auxin-insensitive bodenlos mutation affects primary root formation and apical-basal patterning in the Arabidopsis embryo. *Development* **126**: 1387-1395.
- Hamant, O., Heisler, M.G., Jonsson, H., Krupinski, P., Uyttewaal, M., Bokov, P., Corson, F., Sahlin, P., Boudaoud, A., Meyerowitz, E.M., Couder, Y. and Traas, J. (2008) Developmental patterning by mechanical signals in Arabidopsis. *Science* **322**: 1650-1655.
- Hardtke, C.S. and Berleth, T. (1998) The Arabidopsis gene MONOPTEROS encodes a transcription factor mediating embryo axis formation and vascular development. *The EMBO Journal* **17**: 1405-1411.
- Hareven, D., Gutfinger, T., Parnis, A., Eshed, Y. and Lifschitz, E. (1996) The making of a compound leaf: genetic manipulation of leaf architecture in tomato. *Cell* **84**: 735-744.
- Hasson, A., Plessis, A., Blein, T., Adroher, B., Grigg, S., Tsiantis, M., Boudaoud, A., Damerval, C. and Laufs, P. (2011) Evolution and diverse roles of the CUP-SHAPED COTYLEDON genes in Arabidopsis leaf development. *The Plant Cell* **23**: 54-68.

- Hay, A., Barkoulas, M. and Tsiantis, M. (2006) ASYMMETRIC LEAVES1 and auxin activities converge to repress BREVIPEDICELLUS expression and promote leaf development in Arabidopsis. *Development* **133**: 3955-3961.
- Hay, A., Kaur, H., Phillips, A., Hedden, P., Hake, S. and Tsiantis, M. (2002) The gibberellin pathway mediates KNOTTED1-type homeobox function in plants with different body plans. *Current Biology* **12**: 1557-1565.
- Hay, A. and Tsiantis, M. (2006) The genetic basis for differences in leaf form between Arabidopsis thaliana and its wild relative Cardamine hirsuta. *Nature Genetics* **38**: 942-947.
- Heisler, M.G., Hamant, O., Krupinski, P., Uyttewaal, M., Ohno, C., Jonsson, H., Traas, J. and Meyerowitz, E.M. (2010) Alignment between PIN1 polarity and microtubule orientation in the shoot apical meristem reveals a tight coupling between morphogenesis and auxin transport. *PLoS Biology* **8**: e1000516.
- Heisler, M.G., Ohno, C., Das, P., Sieber, P., Reddy, G.V., Long, J.A. and Meyerowitz, E.M. (2005) Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the Arabidopsis inflorescence meristem. *Current Biology* **15**: 1899-1911.
- Hepworth, S.R., Zhang, Y., McKim, S., Li, X. and Haughn, G.W. (2005) BLADE-ON-PETIOLE-dependent signaling controls leaf and floral patterning in Arabidopsis. *The Plant Cell* **17**: 1434-1448.
- Hersh, B.M. and Carroll, S.B. (2005) Direct regulation of knot gene expression by Ultrabithorax and the evolution of cis-regulatory elements in Drosophila. *Development* **132**: 1567-1577.
- Hibara, K., Karim, M.R., Takada, S., Taoka, K., Furutani, M., Aida, M. and Tasaka, M. (2006) Arabidopsis CUP-SHAPED COTYLEDON3 regulates postembryonic shoot meristem and organ boundary formation. *The Plant Cell* **18**: 2946-2957.
- Hofer, J., Gourlay, C., Michael, A. and Ellis, T.H. (2001) Expression of a class 1 knotted1-like homeobox gene is down-regulated in pea compound leaf primordia. *Plant Molecular Biology* **45**: 387-398.
- Hofer, J., Turner, L., Hellens, R., Ambrose, M., Matthews, P., Michael, A. and Ellis, N. (1997) UNIFOLIATA regulates leaf and flower morphogenesis in pea. *Current Biology* **7**: 581-587.
- Hofmeister, W. (1868) Allgemeine Morphologie der Gewächse. *Handbuch der Physiologischen Botanik*; , Engelmann, W. **1**: 405-664.
- Horiguchi, G., Kim, G.T. and Tsukaya, H. (2005) The transcription factor AtGRF5 and the transcription coactivator AN3 regulate cell proliferation in leaf primordia of Arabidopsis thaliana. *The Plant Journal* **43**: 68-78.
- Hu, Y., Poh, H.M. and Chua, N.H. (2006) The Arabidopsis ARGOS-LIKE gene regulates cell expansion during organ growth. *The Plant Journal* **47**: 1-9.
- Hu, Y., Xie, Q. and Chua, N.H. (2003) The Arabidopsis auxin-inducible gene ARGOS controls lateral organ size. *The Plant Cell* **15**: 1951-1961.
- Huff, P.M., Wilf, P. and Azumah, E.J. (2003) Digital future for paleoclimate estimation from fossil leaves? Preliminary results. *Palaios* **18**: 266-274.
- Hunter, C., Willmann, M.R., Wu, G., Yoshikawa, M., de la Luz Gutierrez-Nava, M. and Poethig, S.R. (2006) Trans-acting siRNA-mediated repression of ETTIN and ARF4 regulates heteroblasty in Arabidopsis. *Development* **133**: 2973-2981.
- Ichihashi, Y., Kawade, K., Usami, T., Horiguchi, G., Takahashi, T. and Tsukaya, H. (2011) Key proliferative activity in the junction between the leaf blade and leaf petiole of Arabidopsis thaliana. *Plant Physiology*: Epub ahead of print.

- Ikezaki, M., Kojima, M., Sakakibara, H., Kojima, S., Ueno, Y., Machida, C. and Machida, Y. (2010) Genetic networks regulated by ASYMMETRIC LEAVES1 (AS1) and AS2 in leaf development in *Arabidopsis thaliana*: KNOX genes control five morphological events. *The Plant Journal* **61**: 70-82.
- Irish, V.F. and Sussex, I.M. (1992) A fate map of the *Arabidopsis* embryonic shoot apical meristem. *Development* **115**: 745-753.
- Jackson, D., Veit, B. and Hake, S. (1994) Expression of maize *KNOTTED-1* related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. *Development* **120**: 405-413.
- Jasinski, S., Kaur, H., Tattersall, A. and Tsiantis, M. (2007) Negative regulation of KNOX expression in tomato leaves. *Planta* **226**: 1255-1263.
- Jasinski, S., Piazza, P., Craft, J., Hay, A., Woolley, L., Rieu, I., Phillips, A., Hedden, P. and Tsiantis, M. (2005) KNOX action in *Arabidopsis* is mediated by coordinate regulation of cytokinin and gibberellin activities. *Current Biology* **15**: 1560-1565.
- Jasinski, S., Tattersall, A., Piazza, P., Hay, A., Martinez-Garcia, J.F., Schmitz, G., Theres, K., McCormick, S. and Tsiantis, M. (2008) PROCERA encodes a DELLA protein that mediates control of dissected leaf form in tomato. *The Plant Journal* **56**: 603-612.
- Jonsson, H., Heisler, M.G., Shapiro, B.E., Meyerowitz, E.M. and Mjolsness, E. (2006) An auxin-driven polarized transport model for phyllotaxis. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 1633-1638.
- Juarez, M.T., Kui, J.S., Thomas, J., Heller, B.A. and Timmermans, M.C. (2004) microRNA-mediated repression of rolled leaf1 specifies maize leaf polarity. *Nature* **428**: 84-88.
- Juarez, M.T., Twigg, R.W. and Timmermans, M.C. (2004) Specification of adaxial cell fate during maize leaf development. *Development* **131**: 4533-4544.
- Jun, J.H., Ha, C.M. and Fletcher, J.C. (2010) BLADE-ON-PETIOLE1 coordinates organ determinacy and axial polarity in *Arabidopsis* by directly activating ASYMMETRIC LEAVES2. *The Plant Cell* **22**: 62-76.
- Kaplan, D.R. (1975) Comparative developmental evaluation of the morphology of Unifacial leaves in the monocotyledons. *Botanische Jahrbücher für Systematik, Pflanzengeschichte und Pflanzengeographie* **95**: 1-105.
- Kasschau, K.D., Xie, Z., Allen, E., Llave, C., Chapman, E.J., Krizan, K.A. and Carrington, J.C. (2003) P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function. *Developmental Cell* **4**: 205-217.
- Katz, A., Oliva, M., Mosquna, A., Hakim, O. and Ohad, N. (2004) FIE and CURLY LEAF polycomb proteins interact in the regulation of homeobox gene expression during sporophyte development. *The Plant Journal* **37**: 707-719.
- Kawade, K., Horiguchi, G. and Tsukaya, H. (2010) Non-cell-autonomously coordinated organ size regulation in leaf development. *Development* **137**: 4221-4227.
- Kawamura, E., Horiguchi, G. and Tsukaya, H. (2010) Mechanisms of leaf tooth formation in *Arabidopsis*. *The Plant Journal* **62**: 429-441.
- Kayes, J.M. and Clark, S.E. (1998) CLAVATA2, a regulator of meristem and organ development in *Arabidopsis*. *Development* **125**: 3843-3851.
- Kazama, T., Ichihashi, Y., Murata, S. and Tsukaya, H. (2010) The mechanism of cell cycle arrest front progression explained by a KLUH/CYP78A5-dependent mobile growth factor in developing leaves of *Arabidopsis thaliana*. *Plant Cell Physiology* **51**: 1046-1054.
- Kepinski, S. and Leyser, O. (2005) The *Arabidopsis* F-box protein TIR1 is an auxin receptor. *Nature* **435**: 446-451.

- Kerstetter, R., Vollbrecht, E., Lowe, B., Veit, B., Yamaguchi, J. and Hake, S. (1994) Sequence analysis and expression patterns divide the maize knotted1-like homeobox genes into two classes. *The Plant Cell* **6**: 1877-1887.
- Kerstetter, R.A., Bollman, K., Taylor, R., Bomblies, K. and Poethig, R.S. (2001) KANADI regulates organ polarity in *Arabidopsis*. *Nature* **411**: 706-709.
- Key, J.L., Barnett, N.M. and Lin, C.Y. (1967) RNA and protein biosynthesis and the regulation of cell elongation by auxin. *Annual New York Academy of Science* **144**: 49-62.
- Kidner, C.A. and Martienssen, R.A. (2004) Spatially restricted microRNA directs leaf polarity through ARGONAUTE1. *Nature* **428**: 81-84.
- Kim, G.T., Fujioka, S., Kozuka, T., Tax, F.E., Takatsuto, S., Yoshida, S. and Tsukaya, H. (2005) CYP90C1 and CYP90D1 are involved in different steps in the brassinosteroid biosynthesis pathway in *Arabidopsis thaliana*. *The Plant Journal* **41**: 710-721.
- Kim, G.T., Tsukaya, H., Saito, Y. and Uchimiya, H. (1999) Changes in the shapes of leaves and flowers upon overexpression of cytochrome P450 in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 9433-9437.
- Kim, M., Pham, T., Hamidi, A., McCormick, S., Kuzoff, R.K. and Sinha, N. (2003) Reduced leaf complexity in tomato wiry mutants suggests a role for *PHAN* and *KNOX* genes in generating compound leaves. *Development* **130**: 4405-4415.
- Kim, Y.S., Kim, S.G., Lee, M., Lee, I., Park, H.Y., Seo, P.J., Jung, J.H., Kwon, E.J., Suh, S. and Paek, K.H. (2008) HDZIP III activity is modulated by competitive inhibitors via a feedback loop in *Arabidopsis* shoot apical meristem development. *The Plant Cell* **20**: 920-933.
- Kim, Y.S., Kim, S.G., Park, J.E., Park, H.Y., Lim, M.H., Chua, N.H. and Park, C.M. (2006) A membrane-bound NAC transcription factor regulates cell division in *Arabidopsis*. *The Plant Cell* **18**: 3132-3144.
- Kinoshita, A., Betsuyaku, S., Osakabe, Y., Mizuno, S., Nagawa, S., Stahl, Y., Simon, R., Yamaguchi-Shinozaki, K., Fukuda, H. and Shinichiro, S. (2010) RPK2 is an essential receptor-like kinase that transmits the CLV3 signal in *Arabidopsis*. *Development* **137**: 3911-3920.
- Klucher, K.M., Chow, H., Reiser, L. and Fischer, R.L. (1996) The AINTEGUMENTA gene of *Arabidopsis* required for ovule and female gametophyte development is related to the floral homeotic gene APETALA2. *The Plant Cell* **8**: 137-153.
- Koch, M., Haubold, B. and Mitchell-Olds, T. (2001) Molecular systematics of the Brassicaceae: evidence from coding plastidic matK and nuclear Chs sequences. *American Journal of Botany* **88**: 534-544.
- Koenig, D., Bayer, E., Kang, J., Kuhlemeier, C. and Sinha, N. (2009) Auxin patterns *Solanum lycopersicum* leaf morphogenesis. *Development* **136**: 2997-3006.
- Kondo, T., Sawa, S., Kinoshita, A., Mizuno, S., Kakimoto, T., Fukuda, H. and Sakagami, Y. (2006) A plant peptide encoded by CLV3 identified by in situ MALDI-TOF MS analysis. *Science* **313**: 845-848.
- Kowalski, E.A. and Dilcher, D. (2003) Warmer paleotemperatures for terrestrial ecosystems. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 167-170.
- Koyama, T., Furutani, M., Tasaka, M. and Ohme-Takagi, M. (2007) TCP transcription factors control the morphology of shoot lateral organs via negative regulation of the expression of boundary-specific genes in *Arabidopsis*. *The Plant Cell* **19**: 473-484.
- Koyama, T., Mitsuda, N., Seki, M., Shinozaki, K. and Ohme-Takagi, M. (2010) TCP transcription factors regulate the activities of ASYMMETRIC LEAVES1 and

- miR164, as well as the auxin response, during differentiation of leaves in Arabidopsis. *The Plant Cell* **22**: 3574-3588.
- Krizek, B.A. (1999) Ectopic expression of AINTEGUMENTA in Arabidopsis plants results in increased growth of floral organs. *Developmental Genetics* **25**: 224-236.
- Kwiatkowska, D. and Dumais, J. (2003) Growth and morphogenesis at the vegetative shoot apex of *Anagallis arvensis* L. *Journal of Experimental Botany* **54**: 1585-1595.
- Kwon, C.S., Hibara, K., Pfluger, J., Bezhani, S., Metha, H., Aida, M., Tasaka, M. and Wagner, D. (2006) A role for chromatin remodeling in regulation of *CUC* gene expression in the Arabidopsis cotyledon boundary. *Development* **133**: 3223-3230.
- Larue, C.T., Wen, J. and Walker, J.C. (2009) A microRNA-transcription factor module regulates lateral organ size and patterning in Arabidopsis. *The Plant Journal* **58**: 450-463.
- Laufer, E., Nelson, C.E., Johnson, R.L., Morgan, B.A. and Tabin, C. (1994) Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79**: 993-1003.
- Laufs, P., Peaucelle, A., Morin, H. and Traas, J. (2004) MicroRNA regulation of the *CUC* genes is required for boundary size control in Arabidopsis meristems. *Development* **131**: 4311-4322.
- Laux, T., Mayer, K.F., Berger, J. and Jurgens, G. (1996) The *WUSCHEL* gene is required for shoot and floral meristem integrity in Arabidopsis. *Development* **122**: 87-96.
- Leibfried, A., To, J.P., Busch, W., Stehling, S., Kehle, A., Demar, M., Kieber, J.J. and Lohmann, J.U. (2005) *WUSCHEL* controls meristem function by direct regulation of cytokinin-inducible response regulators. *Nature* **438**: 1172-1175.
- Lenhard, M. and Laux, T. (2003) Stem cell homeostasis in the Arabidopsis shoot meristem is regulated by intercellular movement of *CLAVATA3* and its sequestration by *CLAVATA1*. *Development* **130**: 3163-3173.
- Lewandoski, M., Sun, X. and Martin, G.R. (2000) Fgf8 signalling from the AER is essential for normal limb development. *Nature Genetics* **26**: 460-463.
- Leyser, H.M., Lincoln, C.A., Timppe, C., Lammer, D., Turner, J. and Estelle, M. (1993) Arabidopsis auxin-resistance gene *AXR1* encodes a protein related to ubiquitin-activating enzyme E1. *Nature* **364**: 161-164.
- Li, C. and Bangerth, F. (1999) Autoinhibition of indoleacetic acid transport in the shoot of two-branched pea (*Pisum sativum*) plants and its relationship to correlative dominance. *Physiologia Plantarum* **106**: 415-420.
- Li, H., Xu, L., Wang, H., Yuan, Z., Cao, X., Yang, Z., Zhang, D., Xu, Y. and Huang, H. (2005) The Putative RNA-dependent RNA polymerase *RDR6* acts synergistically with *ASYMMETRIC LEAVES1* and *2* to repress *BREVIPEDICELLUS* and *MicroRNA165/166* in Arabidopsis leaf development. *The Plant Cell* **17**: 2157-2171.
- Lin, W.C., Shuai, B. and Springer, P.S. (2003) The Arabidopsis *LATERAL ORGAN BOUNDARIES*-domain gene *ASYMMETRIC LEAVES2* functions in the repression of *KNOX* gene expression and in adaxial-abaxial patterning. *The Plant Cell* **15**: 2241-2252.
- Linnaeus, C. (1751) *Philosophica botanica*. Stockholm.
- Long, J.A. and Barton, M.K. (2001) Initiation of axillary and floral meristems in *Arabidopsis*. *Developmental Biology* **218**: 341-353.
- Long, J.A., Moan, E.I., Medford, J.I. and Barton, M.K. (1996) A member of the *KNOTTED* class of homeodomain proteins encoded by the *STM* gene of Arabidopsis. *Nature* **379**: 66-69.

- Lukowitz, W., Gillmor, C.S. and Scheible, W.R. (2000) Positional cloning in Arabidopsis. Why it feels good to have a genome initiative working for you. *Plant Physiology* **123**: 795-805.
- Luschnig, C., Gaxiola, R.A., Grisafi, P. and Fink, G.R. (1998) EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis thaliana*. *Genes & Development* **12**: 2175-2187.
- MacGinitie, H.D. (1953) Fossil plants of florissant beds, Colorado. *Carnegie Institution of Washington Publication* **599**: 1-198.
- Magnani, E. and Hake, S. (2008) KNOX Lost the OX: The Arabidopsis KNATM Gene Defines a Novel Class of KNOX Transcriptional Regulators Missing the Homeodomain. *The Plant Cell* **20**: 875-887.
- Mallory, A.C., Dugas, D.V., Bartel, D.P. and Bartel, B. (2004) MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. *Current Biology* **14**: 1035-1046.
- Mallory, A.C., Reinhart, B.J., Jones-Rhoades, M.W., Tang, G., Zamore, P.D., Barton, M.K. and Bartel, D.P. (2004) MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5' region. *The EMBO Journal* **23**: 3356-3364.
- Marcotrigiano, M. (2001) Genetic mosaics and the analysis of leaf development. *International Journal of Plant Sciences* **162**: 513-525.
- Mariani, F.V., Ahn, C.P. and Martin, G.R. (2008) Genetic evidence that FGFs have an instructive role in limb proximal-distal patterning. *Nature* **453**: 401-405.
- Mayer, K.F., Schoof, H., Haecker, A., Lenhard, M., Jurgens, G. and Laux, T. (1998) Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. *Cell* **95**: 805-815.
- McConnell, J.R. and Barton, M.K. (1998) Leaf polarity and meristem formation in Arabidopsis. *Development* **125**: 2935-2942.
- McConnell, J.R., Emery, J., Eshed, Y., Bao, N., Bowman, J. and Barton, M.K. (2001) Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shoots. *Nature* **411**: 709-713.
- McGinnis, W., Levine, M.S., Hafen, E., Kuroiwa, A. and Gehring, W.J. (1984) A conserved DNA sequence in homoeotic genes of the *Drosophila* Antennapedia and bithorax complexes. *Nature* **308**: 428-433.
- McHale, N.A. and Koning, R.E. (2004) MicroRNA-directed cleavage of *Nicotiana glauca* PHAVOLUTA mRNA regulates the vascular cambium and structure of apical meristems. *The Plant Cell* **16**: 1730-1740.
- Michniewicz, M., Zago, M.K., Abas, L., Weijers, D., Schweighofer, A., Meskiene, I., Heisler, M., Ohno, C., Zhang, J., Huang, F., Schwab, R., Weigel, D., Meyerowitz, E.M., Luschnig, C., Offringa, R. and Friml, J. (2007) Antagonistic regulation of PIN phosphorylation by PP2A and PINOID directs auxin flux. *Cell* **130**: 1044-1056.
- Michos, O., Panman, L., Vintersten, K., Beier, K., Zeller, R. and Zuniga, A. (2004) Gremlin-mediated BMP antagonism induces the epithelial-mesenchymal feedback signaling controlling metanephric kidney and limb organogenesis. *Development* **131**: 3401-3410.
- Mizukami, Y. and Fischer, R.L. (2000) Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 942-947.
- Mukherjee, K., Brocchieri, L. and Burglin, T.R. (2009) A comprehensive classification and evolutionary analysis of plant homeobox genes. *Molecular Biology and Evolution* **26**: 2775-2794.

- Muller, R., Bleckmann, A. and Simon, R. (2008) The receptor kinase CORYNE of Arabidopsis transmits the stem cell-limiting signal CLAVATA3 independently of CLAVATA1. *The Plant Cell* **20**: 934-946.
- Narita, N.N., Moore, S., Horiguchi, G., Kubo, M., Demura, T., Fukuda, H., Goodrich, J. and Tsukaya, H. (2004) Overexpression of a novel small peptide ROTUNDIFOLIA4 decreases cell proliferation and alters leaf shape in Arabidopsis thaliana. *The Plant Journal* **38**: 699-713.
- Nath, U., Crawford, B.C., Carpenter, R. and Coen, E. (2003) Genetic control of surface curvature. *Science* **299**: 1404-1407.
- Nikovics, K., Blein, T., Peaucelle, A., Ishida, T., Morin, H., Aida, M. and Laufs, P. (2006) The Balance between the MIR164A and CUC2 Genes Controls Leaf Margin Serration in Arabidopsis. *The Plant Cell* **18**: 2929-2945.
- Nimchuk, Z.L., Tarr, P.T. and Meyerowitz, E.M. (2011) An Evolutionarily Conserved Pseudokinase Mediates Stem Cell Production in Plants. *The Plant Cell* **23**: 851-854.
- Nimchuk, Z.L., Tarr, P.T., Ohno, C., Qu, X. and Meyerowitz, E.M. (2011) Plant Stem Cell Signaling Involves Ligand-Dependent Trafficking of the CLAVATA1 Receptor Kinase. *Current Biololgy* **21**: 345-352.
- Nissim, S., Hasso, S.M., Fallon, J.F. and Tabin, C.J. (2006) Regulation of Gremlin expression in the posterior limb bud. *Developmental Biology* **299**: 12-21.
- Niswander, L., Jeffrey, S., Martin, G.R. and Tickle, C. (1994) A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* **371**: 609-612.
- Nole-Wilson, S. and Krizek, B.A. (2006) AINTEGUMENTA contributes to organ polarity and regulates growth of lateral organs in combination with YABBY genes. *Plant Physiology* **141**: 977-987.
- Ogawa, M., Shinohara, H., Ogawa-Ohnishi, M. and Matsubayashi, Y. (2008) Arabidopsis CLV3 peptide directly binds CLV1 ectodomain. *Science* **319**: 294.
- Ohno, C.K., Reddy, G.V., Heisler, M.G. and Meyerowitz, E.M. (2004) The Arabidopsis JAGGED gene encodes a zinc finger protein that promotes leaf tissue development. *Development* **131**: 1111-1122.
- Ohyama, K., Shinohara, H., Ogawa-Ohnishi, M. and Matsubayashi, Y. (2009) A glycopeptide regulating stem cell fate in Arabidopsis thaliana. *Nature Chemical Biology* **5**: 578-580.
- Okada, K., Ueda, J., Komaki, M.K., Bell, C.J. and Shimura, Y. (1991) Requirement of the Auxin Polar Transport System in Early Stages of Arabidopsis Floral Bud Formation. *The Plant Cell* **3**: 677-684.
- Ori, N., Cohen, A.R., Etzioni, A., Brand, A., Yanai, O., Shleizer, S., Menda, N., Amsellem, Z., Efroni, I., Pekker, I., Alvarez, J.P., Blum, E., Zamir, D. and Eshed, Y. (2007) Regulation of LANCEOLATE by miR319 is required for compound-leaf development in tomato. *Nature Genetics* **39**: 787-791.
- Ori, N., Eshed, Y., Chuck, G., Bowman, J.L. and Hake, S. (2000) Mechanisms that control knox gene expression in the Arabidopsis shoot. *Development* **127**: 5523-5532.
- Ori, N., Juarez, M.T., Jackson, D., Yamaguchi, J., Banowetz, G.M. and Hake, S. (1999) Leaf senescence is delayed in tobacco plants expressing the maize homeobox gene knotted1 under the control of a senescence-activated promoter. *The Plant Cell* **11**: 1073-1080.
- Otsuga, D., DeGuzman, B., Prigge, M.J., Drews, G.N. and Clark, S.E. (2001) REVOLUTA regulates meristem initiation at lateral positions. *The Plant Journal* **25**: 223-236.
- Overvoorde, P.J., Okushima, Y., Alonso, J.M., Chan, A., Chang, C., Ecker, J.R., Hughes, B., Liu, A., Onodera, C., Quach, H., Smith, A., Yu, G. and Theologis, A. (2005)

- Functional genomic analysis of the AUXIN/INDOLE-3-ACETIC ACID gene family members in *Arabidopsis thaliana*. *The Plant Cell* **17**: 3282-3300.
- Paciorek, T., Zazimalova, E., Ruthardt, N., Petrasek, J., Stierhof, Y.D., Kleine-Vehn, J., Morris, D.A., Emans, N., Jurgens, G., Geldner, N. and Friml, J. (2005) Auxin inhibits endocytosis and promotes its own efflux from cells. *Nature* **435**: 1251-1256.
- Palatnik, J.F., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J.C. and Weigel, D. (2003) Control of leaf morphogenesis by microRNAs. *Nature* **425**: 257-263.
- Parkhurst, D.F. and Louks, O.L. (1972) Optimal leaf size in relation to the environment. *Journal of Ecology* **60**: 505-537.
- Pekker, I., Alvarez, J.P. and Eshed, Y. (2005) Auxin response factors mediate *Arabidopsis* organ asymmetry via modulation of KANADI activity. *The Plant Cell* **17**: 2899-2910.
- Peragine, A., Yoshikawa, M., Wu, G., Albrecht, H.L. and Poethig, R.S. (2004) SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in *Arabidopsis*. *Genes & Development* **18**: 2368-2379.
- Petrasek, J., Mravec, J., Bouchard, R., Blakeslee, J.J., Abas, M., Seifertova, D., Wisniewska, J., Tadele, Z., Kubes, M., Covanova, M., Dhonukshe, P., Skupa, P., Benkova, E., Perry, L., Krecek, P., Lee, O.R., Fink, G.R., Geisler, M., Murphy, A.S., Luschnig, C., Zazimalova, E. and Friml, J. (2006) PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science* **312**: 914-918.
- Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**: e45.
- Phelps-Durr, T.L., Thomas, J., Vahab, P. and Timmermans, M.C. (2005) Maize rough sheath2 and its *Arabidopsis* orthologue ASYMMETRIC LEAVES1 interact with HIRA, a predicted histone chaperone, to maintain knox gene silencing and determinacy during organogenesis. *The Plant Cell* **17**: 2886-2898.
- Piazza, P., Bailey, C.D., Cartolano, M., Krieger, J., Cao, J., Ossowski, S., Schneeberger, K., He, F., de Meaux, J., Hall, N., Macleod, N., Filatov, D., Hay, A. and Tsiantis, M. (2010) *Arabidopsis thaliana* leaf form evolved via loss of KNOX expression in leaves in association with a selective sweep. *Current Biology* **20**: 2223-2228.
- Pines, J. (1994) Protein kinases and cell cycle control. *Seminars in Cell Biology* **5**: 399-408.
- Pinon, V., Etchells, J.P., Rossignol, P., Collier, S.A., Arroyo, J.M., Martienssen, R.A. and Byrne, M.E. (2008) Three PIGGYBACK genes that specifically influence leaf patterning encode ribosomal proteins. *Development* **135**: 1315-1324.
- Poethig, R.S. (1987) Clonal analysis of cell lineage patterns in plant development. *American Journal of Botany* **74**: 581-594.
- Poethig, R.S. and Sussex, I.M. (1985) The cellular parameters of leaf development in tobacco: a clonal analysis. *Planta* **165**: 170-184.
- Pozo, J.C., Timpte, C., Tan, S., Callis, J. and Estelle, M. (1998) The ubiquitin-related protein RUB1 and auxin response in *Arabidopsis*. *Science* **280**: 1760-1763.
- Prigge, M.J., Otsuga, D., Alonso, J.M., Ecker, J.R., Drews, G.N. and Clark, S.E. (2005) Class III homeodomain-leucine zipper gene family members have overlapping, antagonistic, and distinct roles in *Arabidopsis* development. *The Plant Cell* **17**: 61-76.
- Prusinkiewicz, P., Crawford, S., Smith, R.S., Ljung, K., Bennett, T., Ongaro, V. and Leyser, O. (2009) Control of bud activation by an auxin transport switch. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 17431-17436.
- Pufky, J., Qiu, Y., Rao, M.V., Hurban, P. and Jones, A.M. (2003) The auxin-induced transcriptome for etiolated *Arabidopsis* seedlings using a structure/function approach. *Functional & Integrative Genomics* **3**: 135-143.

- Ragni, L., Belles-Boix, E., Gunl, M. and Pautot, V. (2008) Interaction of KNAT2 and KNAT6 with BREVIPEDICELLUS and PENNYWISE in Arabidopsis inflorescences. *The Plant Cell* **20**: 888-900.
- Raup, D.M. and Valentine, J.W. (1983) Multiple origins of life. *Proceedings of the National Academy of Sciences of the United States of America* **80**: 2981-2984.
- Raven, J.A. (1975) Transport of indole acetic acid in plant cells in relation to pH and electrical potential gradients, and its significance for polar IAA transport. *New Phytologist* **74**: 163-172.
- Reddy, G.V., Heisler, M.G., Ehrhardt, D.W. and Meyerowitz, E.M. (2004) Real-time lineage analysis reveals oriented cell divisions associated with morphogenesis at the shoot apex of Arabidopsis thaliana. *Development* **131**: 4225-4237.
- Reddy, G.V. and Meyerowitz, E.M. (2005) Stem-cell homeostasis and growth dynamics can be uncoupled in the Arabidopsis shoot apex. *Science* **310**: 663-667.
- Reinhardt, D., Frenz, M., Mandel, T. and Kuhlemeier, C. (2003) Microsurgical and laser ablation analysis of interactions between the zones and layers of the tomato shoot apical meristem. *Development* **130**: 4073-4083.
- Reinhardt, D., Mandel, T. and Kuhlemeier, C. (2000) Auxin regulates the initiation and radial position of plant lateral organs. *The Plant Cell* **12**: 507-518.
- Reinhardt, D., Pesce, E.R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J. and Kuhlemeier, C. (2003) Regulation of phyllotaxis by polar auxin transport. *Nature* **426**: 255-260.
- Remington, D.L., Vision, T.J., Guilfoyle, T.J. and Reed, J.W. (2004) Contrasting modes of diversification in the Aux/IAA and ARF gene families. *Plant Physiology* **135**: 1738-1752.
- Renaudin, J., Doonan, J.H., Freeman, D., Hashimoto, J., Hirt, H., Inze, D., Jacobs, T., Kouchi, H., Rouze, P., Sauter, M., Savoure, A., Sorrell, D.A., Sundaresan, V. and Murray, J.A.H. (1996) Plant cyclins: A unified nomenclature for plant A-, B- and D-type cyclins based on sequence organization. *Plant Molecular Biology* **32**: 1003-1018.
- Rieckhof, G., Casares, F., Ryoo, H.D., Abu-Shaar, M. and Mann, R.S. (1997) Nuclear translocation of extradenticle requires homothorax, which encodes an extradenticle-related homeodomain protein. *Cell* **91**: 171-183.
- Riou-Khamlichi, C., Huntley, R., Jacqumard, A. and Murray, J.A. (1999) Cytokinin activation of Arabidopsis cell division through a D-type cyclin. *Science* **283**: 1541-1544.
- Robert, S., Kleine-Vehn, J., Barbez, E., Sauer, M., Paciorek, T., Baster, P., Vanneste, S., Zhang, J., Simon, S., Covanova, M., Hayasji, K., Dhonukshe, P., Yang, Z., Bednarek, S.Y., Jones, A.M., Luschnig, C., Aniento, F., Zazimalova, E. and Friml, J. (2010) ABP1 mediates auxin inhibition of clathrin-dependent endocytosis in Arabidopsis. *Cell* **143**: 111-121.
- Rodriguez, R.E., Mecchia, M.A., Debernardi, J.M., Schommer, C., Weigel, D. and Palatnik, J.F. (2010) Control of cell proliferation in Arabidopsis thaliana by microRNA miR396. *Development* **137**: 103-112.
- Roeder, A.H., Ferrandiz, C. and Yanofsky, M.F. (2003) The role of the REPLUMLESS homeodomain protein in patterning the Arabidopsis fruit. *Current Biology* **13**: 1630-1635.
- Rojo, E., Sharma, V.K., Kovaleva, V., Raikhel, N.V. and Fletcher, J.C. (2002) CLV3 is localized to the extracellular space, where it activates the Arabidopsis CLAVATA stem cell signaling pathway. *The Plant Cell* **14**: 969-977.
- Roth-Nebelsick, A. (2001) Computer-based analysis steady-state and transient heat transfer of small-sized leaves by free and mixed convection. *Plants, Cell and Environment* **24**: 631-640.

- Royer, D.L. and Wilf, P. (2006) Why do toothed leaves correlate with cold climates? Gas exchange at leaf margins provides new insights into a classic paleotemperature proxy. *International Journal of Plant Sciences* **167**: 11-18.
- Royer, D.L., Wilf, P., Janesko, D.A., Kowalski, E.A. and Dilcher, D.L. (2005) Correlations of climate and plant ecology to leaf size and shape: potential proxies for the fossil record. *American Journal of Botany* **92**: 1141-1151.
- Rubery, P.H. and Sheldrake, A.R. (1974) Carrier mediated auxin transport. *Planta* **188**: 101-121.
- Rutjens, B., Bao, D., van Eck-Stouten, E., Brand, M., Smeekens, S. and Proveniers, M. (2009) Shoot apical meristem function in Arabidopsis requires the combined activities of three BEL1-like homeodomain proteins. *The Plant Journal* **58**: 641-654.
- Sakamoto, T., Kamiya, N., Ueguchi-Tanaka, M., Iwahori, S. and Matsuoka, M. (2001) KNOX homeodomain protein directly suppresses the expression of a gibberellin biosynthetic gene in the tobacco shoot apical meristem. *Genes & Development* **15**: 581-590.
- Sambrook, J. and Russell, D.W. (2001) Molecular cloning. A laboratory manual. New York, Cold Spring Harbor Laboratory Press.
- Satina, S., Blakeslee, A.F. and Avery, A.G. (1940) Demonstration of the three germ layers in the shoot apex of *Datura* by means of induced polyploidy in periclinal chimeras. *American Journal of Botany* **27**: 895-905.
- Sattler, R. and Rutishauser, R. (1992) Partial homology of pinnate leaves and shoots: Orientation of leaflet inception. *Botanische Jahrbücher für Systematik Pflanzengeschichte und Pflanzengeographie* **114**: 61-79.
- Sawa, S., Watanabe, K., Goto, K., Liu, Y.G., Shibata, D., Kanaya, E., Morita, E.H. and Okada, K. (1999) FILAMENTOUS FLOWER, a meristem and organ identity gene of Arabidopsis, encodes a protein with a zinc finger and HMG-related domains. *Genes & Development* **13**: 1079-1088.
- Scarpella, E., Marcos, D., Friml, J. and Berleth, T. (2006) Control of leaf vascular patterning by polar auxin transport. *Genes & Development* **20**: 1015-1027.
- Schneeberger, R., Tsiantis, M., Freeling, M. and Langdale, J.A. (1998) The rough sheath2 gene negatively regulates homeobox gene expression during maize leaf development. *Development* **125**: 2857-2865.
- Schnittger, A., Grini, P.E., Folkers, U. and Hulskamp, M. (1996) Epidermal fate map of the Arabidopsis shoot meristem. *Developmental Biology* **175**: 248-255.
- Schoof, H., Lenhard, M., Haecker, A., Mayer, K.F., Jurgens, G.A. and Laux, T. (2000) The stem cell population of Arabidopsis shoot meristems is maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. *Cell* **100**: 635-644.
- Schruff, M.C., Spielman, M., Tiwari, S., Adams, S., Fenby, N. and Scott, R.J. (2006) The AUXIN RESPONSE FACTOR 2 gene of Arabidopsis links auxin signalling, cell division, and the size of seeds and other organs. *Development* **133**: 251-261.
- Schubert, D., Primavesi, L., Bishopp, A., Roberts, G., Doonan, J., Jenuwein, T. and Goodrich, J. (2006) Silencing by plant Polycomb-group genes requires dispersed trimethylation of histone H3 at lysine 27. *The Embo Journal* **25**: 4638-4649.
- Schuetz, M., Berleth, T. and Mattson, J. (2008) Multiple MONOPTEROS-dependent pathways are involved in leaf initiation. *Plant Physiology* **148**: 870-880.
- Schwab, R., Palatnik, J.F., Riester, M., Schommer, C., Schmid, M. and Weigel, D. (2005) Specific effects of microRNAs on the plant transcriptome. *Developmental Cell* **8**: 517-527.
- Scott, M.P. and Weiner, A.J. (1984) Structural relationships among genes that control development: Sequence homology between the Antennapedia, Ultrabithorax and fushi

- tarazu loci of *Drosophila*. *Proceedings of the Natational Academy of Science of the United States of America* **81**: 4115-4119.
- Semiarti, E., Ueno, Y., Tsukaya, H., Iwakawa, H., Machida, C. and Machida, Y. (2001) The ASYMMETRIC LEAVES2 gene of *Arabidopsis thaliana* regulates formation of a symmetric lamina, establishment of venation and repression of meristem-related homeobox genes in leaves. *Development* **128**: 1771-1783.
- Serikawa, K.A., Martinez-Laborda, A., Kim, H.S. and Zambryski, P.C. (1997) Localization of expression of KNAT3, a class 2 knotted1-like gene. *The Plant Journal* **11**: 853-861.
- Sessions, A., Weigel, D. and Yanofsky, M.F. (1999) The *Arabidopsis thaliana* MERISTEM LAYER 1 promoter specifies epidermal expression in meristems and young primordia. *The Plant Journal* **20**: 259-263.
- Shani, E., Ben-Gera, H., Shleizer-Burko, S., Burko, Y., Weiss, D. and Ori, N. (2010) Cytokinin regulates compound leaf development in tomato. *The Plant Cell* **22**: 3206-3217.
- Shani, E., Burko, Y., Ben-Yaakov, L., Berger, Y., Amsellem, Z., Goldshmidt, A., Sharon, E. and Ori, N. (2009) Stage-specific regulation of *Solanum lycopersicum* leaf maturation by class 1 KNOTTED1-LIKE HOMEBOX proteins. *The Plant Cell* **21**: 3078-3092.
- Sharma, V.K., Ramirez, J. and Fletcher, J.C. (2003) The *Arabidopsis* CLV3-like (CLE) genes are expressed in diverse tissues and encode secreted proteins. *Plant Molecular Biology* **51**: 415-425.
- Shibaoka, H. and Nagai, R. (1994) The plant cytoskeleton. *Current Opinion in Cell Biology* **6**: 10-15.
- Shleizer-Burko, S., Burko, Y., Ben-Herzel, O. and Ori, N. (2011) Dynamic growth program regulated by LANCEOLATE enables flexible leaf patterning. *Development* **138**: 695-704.
- Sieber, P., Wellmer, F., Gheyselinck, J., Riechmann, J.L. and Meyerowitz, E.M. (2007) Redundancy and specialization among plant microRNAs: role of the MIR164 family in developmental robustness. *Development* **134**: 1051-1060.
- Siegfried, K.R., Eshed, Y., Baum, S.F., Otsuga, D., Drews, G.N. and Bowman, J.L. (1999) Members of the YABBY gene family specify abaxial cell fate in *Arabidopsis*. *Development* **126**: 4117-4128.
- Sinha, N.R., Williams, R.E. and Hake, S. (1993) Overexpression of the maize homeo box gene, KNOTTED-1, causes a switch from determinate to indeterminate cell fates. *Genes & Development* **7**: 787-795.
- Smith, H.M., Boschke, I. and Hake, S. (2002) Selective interaction of plant homeodomain proteins mediates high DNA-binding affinity. *Proceedings of the Natational Academy of Sciences of the United States of America* **99**: 9579-9584.
- Smith, L.G., Greene, B., Veit, B. and Hake, S. (1992) A dominant mutation in the maize homeobox gene, Knotted-1, causes its ectopic expression in leaf cells with altered fates. *Development* **116**: 21-30.
- Smith, R.S., Guyomarc'h, S., Mandel, T., Reinhardt, D., Kuhlemeier, C. and Prusinkiewicz, P. (2006) A plausible model of phyllotaxis. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 1301-1306.
- Snow, M. and Snow, R. (1931) Experiments on phyllotaxis. I. The effect of isolating a primordium. *Philosophical Transactions of the Royal Society of London, Series B*. **221**: 1-43.
- Snow, M.a.S., R. (1937) Auxin and leaf formation. *New Phytologist* **36**: 1-18.
- Sober, E. and Steel, M. (2002) Testing the hypothesis of common ancestry. *Journal of Theoretical Biology* **218**: 395-408.

- Sorefan, K., Girin, T., Liljegren, S.J., Ljung, K., Robles, P., Galvan-Ampudia, C.S., Offringa, R., Friml, J., Yanofsky, M.F. and Ostergaard, L. (2009) A regulated auxin minimum is required for seed dispersal in *Arabidopsis*. *Nature* **459**: 583-586.
- Souer, E., van Houwelingen, A., Kloos, D., Moi, J. and Koes, R. (1996) The NO APICAL MERISTEM gene of petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. *Cell* **85**: 159-170.
- Spinelli, S.V., Martin, A.P., Viola, I.L., Gonzalez, D.H. and Palatnik, J.F. (2011) A mechanistic link between *STM* and *CUC1* during *Arabidopsis* development. *Plant Physiology*: Epub ahead of print.
- Steeves, T.A. and Sussex, I.M. (1989) Patterns in plant development. Cambridge, Cambridge University press.
- Stewart, R.N. and Dermen, H. (1970) Determination of number and mitotic activity of shoot apical initial cells by analysis of mericlinal chimeras. *American Journal of Botany* **57**: 816-826.
- Sugimoto-Shirasu, K., Roberts, G.R., Stacey, N.J., McCann, M.C., Maxwell, A. and Roberts, K. (2005) RHL1 is an essential component of the plant DNA topoisomerase VI complex and is required for ploidy-dependent cell growth. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 18736-18741.
- Sugimoto-Shirasu, K., Stacey, N.J., Corsar, J., Roberts, K. and McCann, M.C. (2002) DNA topoisomerase VI is essential for endoreduplication in *Arabidopsis*. *Current Biology* **12**: 1782-1786.
- Sun, X., Mariani, F.V. and Martin, G.R. (2002) Functions of FGF signalling from the apical ectodermal ridge in limb development. *Nature* **418**: 501-508.
- Sussex, I.M. (1951) Experiments on the cause of dorsiventrality in leaves. *Nature* **167**: 651-652.
- Takada, S., Hibara, K., Ishida, T. and Tasaka, M. (2001) The CUP-SHAPED COTYLEDON1 gene of *Arabidopsis* regulates shoot apical meristem formation. *Development* **128**: 1127-1135.
- Tan, X., Calderon-Villalobos, L.I., Sharon, M., Zheng, C., Robinson, C.V., Estelle, M. and Zheng, N. (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* **446**: 640-645.
- Taylor, S., Hofer, J. and Murfet, I. (2001) Stamina pistilloida, the Pea ortholog of Fim and UFO, is required for normal development of flowers, inflorescences, and leaves. *The Plant Cell* **13**: 31-46.
- Theologis, A., Huynh, T.V. and Davis, R.W. (1985) Rapid induction of specific mRNAs by auxin in pea epicotyl tissue. *Journal of Molecular Biology* **183**: 53-68.
- Theologis, A. and Ray, P.M. (1982) Early auxin-regulated polyadenylylated mRNA sequences in pea stem tissue. *Proceedings of the National Academy of Science of the United States of America* **79**: 418-421.
- Theophrastus (1916) Enquiry into plants (translated from Greek by Sir A. Hort). London, England, William Heinemann.
- Timmermans, M.C., Hudson, A., Becraft, P.W. and Nelson, T. (1999) ROUGH SHEATH2: a Myb protein that represses knox homeobox genes in maize lateral organ primordia. *Science* **284**: 151-153.
- To, J.P. and Kieber, J.J. (2008) Cytokinin signaling: two-components and more. *Trends in Plant Science* **13**: 85-92.
- Traas, J. and Bohn-Courseau, I. (2005) Cell proliferation patterns at the shoot apical meristem. *Current Opinion in Plant Biology* **8**: 587-592.

- Tsiantis, M., Schneeberger, R., Golz, J.F., Freeling, M. and Langdale, J.A. (1999) The maize rough sheath2 gene and leaf development programs in monocot and dicot plants. *Science* **284**: 154-156.
- Ulmasov, T., Hagen, G. and Guilfoyle, T.J. (1999) Activation and repression of transcription by auxin-response factors. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 5844-5849.
- Ulmasov, T., Murfett, J., Hagen, G. and Guilfoyle, T.J. (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *The Plant Cell* **9**: 1963-1971.
- Vierstra, R.D. (2003) The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. *Trends in Plant Science* **8**: 135-142.
- Vieten, A., Sauer, M., Brewer, P.B. and Friml, J. (2007) Molecular and cellular aspects of auxin-transport-mediated development. *Trends in Plant Science* **12**: 160-168.
- Vieten, A., Vanneste, S., Wisniewska, J., Benkova, E., Benjamins, R., Beeckman, T., Luschnig, C. and Friml, J. (2005) Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. *Development* **132**: 4521-4531.
- Vollbrecht, E., Veit, B., Sinha, N. and Hake, S. (1991) The developmental gene Knotted-1 is a member of a maize homeobox gene family. *Nature* **350**: 241-243.
- Vroemen, C.W., Mordhorst, A.P., Albrecht, C., Kwaaitaal, M.A. and de Vries, S.C. (2003) The CUP-SHAPED COTYLEDON3 gene is required for boundary and shoot meristem formation in Arabidopsis. *The Plant Cell* **15**: 1563-1577.
- Waites, R., Selvadurai, H.R., Oliver, I.R. and Hudson, A. (1998) The PHANTASTICA gene encodes a MYB transcription factor involved in growth and dorsoventrality of lateral organs in Antirrhinum. *Cell* **93**: 779-789.
- Wang, H., Jones, B., Li, Z., Frasse, P., Delalande, C., Regad, F., Chaabouni, S., Latche, A., Pech, J.C. and Bouzayen, M. (2005) The tomato Aux/IAA transcription factor IAA9 is involved in fruit development and leaf morphogenesis. *The Plant Cell* **17**: 2676-2692.
- Weir, I., Lu, J., Cook, H., Causier, B., Schwarz-Sommer, Z. and Davies, B. (2004) CUPULIFORMIS establishes lateral organ boundaries in Antirrhinum. *Development* **131**: 915-922.
- Wenkel, S., Emery, J., Hou, B.H., Evans, M.M.S. and Barton, M.K. (2007) A feedback regulatory module formed by LITTLE ZIPPER and HD-ZIPIII genes. *The Plant Cell* **19**: 3379-3390.
- White, D.W. (2006) PEAPOD regulates lamina size and curvature in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 13238-13243.
- Wilf, P. (1997) When are leaves good thermometers? A new case for Leaf Margin Analysis. *Paleobiology* **23**: 373-390.
- Williams, L., Carles, C.C., Osmont, K.S. and Fletcher, J.C. (2005) A database analysis method identifies an endogenous trans-acting short-interfering RNA that targets the Arabidopsis ARF2, ARF3, and ARF4 genes. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 9703-9708.
- Wisniewska, J., Xu, J., Seifertova, D., Brewer, P.B., Ruzicka, K., Blilou, I., Rouquie, D., Benkova, E., Scheres, B. and Friml, J. (2006) Polar PIN localization directs auxin flow in plants. *Science* **312**: 883.
- Wolfe, J.A. (1979) Temperature parameters of humid to mesic forests of Eastern Asia and relation to forests of other regions of the Northern Hemisphere and Australasia. *US Geological Survey Bulletin* **1106**: 1-37.

- Wolfe, J.A. (1993) A method of obtaining climatic parameters from leaf assemblages. *US Geological Survey Bulletin* **2040**: 1-71.
- Wolff, C.F. (1759) *Theoria Generationis*. Halae ad Salam: Litteris Hendelianis.
- Xu, L. and Shen, W.H. (2008) Polycomb silencing of KNOX genes confines shoot stem cell niches in Arabidopsis. *Current Biology* **18**: 1966-1971.
- Xu, L., Xu, Y., Dong, A., Sun, Y., Pi, L., Xu, Y. and Huang, H. (2003) Novel as1 and as2 defects in leaf adaxial-abaxial polarity reveal the requirement for ASYMMETRIC LEAVES1 and 2 and ERECTA functions in specifying leaf adaxial identity. *Development* **130**: 4097-4107.
- Xu, L., Yang, L., Pi, L., Liu, Q., Ling, Q., Wang, H., Poethig, R.S. and Huang, H. (2006) Genetic interaction between the AS1-AS2 and RDR6-SGS3-AGO7 pathways for leaf morphogenesis. *Plant and Cell Physiology* **47**: 853-863.
- Yanai, O., Shani, E., Dolezal, K., Tarkowski, P., Sablowski, R., Sandberg, G., Samach, A. and Ori, N. (2005) Arabidopsis KNOXI proteins activate cytokinin biosynthesis. *Current Biology* **15**: 1566-1571.
- Yang, H. and Murphy, A.S. (2009) Functional characterization of Arabidopsis ABCB, AUX1 and PIN auxin transporters in Schizosaccharomyces pombe. *The Plant Journal* **59**: 179-191.
- Zenoni, S., Reale, L., Tornielli, G.B., Lanfaloni, L., Porceddu, A., Ferrarini, A., Moretti, C., Zamboni, A., Speghini, A., Ferranti, F. and Pezzotti, M. (2004) Downregulation of the Petunia hybrida alpha-expansin gene PhEXP1 reduces the amount of crystalline cellulose in cell walls and leads to phenotypic changes in petal limbs. *The Plant Cell* **16**: 295-308.
- Zhang, J., Chen, R., Xiao, J., Qian, C., Wang, T., Li, H., Ouyang, B. and Ye, Z. (2007) A single-base deletion mutation in SlIAA9 gene causes tomato (Solanum lycopersicum) entire mutant. *Journal of Plant Research* **120**: 671-678.
- Zimmermann, W. (1952) Main results of the 'telome theory'. *Paleobotanist* **1**: 456-470.